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Avian Immunology

Third Edition



Edited by Bernd Kaspers, Karel A. Schat,
Thomas Göbel and Lonneke Vervelde



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Dedication

Dedicated to



Professor Pete Kaiser 1964–2016

Coeditor Second Edition *Avian Immunology*

Valued Avian Immunologist and Sorely Missed by the Editors of the
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Foreword

The fascinating world of avian immunology might be best surmised by the quote from Dr. Jim Kauffman, who stated with obvious eloquence “chickens are not mice with feathers.” The point being that while many immunological attributes are shared between the species, many are also different and that we as immunologists must work to discover them.

In the last two decades, there has been a great increase in our knowledge of avian immunology. Molecular immunologists, utilizing new technologies, have expanded our ability to determine and decipher what is encoded in the avian genome for immunological defense. In addition, strategic funding capabilities provided primarily, but not exclusively, by the United States and EU have resulted in the development of a plethora of monoclonal antibodies against avian immunological proteins (e.g., cytokines and cell markers) through “toolbox” grants. These two driving forces have opened doors and windows in a field that has historically struggled to provide reagents for intricate analysis.

This book arrives at a key time in our need for knowledge to handle the ever-changing pressures for sustainable solutions to poultry production issues on a global scale. The overall health of birds, in particular the immune system, is critical to feed an ever-growing human population. As an example, the loss of antibiotic treatment for poultry flocks in most countries has created a need for immunological intervention strategies to enhance control against bacterial pathogens. In addition to the applied aspect of avian immunology, a comparative analysis, including genomics, function, and evolution, is important to determine how and why the avian immune system works as it does.

The avian genome in general is regarded as condensed in nature to mammalian species in terms of immune gene repertoire. Immunologically speaking, this translates in that birds have to do the same (protect the animal) with less. Despite this, the immune responses between birds and mammals display similar defining characteristics. Both species employ an innate and adaptive immune response to a variety of microbiological incursions from bacteria, viruses, protozoa, and parasites. However, differences also stand out. For example, chickens lack draining

lymph node development, which opens an array of antigen-processing questions for immune recognition and stimulation. Birds also lack several Toll-like receptors found in other vertebras, yet share many that recognize similar molecular motifs. These are but two examples to highlight the differences, but there are many others. Perhaps Winston Churchill, in 1939, was also speaking of avian immunology when he said, “it is a riddle, wrapped in a mystery, inside an enigma.”

It is impossible to discuss the context of this book without acknowledging the current global COVID-19 pandemic caused by the SARS-CoV-2 coronavirus. This relatively novel virus, without existing population immunity, has spread rapidly across transcontinental boundaries. These events are reminiscent of reports in the early 1930s when chicks arrived at the veterinary laboratories of the agricultural college in Fargo, North Dakota. A new disease was raging across poultry farms in Minnesota and North Dakota resulting in sick birds that were undergoing respiratory distress with high mortality. As researchers investigated the disease, it was eventually identified as a viral agent and termed infectious bronchitis virus (IBV) based on disease characteristics. However, it was not until the advent of electron microscopy in the 1960s that pathologists were able to get a visual look at the virus and in 1964 identified “lollipop-shaped” protrusions at the end of the spikes of the virus that resembled a halo of gas surrounding the sun. The relevance of these observations is notable in that approximately a year after these publications, similar morphological characteristics were identified in virus samples obtained from a boy undergoing flu-like symptoms. Scientists utilized these morphological similarities found in both avian and human samples to identify a new viral family, coronavirus.

Parallels are also found in the immunological arena. To combat the SAR-CoV-2 virus, in 2020, vaccines have been developed and approved for use in humans. Of note is that two of the most used at this time in the United States and EU are nucleic acid based on the messenger RNA sequence of the SARS spike (S) gene. The resulting protein is the main coronavirus immunogen and contains epitopes for inducing neutralizing antibodies. The relevance to avian immunology is that over 20 years ago we

and others reported on the development and use of DNA vaccines expressing the S1 from IBV in chickens, which provided protective efficacy and induced both antibodies and T-cell responses.

The future of avian immunology is a bright one. Knowledge gleaned from the release of the avian genome, including conservation of synteny, has already resulted in increased scrutiny of what is and is not available for defense. Functional studies, such as epitope binding of avian MHCs, are shedding new light and possibilities for enhanced immune responses. Research in disease resistance breeding is being accelerated by the development of transgenic technologies, including CRISPR, that allow for manipulation and testing in a more timely manner. As these resources are brought to bear, past barriers in the field of avian immunology will fall.

This book brings together highly distinguished and internationally representative authors who are specialists in their respective fields of avian immunology. This book will be valued by bench scientists, graduate students, poultry veterinarians, commercial industry researchers, and avian ecologists as an “immunological-bible” for a better understanding of avian immunology.

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The importance of the avian immune system and its unique features

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1.1 Introduction

The avian immune system provides an invaluable model for studies on basic immunology. Birds and mammals evolved from a common reptilian ancestor more than 250 million years ago and have inherited many common immunological systems. They also have developed a number of very different, and in some cases remarkable, strategies. Due to their economic importance, and the ready availability of inbred lines, most avian immunology research has involved the domestic chicken, *Gallus gallus domesticus*. A remarkable consequence of this research has been the seminal contributions it has made to understand the fundamental immunological concepts, especially the complete separation of developing bursa- (B-) and thymus- (T-) dependent lymphocyte lineages. Some of these observations have been made by chance, while others have resulted from painstaking work which took advantage of special avian features, such as ease of access to, and precise timing of, all the stages in embryonic development. Some of the avian findings were described before they were recognized as important and subsequently explained in mainstream immunology. The story of avian immunology is a fascinating one and by no means complete, as there is still the need for explanations of a number of unique features and different strategies adopted by birds. In this chapter, some of the “firsts,” rightly attributed to avian immunology, are described and the importance of further studies in avian immunology is highlighted.

1.2 The contribution from avian lymphocytes

The advent of modern cellular immunology, and the fundamental role that lymphocytes play, is generally credited to

the 1950s and 1960s, when lymphocyte function became an active subject for research [1]. The immunological significance of lymphocytes emanated from some seminal studies carried out by Gowans, Chase and Mitchison, Simonsen, and their contemporaries [1,2]. In elegant experiments with laboratory mammals and using cell transfers, these workers demonstrated that lymphocytes are essential for generating immune responses and retaining memory of previous exposure to an antigen. However, evidence that lymphocytes play such a key role in protection against infection and in tumor rejection had, in fact, been discovered almost 40 years earlier [3–6], though little attention was paid to it [7]. This was almost certainly because at the time they were made the observations could not properly be explained and, possibly, because the experimental animal involved was the chicken.

Between 1912 and 1921, James Murphy, an experimental pathologist working at the Rockefeller Institute for Medical Research in New York, performed a series of remarkable experiments using chickens and their embryos to study the growth and rejection of tumor grafts. His experiments appeared to prove beyond question that the lymphocyte is the active component in tissue graft rejection, in protection against infection and, by implication, in innate and acquired immune responses [7]. Murphy [4] observed that fragments of rat tumors would not grow in the adult chicken, just as they did not grow in other species (xenogenic rejection). However, they could be grown on the chorioallantoic membrane (CAM) of developing chick embryos, although only up to about 18 days incubation. In older embryos tumor grafts were rejected, just as they were if grafted onto the newly hatched chick or an adult bird. Interestingly, Murphy [3] observed that the grafts which grew on embryos could be transferred to fresh embryos without any evidence of them being

altered. They also retained their tumorigenic capacity if regrafted onto a rat. Murphy [3] commented that these cellular changes occurring when living tissue is grafted onto an unsuitable host are the same, regardless of the type of host resistance, be it natural resistance because of species differences (allogeneic or xenogeneic rejection) or acquired immunity due to recovery from a tumor implanted earlier. The histological picture consisted of edema surrounded by fibroplasia in the host tissue, the budding out of blood vessels, and infiltration of surrounding host tissues with small lymphocytic cells. The inevitable consequence was that cells in the graft died fairly quickly leaving only a scar. These were quite profound though, at the time, unappreciated observations.

Murphy [4] also performed a series of elegant experiments using adult chicken tissues cogenerated onto the CAM with fragments of rat tumors. He observed that chicken tissues containing an abundant supply of lymphocytes, such as the spleen or bone marrow, caused tumor grafts to be rejected, whereas these were not rejected if the cogenerated tissue lacked a rich supply of lymphocytes. Later on, Murphy [5] showed that after grafting fragments of adult chicken spleen onto the CAM of a 7-day embryo, the embryo's own spleen became grossly enlarged (splenomegaly). This is the first published record of a graft versus host response (GvHR). Much later, it was explained by Simonsen [8] as the immunologically competent lymphocytes of the adult responding to mismatched major histocompatibility complex (MHC) molecules expressed by the embryonic cells. The embryonic cells were recognized as foreign causing the adult cells to replicate and destroy the embryonic host's lymphocytes. Graft versus host disease, in which allogeneic bone marrow transplants recognize the tissues of the graft recipient and cause severe inflammatory disease, is a serious problem for immunosuppressed recipients receiving bone marrow transplants. The phenomenon became a major concern with the introduction of human bone marrow transplantation. Nonetheless, the phenomenon was first described using chick embryos as long ago as 1916.

1.3 Contribution of the bursa of Fabricius

Without doubt, the most significant contribution that studies on the avian system have made to development of mainstream immunology was delineating the two major arms of the adaptive immune system. As already pointed out, in the 1960 the significance of lymphocytes was just becoming appreciated, and it was generally accepted that there are two types of adaptive immune responses: humoral responses involving antibodies and cellular responses mediated by macrophages and lymphocytes. Since antibodies are produced by plasma cells, which themselves are derived from lymphocytes, it was not

understood how such lymphocytes could be the same as those cells involved in cell-mediated functions. The bursa of Fabricius, an obscure sac-like structure attached to the proctodeal region of the bird's cloaca (Fig. 1.1), played a crucial role in unraveling this problem.

The cloacal bursa, takes its name from Hieronymus Fabricius of Aquapendente (1537–1619), is also known as Girolamo Fabrizi d' Acquapendente (Fig. 1.2). He was a professor of surgery at the University of Padua, Italy, from 1565 to 1613 [9] and by all accounts a brilliant anatomist, embryologist, and teacher. For his pioneering work, he was later credited in Italian medical science as the "Father of Embryology." Fabricius not only carried out dissections on human cadavers but also extended his anatomical studies to other species, providing the most beautiful, detailed drawings of his work. From his observations of avian anatomy, he surmised that the cloacal bursa, a hollow structure connected by a duct to the proctodeal region of the cloaca, most likely acts as a receptacle for storing donated semen.

"Since the sac is pervious, so that there is an open passage from the anus to the uterus itself and another from the uterus to the sac, that is, above and below, and since it is closed at the other end, I think it is the place into which the cock introduces and delivers semen so that it may be stored there" [4].

This is not the case, however, but the role of the cloacal bursa continued to puzzle researchers over the following 350 years. Some surmised that since the bursa of Fabricius regresses with sexual maturity, its size having an inverse relationship with the size testes and the adrenals, it must be some sort of endocrine or lymphoid gland associated with growth and sexual development [10].

Over the years many investigated the function(s) of the bursa including one young researcher, Bruce Glick, working at the Poultry Science Department at Ohio State University, United States. Glick surgically removed the bursa from young chicks to investigate the effect on growth. By chance, after one experiment was concluded, a colleague, Timothy Chang, asked if he could use some of the birds for a class demonstration on antibody production. A group of the chickens was injected with *Salmonella* spp. "O" antigen but 1 week later, when the class carried out tests with blood and antigen, there was no evidence of agglutination. Chang, somewhat perplexed, reported the failure to Glick who was able to identify the nonresponder chickens as those that had been bursectomized. At the time they did not seem to fully appreciate the singular importance of this finding [10] but were able to confirm their initial observations in further experiments and wrote up an article entitled: "The role of the bursa of Fabricius in antibody production." This was submitted to *Science* but

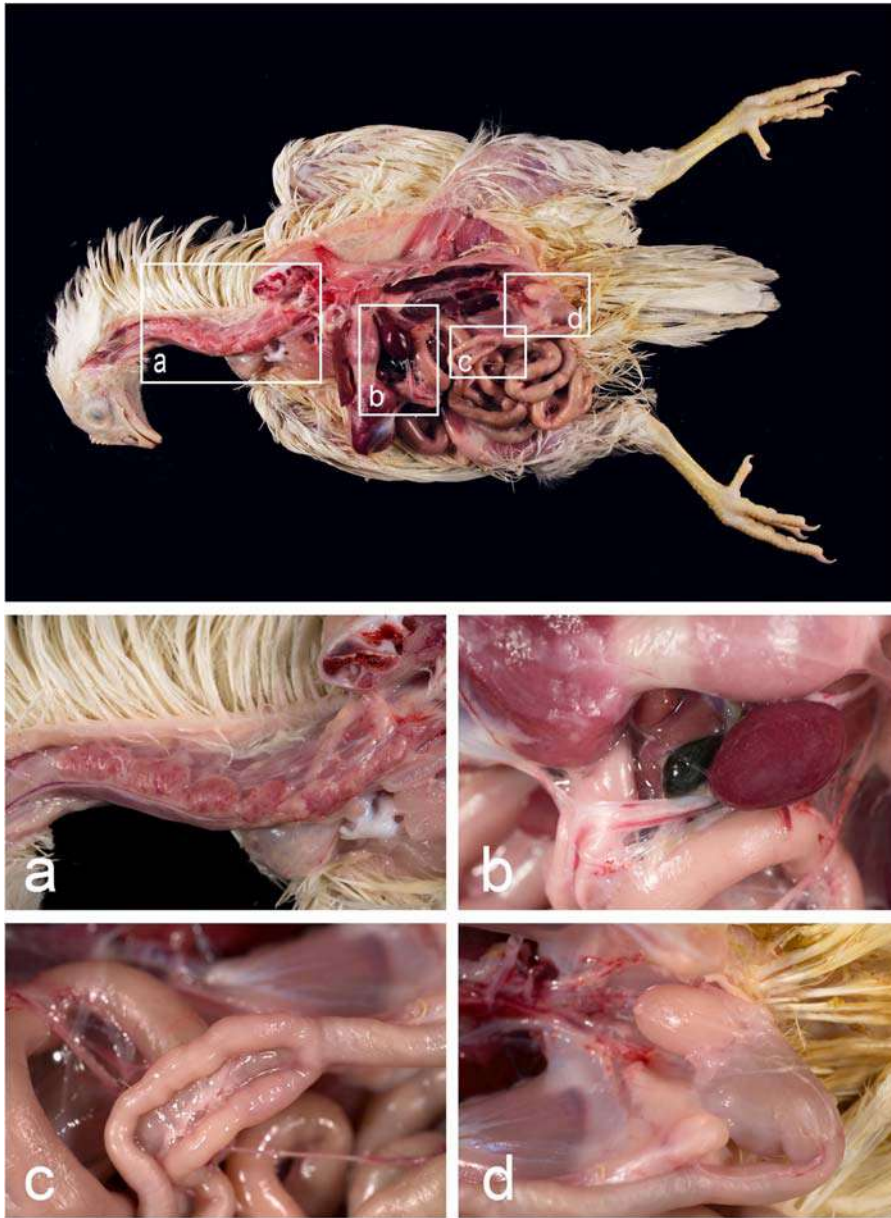


FIGURE 1.1 A dissection showing the positions of the major lymphoid tissues in the chick. Insets provide details of: (A) the seven thymic lobes lying alongside the left jugular vein with the crop lying beneath; (B) the spleen can be seen below the proventriculus, the dark-colored gall bladder lies alongside; (C) cecal tonsils are found at the proximal ends of the two blind caeca, close to the ileo-cecal junction; (D) the ovoid bursa of Fabricius is situated within the pelvic girdle immediately dorsal to the cloaca. From: Dr. Sven Reese, Institute for Anatomy, Histology and Embryology, Faculty of Veterinary Medicine, LMU Munich.

rejected on the grounds that further elucidation of the mechanisms was necessary before the article could be accepted for publication [10]. The article was subsequently submitted to *Poultry Science* [11], where, for a time, it failed to draw much attention. Several years passed before the significance of the work was properly appreciated and mainstream immunologists took an interest in the chicken's immune system. It was eventually concluded that the avian bursa must be essential for antibody-mediated immunity, whereas the thymus, which also undergoes involution during sexual development, is necessary for cell-mediated immunity [12–14]. Almost a decade after Glick and Chang's initial observations [11], Cooper et al. [15]

published their seminal paper on the delineation of the bursal (B) and thymic (T) lymphoid systems in the chicken. These workers proposed that because of the similarities in the lymphoid tissues and immune systems of birds and mammals, a mammalian equivalent for the bursa of Fabricius must exist and provide a source of B-dependent lymphocytes to make antibodies. Later this bursa equivalent was identified as bone marrow. The division of the adaptive immune system into B- and T-dependent compartments has remained a central tenet of immunological thinking ever since. The term B-lymphocyte is derived from "bursa-derived lymphocyte" in honor of that peculiar avian lymphoid structure which provided the original evidence.



FIGURE 1.2 Hieronymus Fabricius of Aquapendente, Professor of Surgery at the University of Padua from 1565–1613. Fabricius was the first to describe the bursa, which is now known to be a primary lymphoid organ, attached by a duct to the proctodeum in birds and essential for the development of bursal-derived (B) lymphocytes and the avian antibody repertoire.

1.3.1 Gene conversion and the bursa

Antibodies recognize specific conformational molecular shapes on their targets through the immunoglobulin (Ig) molecule variable region. Antigenic shapes are legion so an immunocompetent individual must be capable of generating an antibody repertoire with a huge number of Ig specificities [16]. Different B cells produce Ig molecules of different specificities, but each B cell is capable of producing only one Ig specificity. In man and mouse, the antibody repertoire of B cells is generated by a process known as Ig gene rearrangement, which is ongoing throughout life. In the case of the Ig light chain, genes that encode the variable region (V_L gene segment), a joining region (J_L gene segment), and the constant region (C_L gene segment) are spatially separated by noncoding segments in germline DNA. Noncoding segments are spliced to allow the joining of V_LJ_L regions in genomic DNA, while excision of the noncoding section in the RNA transcript allows the $V_LJ_LC_L$ regions to combine permitting translation of a functional Ig light chain molecule [17]. In the case of the Ig heavy chain, a further diversity (D) gene is involved, making a V_HDJ_H rearrangement, otherwise the process is similar. Since multiple copies of V, J, and D genes exist in the mammalian Ig locus (1) random recombination of the different gene segments, (2) different combinations of heavy and light chains paired

together; diversity introduced at the joints between the different gene segments by the variable addition and subtraction of nucleotides and, finally, (3) point mutations introduced into the rearranged sequence diversifying these region further (somatic hypermutation), allow for a vast ($\sim 10^{11}$) antibody repertoire to be generated [16].

In contrast, the cluster of genes encoding the chicken Ig light chain has only a single copy of the functional V_L and J_L genes [18]. Hence, diversity due to V_LJ_L joining is very limited and the effects of VJ rearrangement are minimal (a detailed description can be found in Chapter 4). Likewise, with the Ig heavy chain locus, the presence of single functional V_H and J_H genes means that little diversity can be generated through V_HDJ_H rearrangement. However, clusters of pseudogenes, upstream of the heavy and light chain Ig loci have a critical role in the generation of chicken antibody diversity. By a process known as somatic gene conversion, V_L and V_H sequences are replaced with pseudogene sequences. An enormous amount of diversity is generated by substantial diversity in the hypervariable regions of the donor V pseudogenes and somatic gene conversion events accumulate within single functional V_L or V_H genes. Perhaps, chickens represent the extreme situation with only one functional V_L gene, while other species such as the duck have up to four functional V_L genes, although they still use gene conversion to introduce variability. It seems that, uniquely, birds rely on somatic gene conversion for generating their antibody repertoire, which is the equal of that in immunocompetent mammals. Interestingly, it has recently been shown that if gene conversion is blocked in chicken B cells then somatic hypermutation occurs instead [19]. It has also been observed that gene conversion is not just limited to birds, it also occurs in rabbits [20] pigs, and other mammalian species, though none appear to rely on it as the exclusive means for generating the antibody repertoire.

The striking fact about avian somatic gene conversion is that it only occurs in the bursa of Fabricius (see Chapter 4). For instance, if the bursa is destroyed early in development (60 hours), then those chicks that hatch produce only nonspecific IgM and are unable to mount specific antibody responses. In other words, they do not have an antibody repertoire and are incapable of eliciting typical responses or isotype switching to produce IgG or IgA. However, if the bursa is removed much later during embryonic development, but before 18 days when the B lymphocytes have begun to migrate from the bursa into the peripheral lymphoid tissues, then the hatched chicks lack circulating Ig and are incapable of eliciting specific antibody responses. We know that prebursal stem cells enter the bursa between 8 and 14 days incubation and have already undergone gene Ig rearrangement, probably in the embryonic spleen and bone marrow, for they

express IgM on the surface (see Chapter 4). Within the bursa, they undergo rapid rounds of cell division and only within this unique environment gene conversion occurs. In the absence of the bursa, an antibody repertoire cannot be generated, and a major arm of the immune system becomes nonfunctional. The chicken antibody repertoire is generated during the late embryonic stage and for a short period after hatching. As the chick ages, its B cells undergo additional rounds of somatic gene conversion and the antibody repertoire becomes expanded until a mature repertoire is achieved around 5–7 weeks when the bursa is fully mature. Thereafter, the bursa begins to regress as sexual maturity approaches and the adult probably relies on postbursal stem cells in the bone marrow as the source of B cells.

Of course, generating the antibody repertoire in a burst of activity in the young animal has its risks. Any pathogen that targets and destroys bursal cells will have a devastating effect on antibody-dependent immune responses. One such virus is the small RNA virus that causes infectious bursal disease. Infection of the neonate chick with infectious bursal disease virus (IBDV) may cause no clinical disease but destroys bursal B cells leaving the chick incapable of mounting antibody responses to other pathogens, although paradoxically there is a good response to IBDV itself (see Chapter 14). The insidious nature of IBDV leaves the chick vulnerable to opportunistic infections and unprotected by subsequent vaccinations. So, relying on the generation of the antibody repertoire in a single location and over a relatively short time span is not without danger and, perhaps, represents one of the more “risky” strategies birds have adopted.

1.4 The contribution of the chicken MHC

Pathogens are diverse, cunning, and occupy different niches within the body of the host. Apart from pathogens that are found outside of cells, such as *Clostridium*, *Escherichia coli*, and *Bordetella*, there are intracellular pathogens that can be found in the cytoplasm or cellular vesicles. In addition, retroviruses and herpesviruses integrate into the host’s own genome. To match this diversity, and the different locations where pathogens are found, higher vertebrates have evolved a number of different innate and adaptive immunological mechanisms to improve the chances of survival. Antibodies recognize conformational epitopes on the pathogen’s molecules but need to come into direct physical contact to neutralize a pathogen, as well as recruit cells and other molecules that bring about disposal. Ig molecules are large and cannot easily enter a viable cell, so an intracellular pathogen cannot be recognized by an Ig molecule unless the pathogen’s molecules are expressed on the surface of that infected cells. However, the cellular immune system has

evolved a number of methods for recognizing and destroying cells with intracellular pathogens. These mechanisms allow the host’s effector lymphocytes to recognize infected or neoplastic cells through the expression of proteins that have been proteolyzed within the cell and are expressed on the cell surface as peptide fragments. In mammals, the molecules that present peptides on the surface of cells are encoded by a highly polymorphic genetic region known as the MHC.

The MHC region was originally recognized through its effects on tissue graft rejection and, of course, GvHR. Two major types or classes of MHC molecules are encoded by genes in the mammalian MHC region and expressed on the surface of cells, class I and II MHC molecules (more fully described in Chapter 7). Although both types of molecules are heterodimers: the class I MHC molecule consists of an alpha-chain encoded by the MHC together with an invariant β_2 -microglobulin molecule from a gene outside of the MHC locus; the class II MHC molecule consists of two peptide chains (α and β) whose genes are found in the MHC region. Unlike the MHC class I heterodimer that is present on most types of cells, expression of class II MHC molecules is restricted to antigen-presenting cells such as macrophages, dendritic, and B cells. During synthesis inside the cell, both classes of MHC molecules trap peptide fragments in a cleft on, what is to become, the outer surface of the extracellular domain of the MHC heterodimer. On reaching the cell surface, these peptides are displayed as peptide:MHC complexes to signal T cells through their T-cell receptors. Since even the smallest virus produces a number of proteins, and large pathogens can produce hundreds, there needs to be a large repertoire of T-cell receptors expressed by different T cells to recognize the multiplicity of peptide fragments derived, not from the host’s own cells, but made by pathogens or neoplastic cells. This T-cell repertoire is developed within the thymus (described in Chapter 6), where developing T cells with receptors that recognize self-peptides associated with MHC molecules are eliminated, or inactivated, before they mature to prevent self-recognition and auto-immunity. Mature T cells capable of recognizing “foreign” peptides are released from the thymus into the periphery and become activated if they recognize peptide fragments expressed on MHC molecules. Interestingly, the T-cell repertoire in the chicken is developed in the thymus in a similar way to that of mammals. There is no evidence for a somatic gene conversion mechanism such as occurs in the avian bursa.

In mammals, the MHC is a large and complex region that contains much redundancy [21]. In the human, it consists of about 4 million base pairs encoding at least 280 genes. Separate regions contain several MHC class I and class II genes (there are a vast number of alleles) that are

highly expressed on cells. These regions are separated by the third region that encodes immune response genes (class III). Humans express two or three class I molecules and three or four class II molecules that are highly polymorphic. The high polymorphism is probably driven by the ever-changing variations in pathogens [22,23], although different haplotypes appear to confer approximately the same degree of protection against most of the infectious pathogens. In Chapter 7, Kaufman points out that the associations between the human MHC and infectious disease are, actually, very slight.

The chicken MHC is known as the B locus since it was first identified as a serological blood group locus [24] encoding the polymorphic, and highly immunogenic BG antigen. This BG antigen is highly expressed on blood cells and has no known mammalian equivalent. Later, it was shown that the B locus must constitute the avian MHC, because of its strong association with cell-mediated immune functions, such as graft rejection, mixed lymphocyte reactions, and GvHR. Remarkably, and in marked contrast to the large mammal MHC, the chicken B locus is minute, spanning only 92 kilobases and encoding 19 genes and making it approximately 20-fold smaller than the human MHC [25]. Only two copies each of class I (BF) and class II β (B/L β) genes are found within the chicken B locus. The marked differences between the chicken MHC and its mammalian counterpart have led Jim Kaufman to argue that the chicken B locus represents a minimal essential MHC that must have evolved after birds and mammals separated some 200 million years ago. Another striking feature of the chicken minimal MHC region is that not only does it affect a number of important cell-mediated immune functions but also determines life or death in response to a number of pathogens [26–28]. In Chapter 7, Jim Kaufman develops the argument that chickens, with a minimal essential MHC, appear to have adopted a completely different strategy to that of mammals whose MHC is large and complex. The close association between the chicken MHC and disease resistance is fascinating and at first sight seems to be a suicidal strategy. However, many lessons can be learned from studying avian immunology and its parallel evolution. By investigating the minimal chicken MHC, light should be thrown on the important interactions between pathogens and the immune system and the relevance of the different evolutionary strategies elucidated.

1.5 Contributions to vaccinology

In any review of the “firsts” credited to avian immunology, tribute needs to be paid to pioneering developments in the practical uses of immunology, in other words the use of vaccination. The modern poultry industry relies

heavily on vaccines to protect against a wide range of different infectious agents. Vaccinations are frequent and begin from the day of hatching or even before. Chickens are immunized with live-attenuated vaccines and killed vaccines delivered by various routes (injection, aerosol spray, drinking water, etc.) in mass-vaccination programs that dwarf such programs in human medicine.

Every biology student knows that Edward Jenner is the founding father of vaccination. Jenner discovered that cowpox pustules, obtained from an infected milkmaid, protected an 8-year-old boy, James Phipps, against the related smallpox virus. However, further developments in vaccination, and indeed the term vaccination, only came into use about a century later, following studies by one of the greatest 19th-century scientists, Louis Pasteur. Here again the chicken had a privileged role and serendipity played its part.

In 1878 Pasteur was investigating chicken cholera, a disease with devastating effects, causing chickens to become anorexic, moribund, and usually leading to their death. Pasteur investigated the causative agent, now known as the *Pasteurella multocida*, and succeeded in growing the bacteria in culture. The story goes that Pasteur’s research was interrupted by a holiday and a culture was left in a flask in the laboratory [29]. Upon resuming his research, Pasteur inoculated chickens with this stale culture. The chickens became sick but then recovered within a few days. We now know that the bacteria had become attenuated and no longer capable of causing mortality. Pasteur did not know this and decided to inject new chickens with a fresh bacterial culture but, unfortunately (or fortunately!), his assistant found that chickens at the local market were in short supply. A number of fresh birds were obtained but those that had recovered from the inoculation with the stale culture needed to be reused. As expected, the new chickens all succumbed to the fresh pathogen and died but those that had recovered from the previous treatment with stale inoculum again recovered. Pasteur realized he had achieved with chicken cholera what Jenner had accomplished with smallpox some 100 years earlier, only in this case he had attenuated (weakened) the pathogen by prolonged storage. He called the attenuated culture a “vaccine” [30] in honor of Edward Jenner and then began a, largely successful, search for similar vaccines against other infectious diseases such as pig erysipelas, sheep anthrax, and rabies. The serendipitous discovery of attenuation was another novel finding made using the chicken. Since then, the development of vaccines has had far-reaching implications for the health and welfare of both humans and domesticated animals. The search for better, more effective vaccines still goes on apace.

Another first in vaccine development was in the control of Marek’s disease (MD) a naturally occurring

neoplastic disease of chickens. MD became the scourge of the poultry industry in the 1950s and 1960s, causing major problems for animal health and welfare and becoming a huge financial burden. Before the introduction of MD vaccines, morbidity and mortality in laying flocks ranged from 0%–60% or greater, with losses of 30% being common [31]. The development of an MD vaccine represents the first example of widespread use of vaccination to protect against a natural form of cancer [32]. Over the years it has been remarkably effective [33], although not without problems.

MD was first described as a neurological disease (polyneuritis) by Josef Marek [34]. The condition caused paralysis of the wings and legs and was associated with mononuclear infiltrations and enlargement of the major nerves. Later it was observed [35,36] that in addition to lesions in the nerves and central nervous system, chickens also developed lymphoid tumors in several visceral tissues (visceral lymphomatosis) such as the ovary, liver, kidneys, adrenal, and muscles. With intensification of poultry production, the acute form of MD became a dominant feature. Although the introduction of MD vaccines in the 1970s controlled the disease, in some countries problems with vaccine breaks have continued to occur with regularity and there is now good evidence that the causative herpesvirus (MDV) has been able to evade vaccine-induced immune responses by evolving to greater virulence. Since the 1980s the response of the industry in some countries has been to introduce more aggressive vaccine strategies, using “hotter” vaccines, such as CVI988, either alone or in combination with other MD vaccines (bivalent or trivalent combinations). The most efficacious current MD vaccine CVI988 is derived from a serotype 1 MDV that is weakly oncogenic in genetically susceptible chickens and this has led some to raise the important question [37]: where do we go if hypervirulent MDV pathotypes evolve that can break through the protection of CVI988?

MDV is not the only example of a poultry virus that has changed in response to the introduction of widespread use of vaccines. More virulent isolates of another lymphotropic virus, IBDV, were isolated in the late 1980s. IBDV is a small double-stranded RNA virus that encodes only five viral proteins. As already pointed out, IBDV targets B lymphocytes in the bursa of young chicks causing no clinical signs in neonates but causing clinical disease and some mortality in older chicks (see earlier). Chicks are protected by maternal antibodies derived via the yolk, but it became clear in the late 1980s that the very virulent IBDV being isolated from outbreaks was capable of causing disease in the presence of high levels of maternal antibodies. The response of the industry has been to introduce more aggressive (hotter) vaccines to protect against the more virulent IBDV strains that have evolved under the

pressure of vaccine use. The risk, however, is that these hotter vaccines could themselves be capable of causing bursal damage and immunosuppression in chicks that are poorly protected by maternal antibodies or have a susceptible genotype. Here again, we have an example of a strategy that is holding at present but may not be sustainable long term. More aggressive vaccines or vaccination regimes cannot be introduced without the risk that the vaccines themselves could be harmful.

These issues have been addressed in epidemiological studies on implications of the use of vaccines on the evolution of pathogen virulence. Researchers [38] were chiefly concerned with the use of different vaccines developed against the malaria parasite and its implications for human populations. Mathematical modeling was carried out based on the premise that most vaccines are imperfect and rarely provide full protection from disease. Using various models, these authors predicted that vaccines designed to reduce the growth and/or toxicity of pathogens actually diminished selection pressure against more virulent pathogens. Consequently, subsequent pathogen evolution may lead to higher levels of intrinsic virulence and hence more severe disease in unvaccinated individuals. Such evolution would tend to erode any population-wide benefits, such that overall mortality could increase with the level of vaccination coverage. Interestingly, the authors found evidence of this phenomenon in the practical problems arising from MD vaccination. Current MD vaccines target viral replication and do not prevent infection with MDV, consistent with mathematical modeling that predicts the evolution of pathogen virulence. Yet again, evidence from work on the chicken has proved to be the pathfinder and pointed to important problems to take account of in vaccine design.

1.5.1 Embryonic (in ovo) vaccination

The problems associated with challenge from virulent MDV when chicks are moved into rearing quarters, the vast numbers of chicks requiring vaccinations at the hatchery, as well as the occasional failures caused by manual vaccination, has led to a search for new ways for mass vaccination that can be done at an even earlier stage than the day-old chick. Sharma and colleagues at the East Lansing Regional Poultry Laboratory, United States demonstrated that chick embryos could be successfully vaccinated against MDV at 17–18 days incubation [39,40]. The automated INOVOJECT system, which allows the automated mass application of vaccines to large numbers of eggs (up to 50,000 eggs per hour, [41]), was developed and has been widely applied in the poultry industries of some countries (for a fuller description see Chapter 18). In the United States, almost all broilers (over 7 billion per year) are vaccinated by this method. In ovo vaccination is

achieved by puncturing a small hole through the blunt end of the egg with an oblique pointed needle then passing down a smaller needle to deliver a small amount (usually 50 μ L) vaccine into the amniotic cavity. Since the amniotic fluid is imbibed prior to hatching the vaccine is then taken up by the embryo. Interestingly, the reasons why in ovo vaccination, applied to such an immunologically immature individual, is so effective remains to be fully explained. Nevertheless, the finding that a higher vertebrate can be protected against challenge early after hatching (birth) by vaccinating the embryo is quite revolutionary and its practical application in mass vaccination a great achievement. The immunological explanations will surely come in due course

1.6 Conclusion

Compared to those of mouse and man the avian immune system may seem like a poor relation and yet it has provided important insights into fundamental immunological mechanisms and can claim a number of important “firsts” especially with regard to vaccinology. From the immunological viewpoint, the chicken is, perhaps, the best-studied nonmammalian species. The publication of the chicken genome [42] and recent developments in reverse genetic technology (see Chapter 21) provide the opportunity for a “quantum shift” in the search for new chicken genes and exciting possibilities for developing new tools and reagents to study immune responses and immunogenetics. Interest in studying immune responses of other avian species is increasing, including species of wild birds. Ecologists are now taking an interest in measuring immunocompetence and determining its importance as a heritable trait for the survival, both of the individual and the population. Avian immunology is a fascinating and growing field and will surely provide new and exciting insights for mainstream immunology in the future.

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Structure of the avian lymphoid system

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2.1 Introduction

Understanding the physiology and immunology of the lymphoid system is handicapped without knowledge of its basic structure. Lymphomyeloid tissues develop from epithelial (bursa of Fabricius and thymus) or mesenchymal (spleen, lymph nodes, bone marrow) anlagen, which are colonized by blood-borne hemopoietic cells. In the case of central lymphoid organs, hematopoietic stem cells enter the thymic or bursal anlagen and develop to become immunologically competent T and B cells, respectively. Hence, T and B cells are of extrinsic origin, as proposed by Moore and Owen [1,2].

Immunologically mature cells enter the circulation and colonize the peripheral lymphoid organs: spleen; Harderian gland; and gut-, bronchus-, and skin-associated lymphoid tissues. This peripheralization process can be experimentally manipulated by surgical or chemical interventions. Homing of lymphocytes may take place through the high endothelial venules (HEV), which are located in the T-dependent zones of the peripheral lymphoid organs. In these organs, the T and B cells occupy different compartments, called T- and B-dependent zones, respectively. In the avian spleen, the T-dependent zone—called the periarterial lymphatic sheath (PALS)—surrounds the splenic central artery. In other lymphoid organs, this is not well defined, and the interfollicular region is regarded as a T-dependent area. B-cell compartments are the germinal centers (GCs). In addition to the GC, the periellipsoidal white pulp (PWP) of the spleen is also a B-dependent region, like the marginal zone of the mammalian spleen. Possibly, this B-cell population of the PWP is responsible for antibody production against bacterial capsular antigens (e.g., *Pneumococcus*). Splenectomy of the pigeon abrogates the antibody response, and the birds die soon after *Pneumococcus* infection [3].

The splenic anlage develops from the dorsal mesogastrium, ventral to the notochord, and its ablation at

the lower thoracic level abrogates splenic development, suggesting a notochordal inductive effect. Hematopoietic colonization of the splenic anlage starts at around 6.5 days of incubation, identical to that of the thymus. It is worth mentioning that, in the spleen, not only the cells of the hematopoietic compartment but also other resident cells, such as the ellipsoid-associated cells (phagocytic) and supporting cells of the ellipsoid (capable of collagen production), are of blood-borne origin [4,5].

Several peripheral or secondary lymphoid tissues can be distinguished in the chicken, most notably the spleen and all mucosa-associated tissues, such as the eye-associated lymphoid tissues (the Harderian gland (HG) and the conjunctiva-associated lymphoid tissue) of the lower eyelid; the nasal-, bronchus-, genital- and gut-associated lymphoid tissues (esophageal and pyloric tonsils, Peyer's patches (PP), cecal tonsils, and Meckel's diverticulum); and the skin- and pineal-associated lymphoid tissues.

Secondary lymphoid organs begin to develop in preselected sites before hatching. Their formation requires the so-called lymphoid tissue inducer cells (Lti), which have been identified in the mouse and the human [6] but not yet in the chicken. Further maturation of the lymphoid tissues is antigen driven, as can be demonstrated in germ-free chickens [7].

Lymphoid organs are highly compartmentalized. Each anatomical compartment has its own arrangement of Lti cells in B cell- and T cell-dependent areas. The different types of nonlymphoid cells create a specific microenvironment in each of the compartments. Separate areas exist where antigen presentation to T cells by nonlymphoid cells occurs, T cells interact with B cells, and immunoglobulin production takes place. It is therefore important to realize that in vitro studies generate information on the possible interactions between cells, though some of these

interactions are prevented in vivo because of compartmentalization. Appreciation of the detailed structure of an organ is essential for complete understanding of its functioning.

The development of secondary lymphoid organs begins when T and B cells intermingle and then separate into distinct T and B cell areas, followed by the induction of GCs in T cell areas. With separation into T and B cell areas, nonlymphoid cell populations develop at their characteristic sites.

Nonlymphoid cells can be classified into two groups. The first consists of epithelial cells, endothelial cells and connective tissue cells, including reticular cells. Endothelial cells form the inner lining of arteries, veins and capillaries, and lymphocytes cross this endothelium to enter lymphoid tissue. Specialized vessels with high endothelial cells that bulge into the lumen are the so-called HEV, which facilitate the entry of lymphocytes using specific adhesion molecules that interact with tissue-specific homing receptors on lymphocytes. These endothelial cells have a high number of mitochondria and ribosomes, suggesting an increased state of activity which may be related to their function, transporting both cells and antigens. Reticular cells and their extracellular matrix form the reticulum, the basic framework of lymphoid tissues. Little is known about the reticular cells, but they could have direct involvement in the regulation of immune functions by guiding the migration and anchorage of lymphocytes to their respective compartments. Reticular cells contain glycoproteins for which many lymphocytes express adhesion molecules.

The second group consists of macrophages [8] and antigen-presenting dendritic cells (DC), which belong to the mononuclear phagocyte system. This system includes all cells derived from monoblasts in the bone marrow. This group of cells is described in detail in [Chapter 8.1](#).

2.2 The thymus

2.2.1 Anatomy and histological organization

The avian thymus lies parallel to the vagus nerve and internal jugular veins [9]. On each side of the neck are 7–8 separate lobes, extending from the third cervical vertebra to the upper thoracic segments [10]. Each lobe is encapsulated with a fine fibrous connective tissue capsule and embedded in adipose tissue (refer to [Chapter 1](#), Fig. 1.1). From the capsule, septae invade the thymic parenchyma and incompletely divide the lobe into lobules. The thymic lobes reach a maximum size of 6–12 mm in diameter by 3–4 months of age, before physiological involution begins [11]. The parenchyma of each lobe consists of a centrally located uniform medulla surrounded by a lobulated cortex. Septae end at the

corticomedullary border, leaving the medulla undivided ([Fig. 2.1A](#) and [B](#) and [Fig. 1.1](#)). Arteries travel in the septum, entering the thymic parenchyma at the end. Besides the arteries, capillaries, veins, and efferent lymphatics are

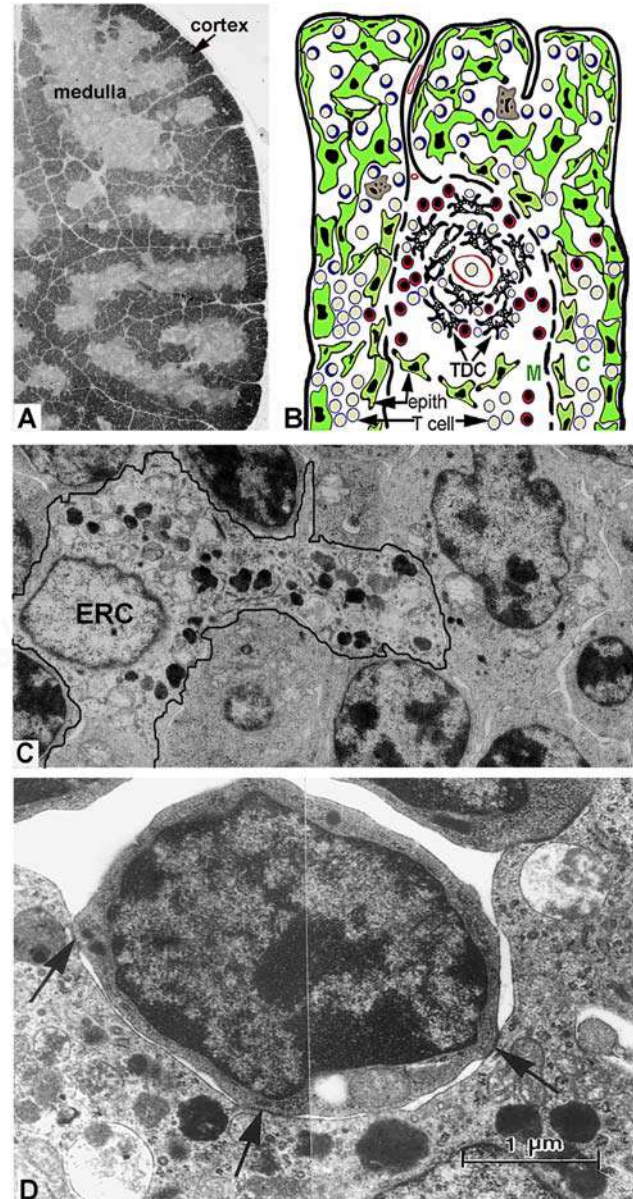


FIGURE 2.1 The detailed structure of the thymus. (A) Hematoxylin-eosin-stained thymic lobe. The undivided pale-stained medulla is surrounded by lobulated heavily stained cortical lobules. (B) Schematic illustration of the avian thymus. Thymic dendritic cells are located in the epithelial-free zone of the thymic medulla, concentrated around the blood vessels. (C) The densely packed cortical thymocytes result in basophilic staining. The large, pale nuclei belong to the epithelial reticular cell (ERC) (outlined). Granules show different electron densities, and the membrane-bound vesicles contain a fine flocculated substance. (D) Attachment spots between the cortical thymocytes and the ERC. *epith*, Epithelial reticular cells, *M*, medulla, *C*, cortex. (B) Adapted from Nagy et al., *Dev Comp Immunol*; 2016. 58: 47–59

also found. Septae have a rich cellular composition consisting of fibroblasts, plasma cells, lymphocytes, and, occasionally, a few granulocytes. The surface of the lobules is isolated from the capsule and septae by a basal lamina (BL). The connective tissue of the thymic capsule and septae develop from the cells of the cranial neural crest. Lack, or impaired migration, of neural crest cells to the third and fourth brachial arches results in a thymic condition such as Di George syndrome in humans and nude mice.

During embryonic development, the thymic mass gradually increases with the colonization of hematopoietic stem cells, and this rapid increase, a few days before hatching, results in the appearance of the medulla. During the formation of the thymus, invasion of the connective tissue septae results in cortical lobulation, which coincides with the emigration of T cells from the thymus. The thymic reticulum develops from the endoderm of the third and fourth branchial pouches [12]. In the epithelial anlage of the thymus, immature T lymphocytes proliferate in the subcapsular zone, from where they migrate toward the medulla during T-cell maturation. Immunologically competent cells leave the thymus via medullary postcapillaries.

2.2.2 Thymic cortex

Pale-stained, fine cytokeratin meshes of the epithelial reticular cells (ERC) in the cortex are densely packed with thymocytes, contributing to cortical basophilia (Fig. 2.1A and B). Moderate numbers of macrophages also occur in the network of cortical epithelial cells. Large- and medium-sized lymphocytes are situated under the surface epithelial cells, which may proliferate, suggesting that the subcapsular zone of the cortex is the major site of proliferation. During T-cell maturation, the cells migrate toward the corticomedullary border, where macrophages and thymic DC sentinel and select the thymocytes before these enter the medulla and circulation via the medullary postcapillaries.

The fine cytokeratin network of the cortex is formed by the cortical ERC (Fig. 2.1C). Two types of ERC can be identified, though it is difficult to distinguish between them cytologically. One is located on the surface of the lobe and contains few, or no, cytoplasmic granules, but expresses more cytokeratin than cortical ERC. Possibly, its major function is to isolate the cortical parenchyma from the mesenchyme [13,14], which surrounds the thymic lobe (Fig. 2.1B) and participates in the cortical blood–thymus barrier. These cells are connected by desmosomes to one another and to the cortical ERC. The latter cells have large, irregular-shaped nuclei with evenly dispersed chromatin substances (Fig. 2.1C). The cytoplasm is rich in mitochondria and loaded with granules,

which exhibit variations in shape and electron density (Fig. 2.1D). The ends of the cytoplasmic processes contain moderately developed smooth- and rough-surfaced endoplasmic reticulum. DEC205 (CD205) is expressed by the cytokeratin⁺ reticular epithelial cells of the cortex. Their relationship to the cortical thymocytes is a remarkable cytological phenomenon. Between each epithelial cell and thymocyte, several adhesion points occur, indicating that cell-to-cell contact may be significant in T-cell maturation (Fig. 2.1D), which suggests a nursing function for ERC [15]. Several granules contain very loose, low-density material, suggesting partial release of their granular contents (Fig. 2.1D) and thus giving the appearance of vesicles. This type of cell occurs exclusively in the cortex. Macrophages, identified by the CVI-ChNL-74.2 and KUL01 (MRC1L-B) monoclonal antibodies [16], are scattered over the cortex and medulla (Fig. 2.2F). The cortex is loaded with CD8⁺ cells, while the medulla has far fewer CD8⁺ cells (Fig. 2.2D).

2.2.3 Thymic medulla

The large number of epithelial and highly variable epithelial-like cells and the smaller number of thymocytes in the medulla result in less basophilic staining (Fig. 2.1A). Epithelial cells can be classified as several types, but it is not known whether they represent actual cell types or stages of differentiation. The functional significance of epithelial cells is not known, but an endocrine function has been suggested [17,18]. The two medullary regions can be distinguished in sections stained with anticytokeratin (Fig. 2.2A). Over the medulla, rough cytokeratin positive “cords” form a 3D network, which initiates at the corticomedullary border joining to the fine cortical cytokeratin⁺ meshwork. The larger part of the medulla among the cords is keratin-free and shows direct connection with the connective tissue of the interlobular septae. This may indicate a common developmental origin for the medulla and the connective tissue of the thymic capsule and interlobular septae. The blood vessels of the medulla locate in the keratin-free regions, and around the postcapillaries of the medulla, the CVI-ChNL-74.3⁺ DC are localized (Figs. 2.1B and 2.2C) [13].

The classical histological structure in the thymic medulla is Hassall’s corpuscle, an epithelial cell aggregation (Fig. 2.2B). In chickens, Hassall’s corpuscles are small, poorly developed structures, unlike their human counterparts. Their origin remains an enigma, although it has been proposed that they are the result of the turnover of epithelial cells since the center frequently shows keratinization like that of the skin epidermis. Some regard Hassall’s corpuscles as a place of thymocyte degeneration, though thymocytes are rarely observed in them, unlike neutrophils (mammals) or heterophils (birds).

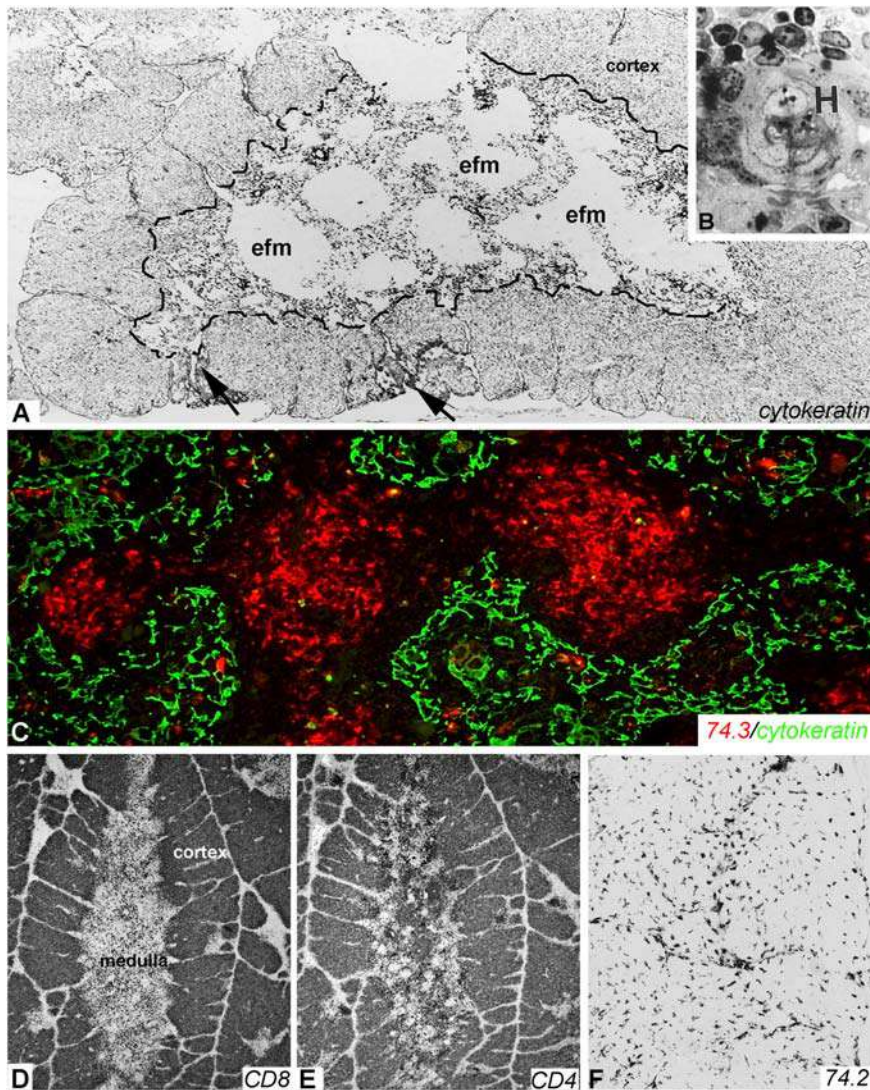


FIGURE 2.2 The detailed structure of the thymus. (A) Pan-cytokeratin immunostaining shows a very fine network in the cortex, while the space of the septae (arrow) is continuous with the epithelium-free medullary (efm) region. The medullary epithelium has a rough cytokeratin network. (B) Hassall's body (H) shows a concentric appearance. (C) 74.3 antigen is expressed by thymic dendritic cells (red) located in the reticular epithelial-free zone (green) of the medulla. (D) The pattern of the $CD8\alpha^+$ cytotoxic T cells in the thymus. (E) $CD4^+$ helper T cells in the thymus. (F) The CVI-ChNL-74.2 monoclonal antibody shows uniformly scattered macrophages over the cortex and medulla.

These granulocytes are probably scavenger cells, eliminating Hassall's corpuscles. Over the past quarter-century, several researchers have reported the production of biologically active substances, such as alpha-naphthylesterase and leucinaminopeptidase [19], interleukin (IL)-7 [20], transforming growth factor- α [21], CD30 ligand [22] and an IL-7-like cytokine called thymic stromal lymphopoietin (TSLP) [23,24]. TSLP is produced by Hassall's corpuscles in the human thymus, and TSLP receptors are preferentially expressed by immature medullary myeloid DC. TSLP activates myeloid DC, which generates regulatory T cells in the thymic medulla [24]. The high-affinity receptor for TSLP and the IL-7 receptor (IL-7R) share the IL-7R α chain. The chicken IL-7R α chain is expressed on many cortical thymocytes (less so toward the capsule) and some medullary cells, suggesting a T cell maturation-dependent expression [25] similar to that of the mammalian thymus [26]. The location and

pattern of the $CD8^+$ and $CD4^+$ cells of the thymus are shown in Fig. 2.2D and E.

Skeletal muscle cells are a common feature in the thymic medulla (Fig. 2.3A). Some are round or ovoid in shape with striated myofibrils encircling the nucleus [27]. Others are elongated with their ends appear Y-shaped. There are no signs of innervation to these skeletal muscles.

Thymic corticomedullary border

The corticomedullary border contains a DC barrier which expresses the major histocompatibility class (MHC) II antigen and may play a role in the negative selection of T cells [28]. However, our observations, based on immunocytochemistry with anti-MHC II and CVI-ChNL-74.3, indicate that the thymic DC accumulate in the cytokeratin-free medullary region (Fig. 2.2C) [13]. The CVI-ChNL-74.3 $^+$ thymic DC coexpress CD83 [14,29]. In

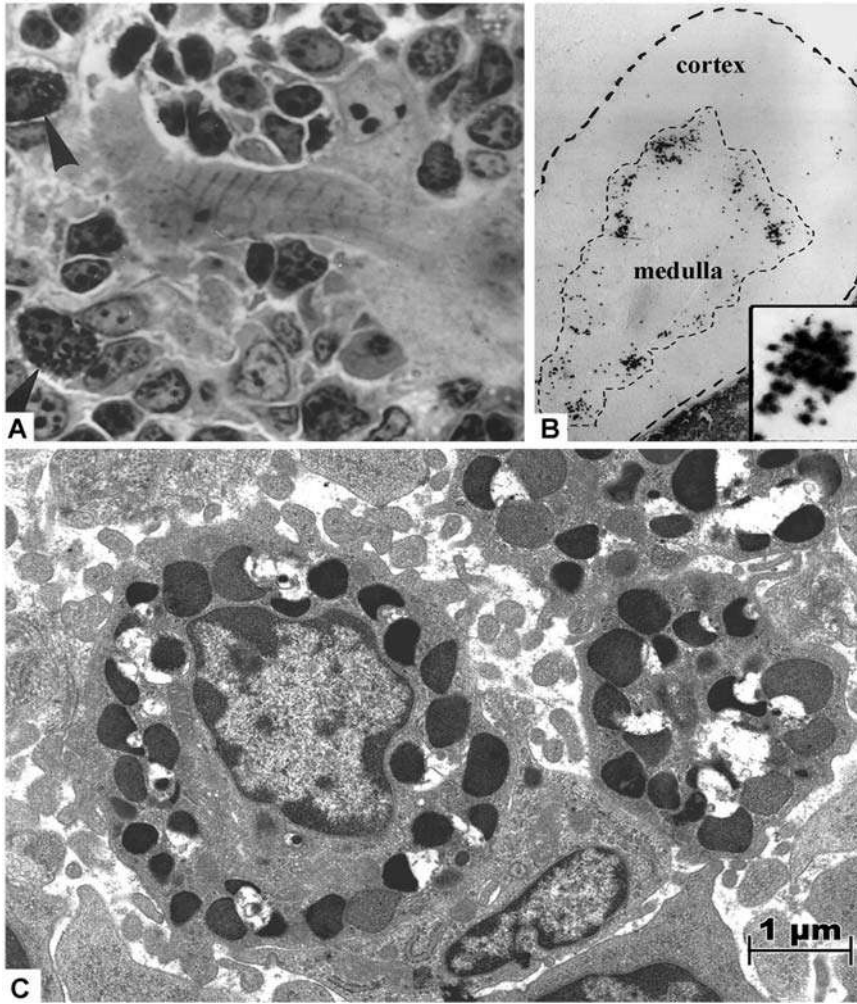


FIGURE 2.3 The detailed structure of the thymus. (A) A skeletal muscle cell (myoid) in the medulla shows regular striations (I and A bands of the sarcomere). Peroxidase-positive cells (arrow). (B) At the corticomedullary border, there is a peroxidase⁺ cell barrier. The lobe and the corticomedullary border are outlined. The peroxidase⁺ cells form small groups, suggesting their clonal origin (inset). (C) An electron micrograph of the peroxidase-positive cell. The irregularly shaped nucleus is surrounded by granules. Many of them show granulolysis.

addition to thymic DC, a large number of peroxidase-positive cells occur at the corticomedullary border and may also contribute to the negative selection of T cells [30]. These endogenous peroxidase-positive cells generally form groups, and single cells occur between them (Fig. 2.3B). Their irregularly shaped granules show granulolysis, and the cell surface is highly ruffled (Fig. 2.3C). Peroxidase-positive cells do not have segmented nuclei, and the ultrastructure of their cytoplasmic granules is not comparable to that of eosinophilic granulocytes. Granulocyte-specific mAbs (Grl-1 and Grl-2) do not recognize these thymic peroxidase-positive cells.

2.3 The bursa of Fabricius

2.3.1 Anatomy and histology

The chicken bursa of Fabricius has the shape and size of a chestnut and is located between the cloaca and the sacrum (Fig. 1.1). A slot-like bursal duct provides a continuous and free communication between the proctodeum

and the bursal lumen. As a diverticulum of the cloaca, the bursa is lined with a cylindrical epithelium thought to be of endodermal origin; however, a recent experimental study proposed an ectodermal origin [31]. The bursa reaches its maximum size at 8–10 weeks of age; then, like the thymus, it undergoes involution. By 6–7 months, most bursae are heavily involuted [11].

The bursa, like other hollow organs, is surrounded by a thick, smooth muscle layer. Studies generally neglect this muscle coat, and its contractility is not considered in bursal function. During muscle contraction, compression of the follicles can promote the flow of cells within the medulla and contribute to the emptying of lymphatics situated in the axis of the folds. In the bursal lumen, 15–20 longitudinal folds emerge, resulting in a slit-like space. During muscle contraction, the surfaces of the folds come into contact with one another, so the bursal lumen is almost a virtual space. Inside each fold, follicles are organized into two layers separated by axial structures (arteries, veins, lymphatics, and connective tissues) (Fig. 2.4). Consequently, follicles are in contact with blood and

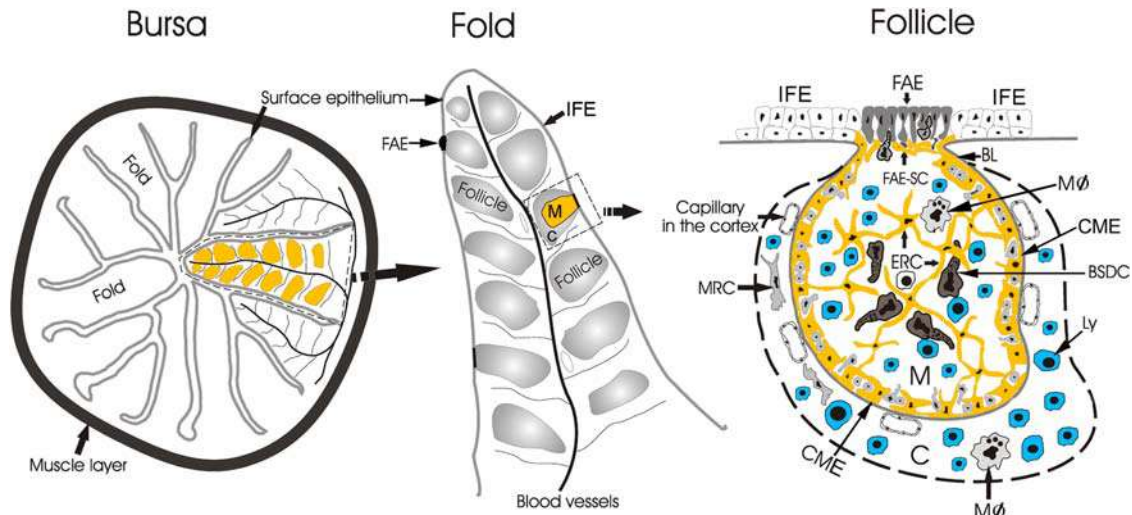


FIGURE 2.4 The structure of the bursa of Fabricius. This schematic of the histological organization of the bursa of Fabricius shows the corticomedullary epithelium (CME) (shaded) in the medulla; the follicle-associated epithelium supportive cell (FAE-SC); the epithelial reticular cell (ERC); the bursal secretory dendritic cell (BSDC); the interfollicular epithelium (IFE). BL, basal lamina; Ly, lymphocyte; MØ, macrophage; M, medulla; C, cortex; MRC, mesenchymal reticular cell in the cortex.

lymphatic vessels as well as the bursal lumen. In one of the ventral folds, peripheral (secondary) lymphoid tissue can form.

2.3.2 Bursal surface epithelium

The surface epithelium of each fold consists of an interfollicular epithelium (IFE) and a follicle-associated epithelium (FAE) [32] that form about 90% and 10% of the surface, respectively (Fig. 2.5A) [33]. IFE is columnar and produces a mucin-like substance, which is released into the bursal lumen and lubricates the surface of the folds. The columnar epithelium is pseudostratified, since there is a layer of cuboidal basal cells with densely stained cytoplasm. Their function is not known. Perhaps these cells are predestined for the IFE, since the surface epithelium does not proliferate and no epithelial stem cell has been identified. The IFE rests on a basement membrane (BL), continuous with the corticomedullary border separating the medulla from the cortex (Figs. 2.4 and 2.5B).

FAE covers the bursal fold above the follicles and provides a direct connection between the follicular medulla and the bursal lumen (Figs. 2.4 and 2.5B). Gamma-actin recognized by GIIF3 monoclonal antibody (Fig. 2.5C) is expressed by guinea fowl bursa of Fabricius FAE [34,35]. Using *CSF1R*-transgenic chickens, Balic et al. showed that FAE comprised a mixture of *CSF1R*⁺ epithelial cells (Fig. 2.5C') and *CSF1R*⁺ macrophage-like cells expressing phosphatidylserine receptor T-cell immunoglobulin and mucin domain-containing 4 antigen (TIM4) [36]. A special smooth-surfaced vesicular system in the apical

zone of the FAE cells may contribute to bidirectional transport by these cells (as proposed by Bockman and Cooper [32]). The luminal or apical surface of the FAE, unlike that of the IFE, is adhesive because *Salmonella* can enter the bursal lumen [36] and attach exclusively to the *CSF1R*⁺ FAE (Fig. 2.5D). Antigen or particles can gain access to the medulla from the bursal lumen [33,37,38], and in the opposite direction products of bursal secretory DC (BSDC) are also taken up by the FAE [34,38]. During late embryonic life, such products appear in large quantities in the medulla and enter the FAE's intercellular space. FAE cells take up and possibly secrete these products into the bursal lumen [39]. Some FAE cells express TIM4, CD44, and nonspecific esterase, like macrophages, which suggests their extrabursal origin. Interestingly, these cells do not stain with typical monocyte/macrophage markers, like CVI-ChNL-68.1, CVI-ChNL-68.2, CVI-ChNL-74.2) or KUL01 (mannose receptor MRC1L-B), and may represent senescent BSDC that have migrated from the medulla [40]. Anti-pan-cytokeratin staining indicates the reduced level or absence of keratin-intermediate filaments in the FAE, although each epithelial medullary component expresses cytokeratin (Fig. 2.5E).

The FAE is an attachment point between the follicle and the surface epithelium, so the number of FAE attachments is identical to the number of follicles (Figs. 2.4 and 2.5F). Under each FAE, the BL is absent and (unusual for an epithelium) replaced by 2–3 layers of squamous epithelial cells, known as FAE-supportive cells (Figs. 2.4 and 2.5D). The FAE cells are connected to the FAE-supportive cells by desmosomes. The flat FAE-supportive cells, together with the corticomedullary epithelial cells,

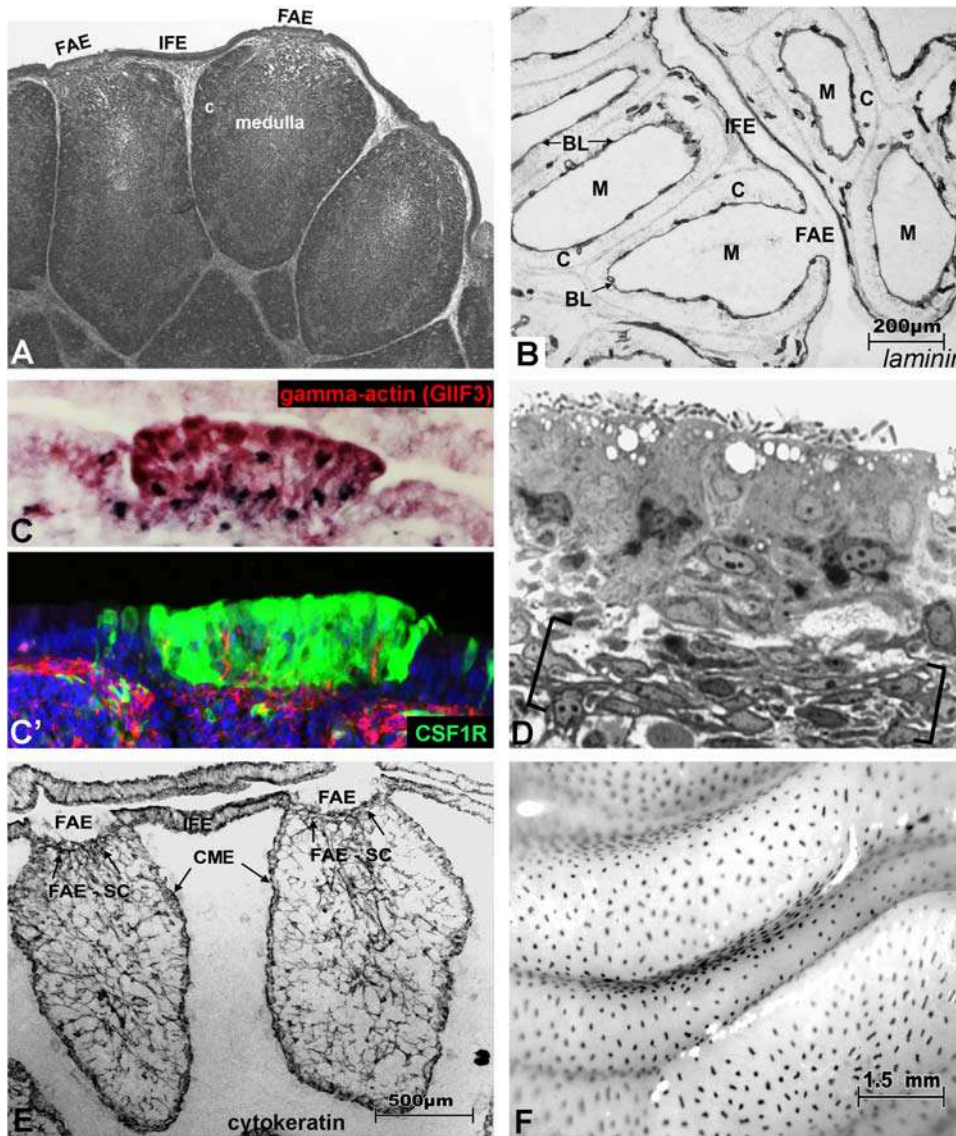


FIGURE 2.5 The structure of the bursa of Fabricius. (A) Hematoxylin-eosin staining indicates the follicle-associated epithelium (FAE) and the interfollicular epithelium (IFE). The FAE is above the medulla. (B) Antilaminin immunostaining indicates that the basal lamina (BL) under the interfollicular epithelium (IFE) continues at the corticomedullary border (arrow). The FAE, the medulla (M), and the cortex (C). (C, C') Gamma-actin⁺ and CSF1R⁺ FAE cells. (D) The FAE sits on top of the follicle and is separated from the IFE by a shallow epithelial trough (bracket). In the FAE, three macrophage-like cells can be seen. Under the FAE, the basal lamina is absent. A large number of bacteria are attached to the FAE. (E) Anticytokeratin staining identifies IFE, FAE-supportive cells (epithelial trough), and ERC. (F) A carbon particle suspension dropped onto the anal lips is sucked up into the bursal lumen. The carbon is absorbed by the FAE, allowing counting of the number of follicles. Each black dot covers one FAE.

envelop the medulla, separating it from the FAE and cortex, respectively. The FAE is devoid of lymphocytes (Fig. 2.5D). However, because classical M cells can stimulate lymphocytes, the absence of any lymphocytes in the FAE questions whether these FAE cells are in fact classical M cells.

Soluble substances dropped onto the cloacal lips are rapidly sucked into the bursal lumen [33,36,38,41,42]. The mechanism of this suction is not known, although it may be due to negative pressure within the bursal lumen. The bursal duct is flat, not circular, and therefore generally closed. Absorption of the air from the bursal lumen can result in negative pressure, with contraction of the cloacal sphincter opening up the bursal duct. By this mechanism, bacteria, viruses, and tracers can gain access into the bursal lumen, to be absorbed by the FAE (Fig. 2.5D and F). The FAE takes up colloidal carbon,

providing a means to count the number of follicles, which is estimated to be 20,000 per bursa. During folliculogenesis, there is no cell migration between follicles. Therefore assuming a minimum of 1 B cell precursor per follicle, this means a minimum of 20,000 pre-B cells are required for bursal colonization [33].

2.3.3 Bursal follicle

Each bursal follicle consists of a medulla and a cortex with a closely associated (both structurally and functionally) but not integral FAE [32]. During bursal ontogeny, the medullary anlage emerges on embryonic day 11–12 (E11–12) and is soon followed by FAE formation (E14–15), with the first cortical cells appearing around hatch [43–45]. The cortex is fully developed by 2 weeks post-hatch (see Chapter 3).

Bursal folds are filled with follicles, which are flattened, oval-shaped and $\sim 0.2\text{--}0.4\text{ mm}$ in diameter [33]. A collagen-rich capsule surrounds each follicle, which represents the structural, functional, and pathological bursal unit. Each follicle has its own blood supply independent of neighboring follicles. Small precapillaries branch from the main artery in the fold and cross the follicular cortex, creating a dense capillary network at the corticomedullary border (Fig. 2.6A). Blood vessels never enter the medulla. A blood–bursa barrier may exist in the medulla, though not in the cortex. Early studies concerning phagocytic capability in the bursa support the existence of a blood–bursa barrier, as the bursa contains no phagocytosed substances [46].

The corticomedullary BL provides a complete separation of the medulla from the cortex (Fig. 2.5B); these two regions are different histologically, ontogenically, and possibly functionally. Supporting cells in the medulla and the cortex are respectively of epithelial and mesenchymal origin.

2.3.4 Medulla

The medulla, like the thymus, is a classical lymphoepithelial tissue. It consists of epithelial cells, which originate from the anal invaginations of ectoderm [31], and blood-borne hematopoietic cells, including lymphoid cells, DC, and perhaps macrophages; few plasma cells occur in the involuting bursa.

2.3.5 Bursal medullary epithelial cells

The ERC form a cellular network in the medulla, and there is no immunocytochemically identifiable extracellular matrix (ECM). This is unlike the cortex, where the desmin⁺ mesenchymal reticular cells (MRC) produce an ECM. Epithelial cells can be classified according to their location and cellular structure. The superficial epithelial cells form a layer on the surface of the medulla. At the corticomedullary border, they form arches on the medullary side and are covered by a BL on the cortical side. Under the FAE, the arch-forming cells become flat and

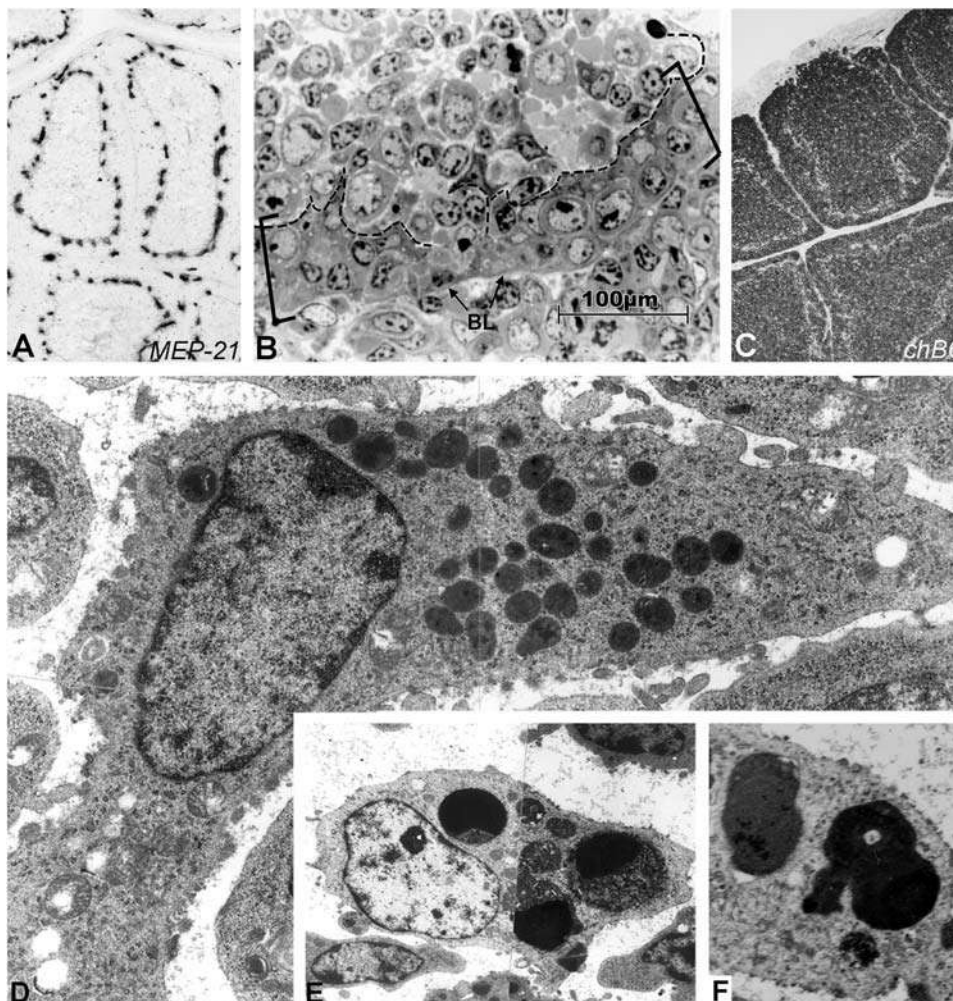


FIGURE 2.6 The structure of the bursa of Fabricius. (A) The MEP-21 (antithrombomucin) monoclonal antibody recognizes thrombocytes and endothelial cells. There is a dense capillary network along the corticomedullary basal lamina in the cortex; there are no blood vessels inside the medulla. (B) Epithelial cells of the corticomedullary border form arches, which are filled with blast-like cells (bracket). The arch opening toward the medulla and blast-like cells join to the medulla. BL, basal lamina; (C) Both medulla and cortex are densely packed with chB6⁺ cells, but the corticomedullary border appears as an unstained, light line. (D) An electron micrograph of the medulla showing that the mature dendritic cell (DC) has an eccentrically located nucleus with a large cell process that is loaded with electron-dense granules. The moderately ruffled cell surface is covered by a fine filamentous substance. (E) A macrophage-like cell in the medulla, loaded with apoptotic lymphocytes in different stages of digestion. (F) A detail of a macrophage-like cell. The surface membrane is covered by a fine flocculated substance like the DC.

serve as supportive cells for the FAE, replacing the BL. Hence, the interior of the medulla is separated from the cortex and FAE. The corticomedullary epithelium enclose CD45⁺ lymphoblast-like cells (Fig. 2.6B), which express neither B cell (e.g., chB6, CXCR4, and CD1) nor BSDC (e.g., vimentin, IgY, CSF1R and 74.3) or macrophage (TIM4, 74.2, 68.2) antigens (Fig. 2.6C). Delta-Notch signaling in the corticomedullary border suggests that the function of the arch-forming cells differs from that of the medullary ERC [47]. These corticomedullary epithelia may have nursing or regulatory function for the hematopoietic cells. Recently, Wu et al. showed that epithelial cells of the corticomedullary border produce macrophage colony-stimulating factor (CSF1) [48]. Ramm et al. [49] suggested that the cells at the corticomedullary junction are resistant to infectious bursal disease virus (IBDV) and represent a macrophage subpopulation. If the blast-like cell population is exhausted after IBDV infection or testosterone treatment, regeneration of the follicle, but not the entire bursa, is handicapped (N. Nagy, unpublished observation).

Medullary epithelial cells have stellate morphology and form a 3D network (Fig. 2.5E) for B cells (Fig. 2.6C), bursal secretory dendritic cells (BSDCs) (Fig. 2.6D), and macrophages (Fig. 2.6E). Epithelial cells are connected by desmosomes to one another and to the cells of the FAE-supportive and corticomedullary arch-forming cells. Their cytological structure is comparable to that of FAE-supportive cells; namely, they are rich in cytokeratin and have poorly developed cytoplasmic organelles, suggesting a supportive rather than secretory function.

2.3.6 Bursal secretory dendritic cells

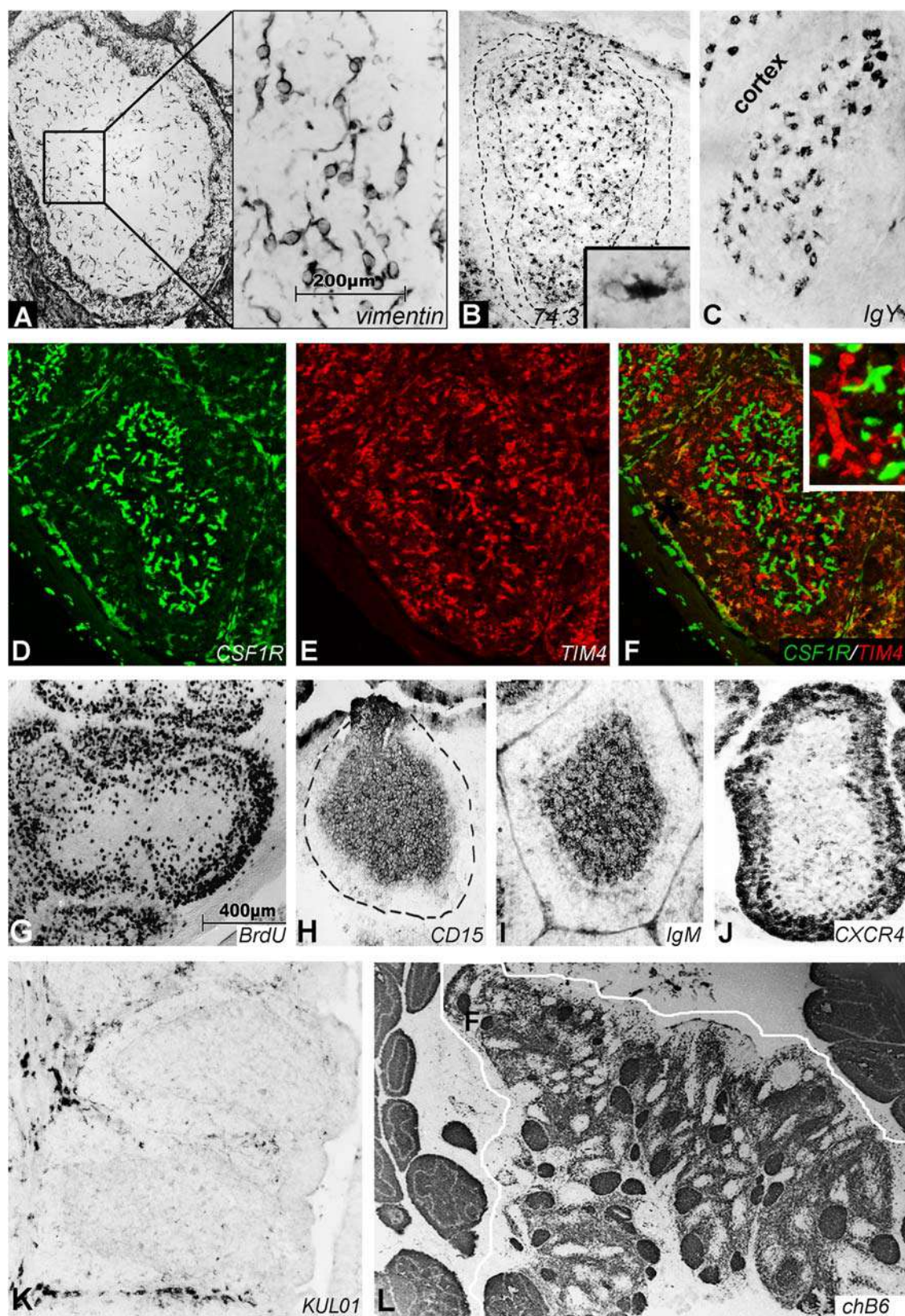
BSDCs, first identified by Oláh and Glick [50,51], are found only in the medulla (Fig. 2.6D). Cyclophosphamide eliminates B cells from both the medulla and cortex but does not affect BSDCs. However, B cell depletion results in aggregation of these cells in the center of follicles. BSDCs have two cellular processes, which are different in size and cytological structure. The smaller one contains a few rough-surfaced endoplasmic reticulum cisternae, free ribosomes, and a large number of vimentin-intermediate filaments (Fig. 2.7A). The larger one contains many membrane-bound electron-dense granules around a well-developed Golgi region, or it lines up along the cell membrane (Fig. 2.5D). The arrangement of the cytoplasmic organelles in the cell gives the mature BSDC a polarized appearance (Figs. 2.6D and 2.7A). The mature BSDC is highly elongated in shape and has a nuclear chromatin structure similar to that of the lymphocyte. Granular contents are released and attach to the outer surface of the cell membrane [34,52,53]. Cell adhesion sites

can be observed between BSDCs and lymphocytes [52]. The senescent BSDC seems to be phagocytic; the cell loses the vimentin-intermediate filament and surface receptor for IgY, but the membrane-bound electron-dense substance is preserved for a while (Fig. 2.6D and E).

The biochemical composition of the granules is not known, but the mAb CVI ChNL-74.3 recognizes intracellular BSDC antigens, possibly granules (Fig. 2.7B) [54]. IBDV infection eliminates the 74.3⁺ staining, suggesting that BSDCs are an IBDV target. The antigen recognized by mAb 74.3 has not been characterized, but double-staining with vimentin clearly indicates colocalization [39]. Surface IgY (sIgY) appears on BSDC just before hatching and persists throughout life, so anti-IgY can also be used for their identification (Fig. 2.7C). IgY appears on the cell membranes of BSDC before hatching and therefore should be of maternal origin. IBDV infection eliminates not only the B cells but also sIgY, confirming the involvement of BSDC in IBDV pathogenesis. Besides IgY, vimentin and 74.3 antigen avian BSDCs express putative CD11c, DEC205, and CSF1R (Fig. 2.7D) in chicken, NIC2, CSF1R in quail, gray partridge, and guinea fowl, respectively [29,34,52,55,56]. These cells show no reaction with the classical avian monocyte/macrophage-specific mAbs (Fig. 2.7D–F), thus, these mAb are convenient tools for monitoring the BSDC's condition [14]. Corticomedullary epithelium promote the proliferation (Fig. 2.7G) of BSDC precursors and B cells (Fig. 2.7H–J), which are nearby, but inhibit their differentiation, suggesting a regulatory function; the ERC allow them to differentiate [57]. At hatching, 5–8 vimentin-positive BSDC per follicle are present, increasing to 65–70 by 4–6 weeks of age.

2.3.7 Bursal macrophages

In histological sections, macrophages with phagocytic substances are easily identified in both the cortex and medulla; however, no positive cells are recognized using mAb against monocyte/macrophage markers, such as CVI-ChNL-74.2, 68.1, 68.2, and KUL01 (Fig. 2.7K). This suggests either that the bursal follicle does not have macrophages or that its macrophage population is unique. Recently, it has been shown that the bursal medullary macrophages express lysosome-specific Lep100 antigen (Lamp-1) and TIM4 [14,36,48]. After IBDV infection, large cells loaded with cellular debris and virus particles appear first in the medulla and later in the cortex, while BSDC cannot be identified using any cell marker. Electron micrographs show a surface substance on several macrophages, comparable to that on BSDC (Fig. 2.6E and F). This finding, together with the MHC class II⁺ macrophage-like cells in the FAE, indicates that the medullary macrophages are possibly senescent BSDC [40].



(Continued)

2.3.8 Bursal lymphocytes

At least 98% of bursal lymphocytes are B cells. Scattered T cells occur in the cortex, but few of them enter the medulla. B cells proliferate in both cortex and medulla (Fig. 2.7G). After 8–10 weeks of age, the number of lymphocytes in the interior of the medulla decreases, indicating the initiation of bursal involution, which is a process completed by 6–7 months of age. A few plasma cells may develop in the cortex and medulla with age, but generally their number remains very low, suggesting some kind of inhibition of B-cell terminal maturation. However, in birds surviving IBDV infection, the bursa has a remarkably large number of plasma cells before fibrosis is complete.

One perennial question remains: Are there any differences between cortical and medullary B cells? Trafficking of bursal cells between the cortex and medulla occurs, and there are some differences in phenotype; however, the functional differences are not fully understood (see Chapter 4 for further discussion). Medullary chB6⁺ B cells express surface IgM [58] and CD15 (Lewis X) [59], but the Ov antigen and CXCR4 appear only on cortical B cells [60] (Fig. 2.7H–J). This makes it difficult to determine the B-cell maturation process. During acute involution of the bursa, caused by IBDV treatment, T lymphocytes accumulate in the follicles. The more severely damaged follicles have more T cells, suggesting a delicate balance between T and B cells. If B cells are destroyed or emigrate from the follicle, T cells can temporarily replace them [61]. The accumulation of T cells in bursal follicles seems to depend on B cell depletion and not just infection.

2.3.9 Cortex

The cortex begins to develop around hatching, and it is separated from the medulla by the BL of the corticomedullary border, its surface is covered by fine collagen-rich capsules. The cortex, unlike the medulla, develops exclusively from the mesoderm. MRC form the supporting cells of the cortex and express vimentin- and desmin-intermediate filaments [62]. The 3D meshwork of the MRC is filled with chB6⁺/CXCR4⁺ B lymphocytes (Fig. 2.7J) and CSF1R⁺/Lep100⁺/TIM4⁺ macrophages. BSDC are lacking in the cortex. Cortical B cells are heterogeneous, chB6⁺ cells are

dispersed uniformly throughout the cortex but only the chB6⁺ B cells located in the outer region of the follicular cortex coexpress CXCR4. It is suggested that B cells have to downregulate CXCR4, express Ov antigen before they can leave the CXCL12-rich environment of the follicle cortex and migrate out to colonize the peripheral lymphoid organs [60]. The ECM is rich in collagen III and fibronectin, indicating intensive cell migration through the cortex. The complete absence of ECM in the medulla means that cell migration is questionable, but the ERC surface serves as a scaffold for it. The cortex has a rich capillary network adjacent to the corticomedullary BL (Fig. 2.6A) and is drained by postcapillaries, which conjoin to form veins in the connective tissue of the fold where the lymphatics emerge.

2.3.10 Peripheral lymphoid tissue of the bursa of Fabricius

Close to the bursal duct, one of the ventral folds produces peripheral (secondary) lymphoid tissue (Fig. 2.7F). This tissue is not likely to be a simple continuation of cloacal lymphoid infiltration because the fold is flat, it is wider than others and its colonization by lymphoid precursors is delayed about 1 day. The peripheral lymphoid tissue consists of GC and an interfollicular dense lymphatic substance that represents B- and T-dependent regions, respectively. This fold may provide communication between the cloacal contents and the bursa of Fabricius [37,63,64].

2.3.11 Germinal center of the peripheral lymphoid organs

Histologically, the GC is defined by aggregates of blast cells which, after antigenic stimulation, occur in defined areas within peripheral tissues. In chickens, two types of GC, fully and partially encapsulated, have been described. Although their cell populations do not differ cytologically, there is speculation that the two types represent functionally different structures [65]. Alternatively, they may represent different stages of GC development [66]. Mature GC are surrounded by a capsule of connective tissue and completely lack blood vessels (Fig. 2.8A, G, and H).

FIGURE 2.7 The structure of the bursa of Fabricius. (A) A vimentin-intermediate filament is expressed by the bursal secretory dendritic cells (BSDC). Inset: magnified from the outlined area. The BSDC generally have two well-developed cell processes, giving them a highly polarized appearance. (B) CVI-ChNL-74.3 mAb recognizes cytoplasmic antigen in the BSDC. The 74.3⁺ cells occur in the medulla, but occasionally they show up in the FAE. (C) The anti-IgY-specific antibody identifies strong membrane-bound IgG immunoreaction on the cell surface of the BSDC. (D–F) Double staining with CSF1R (green) and TIM4 (red) highlights the BSDC in bursal follicles. TIM4 is expressed by the macrophages-like cells of the follicular medulla, but not the CSF1R⁺ BSDC. (G) BrdU incorporation shows that proliferating cells are closely associated with the marginal zone of the medulla and cortex. (H, I) Medullary B cells express CD15 and IgM. (J) In adult bursa only cortical B cells express CXCR4 antigen. (K) The macrophage marker KUL01 does not stain cells in the follicles. MRC1L-B⁺ cells occur only in the interfollicular connective tissue. (L) Between two bursal folds (B) the peripheral lymphoid tissue (outlined) consists of germinal centers and dense lymphoid tissue, as shown by chB6 (Bu-1) antigen immunostaining.

In the spleen, the induction sites of GC seem to be directed by clusters of stromal cells, recognized by the mAb CVI-ChNL-74.3, which are located close to the T cell areas, adjacent arteries and arterioles. During a humoral response, newly developing GC, consisting of bromodeoxyuridine-incorporating proliferating (BrdU⁺) B cells, are located adjacent to clusters of 74.3⁺ cells [67]. During development, the B cells grow around the clusters until they completely surround them. Cells in the middle of the GC do not incorporate BrdU; thus they are not mitotically active and resemble centrocytes, while the outer ring of proliferating cells represents centroblasts (Fig. 2.8B). Although a light zone of centrocytes and a dark zone of centroblasts, as described in mammalian GC, can be less easily discriminated morphologically in the chicken, functional homology seems likely [65,66,68]. Structurally similar to mammalian metallophilic macrophages, a mature GC is surrounded by TIM4⁺ macrophages [69]. In mature GC, 74.3⁺ CSF1R⁺ cells, called follicular dendritic cells (FDC), can trap immune complexes on their surface. These trapped immune complexes are important in memory formation. Specific antibodies or antibody-containing cells are detected in serum and spleen, respectively, before the formation of GC. Therefore the site of GC formation is unrelated to that of the simultaneously induced antigen-specific plasma cells, as reported for the mouse spleen [66,70].

Transmission electron microscopy shows a dense intercellular substance around the FDC. The structure of the FDC's nuclear chromatin resembles that of small lymphocytes, and the cytoplasm contains a variable number of electron-dense granules of various sizes (Fig. 2.8C). The surface membrane of 74.3⁺/CSF1R⁺ FDCs (Fig. 2.8D and E) binds (but does not express) IgY (Fig. 2.8F), and the FDC's cytoplasm contains vimentin-intermediate filaments (Fig. 2.8G). The anti-vimentin-specific antibody and anti-CSF1R are convenient markers to identify avian FDC [14,55,56,62]. In addition to FDC, the GC contains other types of nonlymphoid cells such as supporting reticular cells, which express desmin-intermediate filaments (Fig. 2.8H). Tingible body macrophages are not stained by classical monocyte-macrophage markers, such as 74.2 (Fig. 2.8I), 68.2, and KUL01 (MRC1L-B), but they express TIM4 [69] and lysosomal membrane protein LAMP-1 (Lep100 antibody) (Fig. 2.8J). This may indicate a different developmental stage or a different origin. The tingible body macrophages engulf apoptotic B cells and cell debris. A mature GC consists of proliferating Bu-1⁺ (chB6 antigen) B cells (Fig. 2.8K), a few T cells expressing CD3, CD4, and TCR α V β 1 (Fig. 2.8L), and 74.3⁺ FDC. Arakawa et al. [71] and Igyarto et al. [67] demonstrated that the antigen-activated B cells of the spleen migrate into the GC, where they expand, as proposed for human GC development

[63]. Arakawa et al. [71] estimated the clonal complexity in each GC of the spleen, suggesting that 6–12 B cell clones are expanding, with each producing a different antibody.

Contradictory results concerning Ig expression in GC have been reported. Jeurissen et al. [70] described follicle B cells expressing sIgM but not sIgY. Yasuda et al. [72] isolated individual GC from the spleen and analyzed GC cell populations at the individual cell level. They described considerable differences in sIgM and sIgY expression between GC. Ig-expressing cells were found in the center of the GC but not in the outer rim, where proliferating B cells were found. The relative proportions of sIgM⁺ and sIgY⁺ cells changed after intravenous inoculation of DNP-KLH, with the highest proportion of sIgM⁺ cells detected on day 7. The proportion of sIgY⁺ cells increased during the first 14 days, but, as observed for sIgM⁺ cells, large individual variations were evident. In contrast, the proportion of CD3⁺ cells remained relatively constant at 10%–20% in each GC. Double-staining studies of GC have elucidated the nature of the isolated sIg⁺ cells, where the FDC appeared to possess IgG on their surface [62].

2.4 The spleen

2.4.1 Origin and anatomy

The splenic primordium first appears as a mass of mesenchymal cells in the 48-hour embryo [73]. Sinusoids with erythrocytes appear in the mesenchyme at E5, granulopoiesis begins from E7 and erythropoiesis follows at E11. In contrast to that of mammals, the avian spleen is not considered a reservoir of erythrocytes for rapid release into the circulation [74]. Although not a primary site for lymphocyte antigen-independent differentiation and proliferation, the spleen has an important role in embryonic lymphopoiesis, for it is here, though not solely here, that B cell progenitors undergo rearrangement of their Ig genes before colonizing the bursa of Fabricius [75] (see also Chapter 4). At the time of hatching, the spleen becomes a secondary lymphoid organ which provides an indispensable microenvironment for interaction between lymphoid and nonlymphoid cells [14]. The contribution of the avian spleen to the immune system as a whole may be more important than in mammals because of the poorly developed avian lymphatic vessels and lymph nodes.

The chicken spleen is a round or oval structure lying dorsal to and on the left side of the proventriculus (Fig. 1.1C). One or more small accessory spleens sometimes occur nearby [76]. Major splenic development occurs after hatching, following exposure to antigens [77]. The main blood supply to the spleen is provided by the Arteria lienalis cranialis and caudalis, with some small

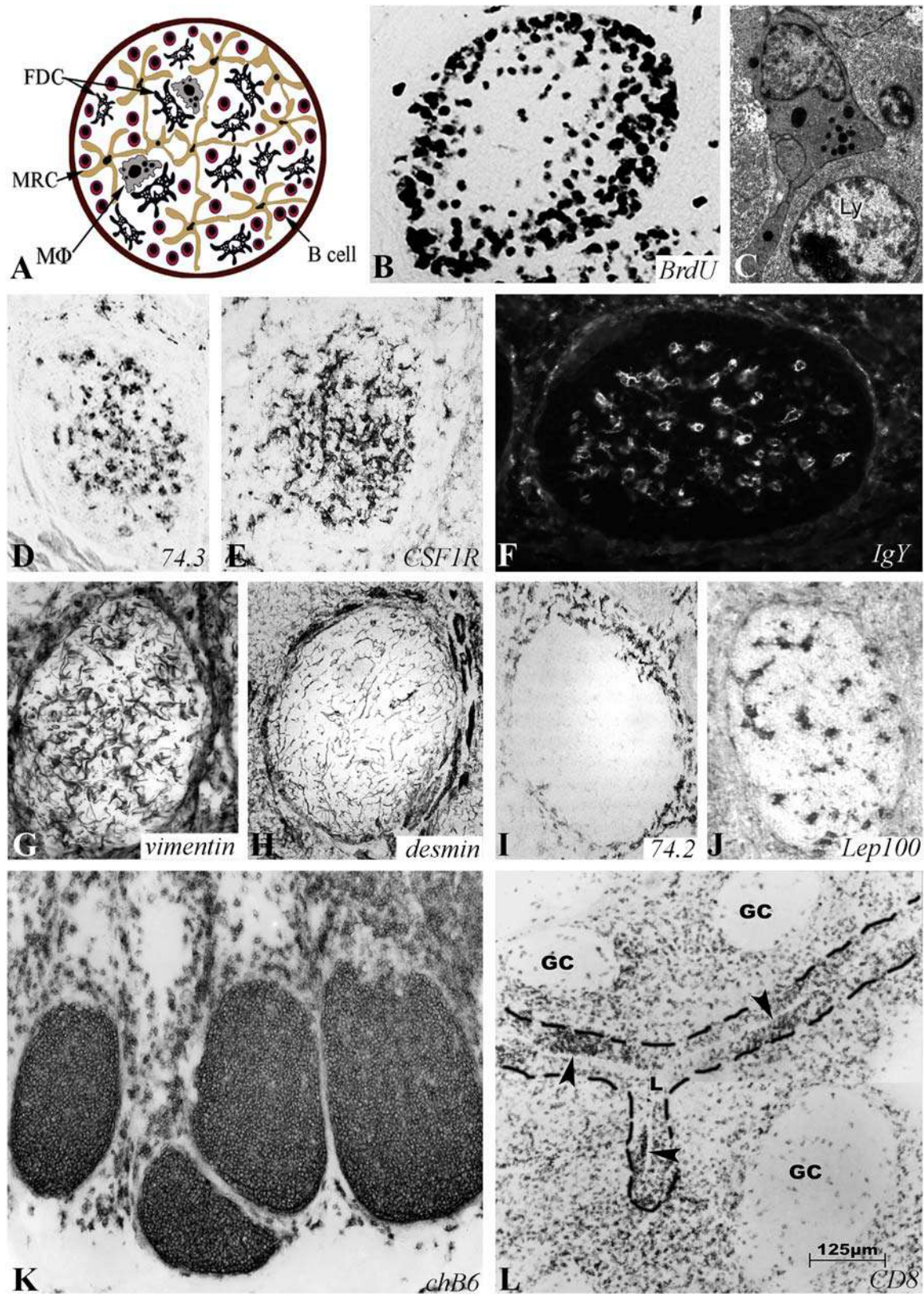


FIGURE 2.8 The structure of the germinal centers. (A) Schematic illustration of the germinal centers. Follicular dendritic cells (FDC) and macrophages are permanent constituents of the GC. Majority of the tingible-body macrophages occur in the outer layer of the GCs. (B) BrdU (Continued)

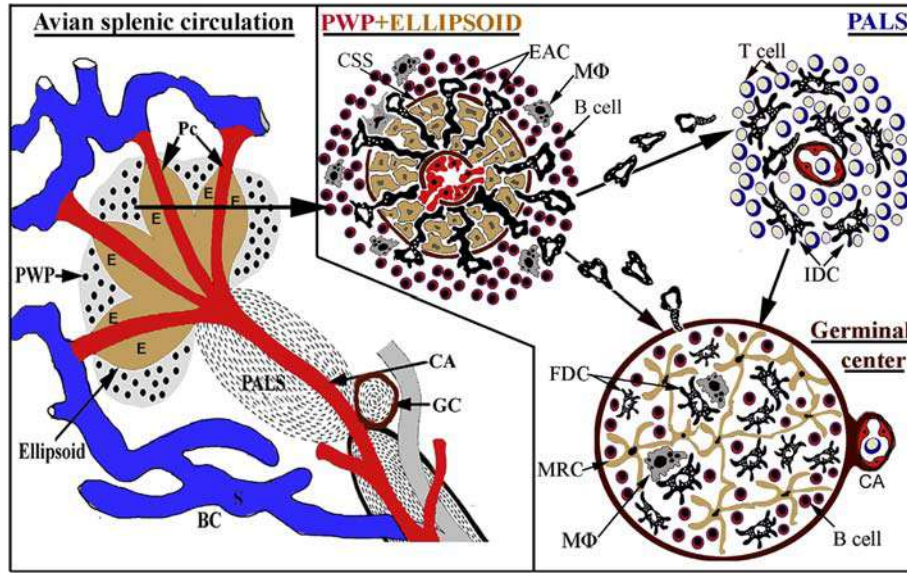


FIGURE 2.9 Schematic structure of the chicken spleen. Schematic illustration of the avian spleen and ontogeny of splenic dendritic cells. Left panel: the ellipsoid (E) also called Schweigger-Seidel sheath surrounds the penicilliform capillaries. The penicilliform capillary inside the ellipsoid has a discontinuous thick basement membrane. Through these stomata antigens can leave the circulation and make contact with the ellipsoid-associated cells (EAC). The ellipsoid is surrounded by EAC and embedded in the periellipsoidal white pulp (PWP) formed by the mixture of B cells and macrophages. The CA is surrounded by T lymphocytes, which form the PALS. GC develop in close vicinity to the central artery. Right panel: anatomical localization of splenic DC and two possible mechanisms for splenic DC development: according to the first model follicular dendritic cells (FDCs) and interdigitating dendritic cells (IDCs) are derived from a common precursor (EAC) located in the ellipsoid. After antigen trapping, EACs detach from the ellipsoid, migrate to the PALS where they first transform to IDC and later to FDC. The second model suggests that EACs directly differentiate to FDC and initiate GC formation. BC, Billroth or pulp cord; CA, central artery; E, ellipsoid; FDC, follicular dendritic cell; FRC, follicular reticular cell; GC, germinal center; PALS, periaarteriolar lymphoid sheath; Pc, penicilliform capillary; S, sinusoid. Adapted from Nagy et al., *Dev Comp Immunol*, 2016, 58: 47–59.

branches of the A. gastrica and hepatica also entering. The spleen is surrounded by a thin capsule of collagen and reticular fibers; poorly developed connective tissue trabeculae enter the splenic tissue from the capsula. Branches of the splenic artery travel in these trabeculae and then enter the splenic pulp. The central artery divides into smaller central arterioles, which also possess a single muscle layer, and from this arise several penicillar capillaries, which lack muscular layers and have stomata. They are surrounded by Schweigger-Seidel sheaths (CSSs) [78] or ellipsoids (Fig. 2.9).

There has been much discussion concerning the junction of the penicillar capillaries with the venous sinuses [4]. The “closed-circulation theory” claims that the penicillar capillary is directly connected with venous sinuses, whereas the “open-circulation theory” claims that they can open freely into the pulp cords, from which blood enters

the sinuses between their endothelial cells. Thus the pulp, or Billroth cords, can be regarded as a portion of the circulation between the arterial and venous system. This kind of circulation requires a unique structure that regulates blood flow through the spleen. The chicken spleen was once considered to have an open circulation system [79]. However, perfusion fixation and light and electron microscopic studies [80] showed that the capillaries which enter the red pulp are continuous with the sinuses and gradually take on their structure. Therefore the chicken spleen has a closed circulation system in contrast to the mammalian spleen, which has an open circulation. The closed circulation system may be a structural feature of the avian spleen because more recently the closed circulation system, blood spleen barrier, was demonstrated in the duck [81].

The basic structure of the chicken spleen is the same as that of the mammalian spleen. It consists of red and

immunocytochemistry shows the proliferating zone is in the periphery of the GCs, where FDCs are absent. FDCs are localized in the center, where the rate of B cell proliferation is low. (C) An electron micrograph of chicken FDC. The dense cytoplasmic granules and the surface “substance” (immunocomplex) outline the FDC. (D) 74.3 mAb shows cytoplasmic granules in the FDC. (E) Anti-CSF1R and (F) anti-IgY shows surface labeling on the FDC. (G) Vimentin-specific antibodies recognize the intermediate filament of the FDC. (H) Desmin expression reveals the reticular cells. (I) 74.2 mAb does not show positive cells in the GC. (J) Lep100 (lysosomal membrane protein) mAb shows cells with lysosomal activity. (K) chB6 recognizes B cells of the GC. (L) A CD8 α immunohistochemistry of GC reveals scattered CD8 $^{+}$ cells, while the lymphoepithelial tissue (the surface epithelium of the cecal tonsil) is heavily loaded with CD8 $^{+}$ cells (arrow).

white pulp, the latter being devoid of erythrocytes and predominantly populated by lymphocytes. Consistent with its less prominent contribution to oxygen circulation, avian red pulp occupies less space than does its mammalian counterpart: 40%–45% in birds compared with 76%–79% in humans [82]. Together with the meager capsule and trabeculae, this helps explain the relatively smaller size. Some divergence in splenic architecture is evident in the chicken, where the distinction between red and white pulp is much less clearly defined. The differences are discussed in the next subsections.

Red pulp

The avian spleen begins to function as a hematopoietic organ shortly after the splenic anlage emerges. In most species, this function is transient and restricted to the red pulp. Once hematopoiesis ceases, the red pulp function changes to filtering out circulating senescent erythrocytes. To achieve this, splenic stromal elements develop a unique communication with the circulation. Immunohistochemical investigations show that the splenic ECM is highly complex, with each compartment having a specific composition that contributes to the adhesion and/or migration of leukocytes and resident cells. Reticular fibers, whose basic constituents are types I and III collagen, form a fine supporting network [4].

Since the chicken spleen has a closed circulation system, capillaries enter the red pulp and go onto the sinuses (Fig. 2.9). These are lined with a flat endothelium and connected to one another. The sinuses are drained by collecting veins, which fuse to become large trabecular veins that leave the spleen.

Both lymphoid and nonlymphoid cells are recognized in the red pulp. Single T cells are abundant in the sinuses and are CD8⁺ and TCR $\gamma\delta$ ⁺ (recognized by mAb TCR1), although some CD4⁺ TCR $\alpha V\beta 1$ or TCR $\alpha V\beta 2$ cells are also present (Fig. 2.10A–E). In adult birds, most TCR $\gamma\delta$ ⁺ cells have a heterodimeric CD8 $\alpha\beta$ receptor; however, in embryonic and neonatal spleens, a large proportion of CD8⁺ TCR $\gamma\delta$ ⁺ cells express only a homodimer of the CD8 α chain [83]. From the embryonic spleen, a natural killer (NK) cell population of CD8 α cells coexpressing CD25 and 28–4 can be isolated [84]. Plasma cells expressing each of the Ig isotypes are also present in the red pulp, especially near the large blood vessels. They show strong intracytoplasmic Ig staining and are CD57⁺ but chB6[–] [67]. In the red pulp, many macrophages are present. These are strongly acid-phosphatase positive and stain with mAb 68.1 and with 74.2 [54], KUL01 [85], and EIV-E12 [86]. Expression of MHC class II antigen has been demonstrated on MRC1L-B⁺ cells, suggesting a role in antigen presentation. Moreover, these

macrophages are capable of phagocytosis since colloidal carbon (Indian ink) is detected in the cells within 24 hours after intravenous injection. Other nonlymphoid cells, such as heterophils, are also scattered throughout the red pulp sinuses.

White pulp

The spleen is the largest lymphoid organ lacking afferent and efferent lymphatics, like “true” lymph nodes (see later), and it can obtain antigens only from the blood circulation. White pulp follows and surrounds the splenic vascular tree [80]. Chicken white pulp contains morphologically distinct areas: periaarteriolar lymphoid sheaths (PALS) that surround the central arteries, which have visible muscular layers, and PWP that surrounds the penicillary capillaries. SCCs or ellipsoids are embedded in the PWP (Fig. 2.9). The PWP is sometimes described as the periellipsoid lymphocyte sheath, analogous to the mammalian marginal zone. GC surrounded by a capsule of connective tissue develop at the bifurcation of the arteries, at the origin of the PALS.

2.4.2 Periaarteriolar lymphoid sheath

Arterioles are surrounded by dense sheaths of T lymphocytes, the PALS. These T cells are CD3⁺, and most express CD4 and TCR $\alpha V\beta 1$ molecules. The remaining T cells express CD4 and TCR $\alpha V\beta 2$ or CD8 and TCR $\alpha V\beta 1$ (Fig. 2.10A–F). The dynamics of the T cells in the PALS was demonstrated in thymectomized chicks as upon depletion of TCR $\alpha\beta 1$ ⁺ cells, the number of TCR $\alpha\beta 2$ ⁺ cells in the PALS increases which could compensate for the deficit in TCR $\alpha V\beta 1$ ⁺ cells. [87]. As in mammals, the CD4⁺ helper cells play a central role in immune responses, and their activation is a prerequisite for the responses of cytotoxic T lymphocytes and B cells. Their central localization around and in GC indicates their role in B cell maturation.

In the PALS, interdigitating DC (IDC) are dispersed between the T cells and are MRC1L-B⁺ and MHC II⁺ but CD57[–] [67]. In the PALS, the IDC can express S-100 and 74.3 antigens (Fig. 2.10G). These cells are likely to be the precursors of FDC, since they are found either scattered or outside of PALS [54,67,80,88].

Various compartments of the chicken spleen are involved in humoral responses. The chB6⁺ cells in the PWP (Fig. 2.10I) are CD57[–], and most cells express IgM or IgA but less so IgY. The antigen-specific antibody-producing cells are first located in the PWP, but later most are found in the red pulp [89–91].

2.4.3 Ellipsoids and periellipsoid white pulp

Penicillar capillaries lack muscular layers, have stomata, and are lined with cuboidal endothelium. The mid-portion

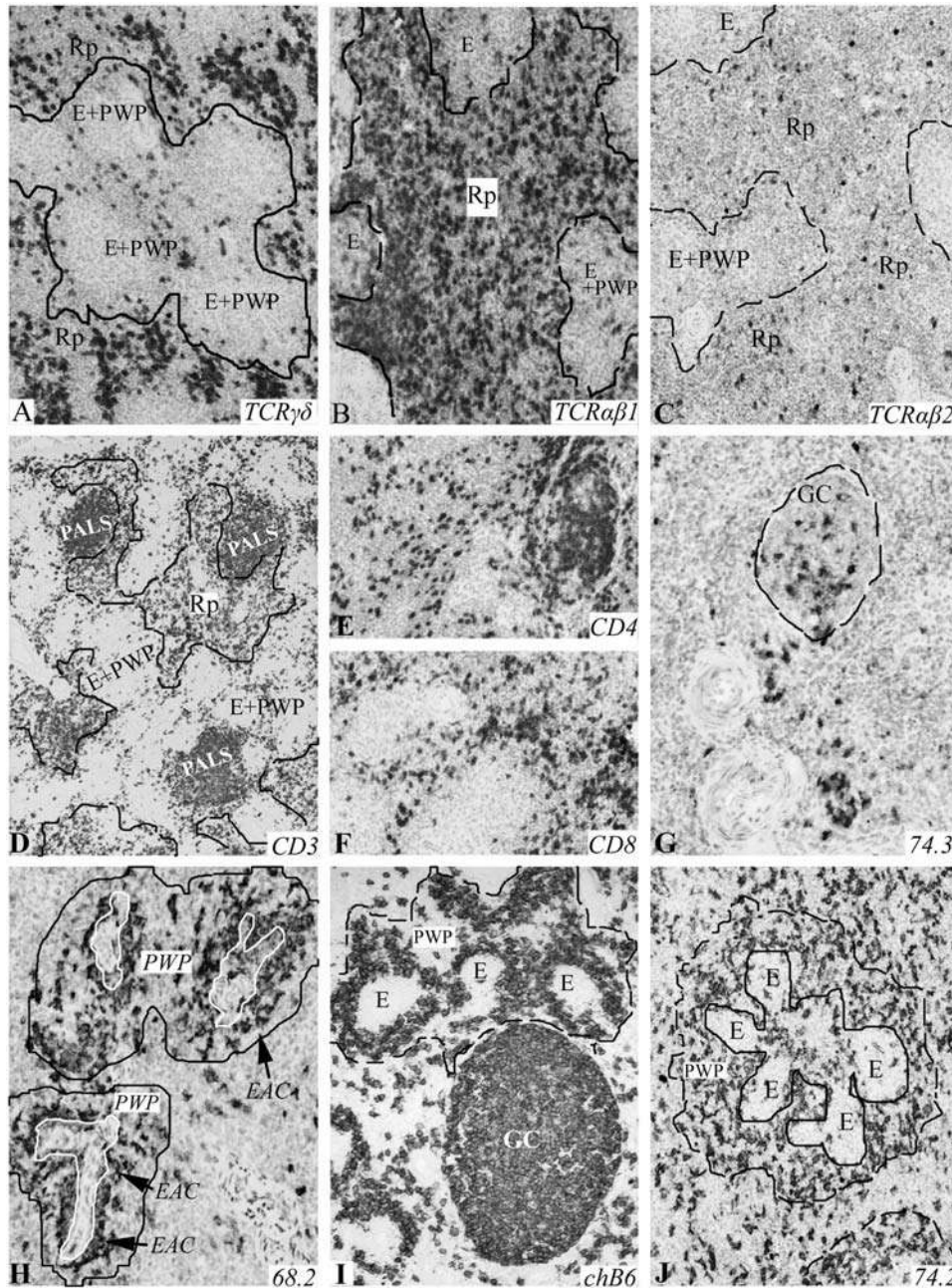


FIGURE 2.10 Detailed structure of the chicken spleen. The spleen consists of red pulp (RP) and white pulp surrounding the vascular tree containing morphologically distinct areas; the periarteriolar lymphatic sheaths (PALS) and ellipsoids (E) are surrounded by periellipsoidal white pulp (PWP). T cells are abundant in the RP and express mostly $TCR\gamma\delta$ (A) or $TCR\alpha\beta1$ (B); few $TCR\alpha\beta2^+$ cells (C) are found. Arteries and arterioles are surrounded by PALS consisting of $CD3^+$ T cells (D) expressing either CD4 (E) or CD8 α (F). (G) Germinal centers (GC) contain the 74.3^+ follicular dendritic cells. The PWP consists of ellipsoids (H) surrounded by 68.2^+ ellipsoid-associated cells (EAC), $ChB6^+$ B cells (I) and (J) 74.2^+ macrophages.

of the capillary is surrounded by the ellipsoid, an efficient filtration apparatus in which mechanical filtration is carried out by the ECM and phagocytic filtration is carried out by cellular components. Cells of the ellipsoid are surrounded by the discontinuous capsule of the CSS (Fig. 2.9). The basal membrane and the CSS are interconnected with fine reticular fibers, forming a 3D network. Intravenously injected extracellular tracers or antigens leave the circulation through the capillary stomata entering the ellipsoid, whose cells are the first to be exposed to antigens. The CSS provide a mechanical filtering system, preventing tracers and antigens from spreading freely into the PWP.

Penicillar capillaries are surrounded by the cells of the ellipsoid, which are reticular cells that are round or ovoid in shape [80]. These cells are also referred to as ellipsoid-associated reticular cells [92], ellipsoidal reticular cells [93], or ellipsoid-supporting cells [4]. They express the antigen CSA-1 and contain heat-shock protein 47 (HSP47) in their cytoplasm, similar to the endothelial cells of the penicillar capillaries [93]. HSP47 binds to procollagen and may play a chaperone role, especially for types I, II, and IV collagen. Nagy et al. have confirmed that collagen types I and III are produced in the ellipsoids [4]. With the use of specific

markers, the ellipsoidal cells have been identified as $CD57^+$ and $MRC1L-B^-$ [92].

Ellipsoid-associated cells (EAC) are phagocytic and found at the surface of the ellipsoid [80]. They express markers recognized by mAb 68.2 [94], S-100 [88], and E5G12 [67] and are responsible for the clearance of antigens from the circulation (Fig. 2.10H). Using *CSF1R*-transgenic reporter chickens it was shown that these cells also express *CSF1R* (Fig. 2.11) [56].

The ring of EAC is surrounded by B lymphocytes within the PWP; these cells express *chB6* (Fig. 2.10I) [4,92,95,96]. Around the EAC, another ring is formed by acid phosphatase-containing macrophages, which can be identified using the mAb 74.2 (Fig. 2.10J), *MRC1L-B* and the phosphatidylserine receptor *TIM4* [4,16,69,85].

2.4.4 The marginal-zone equivalent and antigen handling

In the mammalian spleen, the marginal zone plays a crucial role in the initiation of humoral immune responses. After intravenous injection, antigens are discharged from arterioles into the marginal sinus and localize within the marginal zone. Soluble T cell-independent type-2 antigens, such as Ficoll, are selectively absorbed by marginal-zone macrophages. In mice, marginal-zone macrophages express pattern recognition receptors (such as Toll-like receptors, TLR), the C-type lectin *SIGNR1* (the murine equivalent of *DC-SIGN*), and the type-I scavenger receptor *MARCO*. These receptors show a markedly complementary recognition of pathogens [97]. It has been suggested that these macrophages play a critical role in processing particulate antigens into smaller fragments. T cell-dependent antigens can be redistributed from the marginal zone to the PALS by marginal-zone B cells that, in reacting to antigens, migrate to T cell areas.

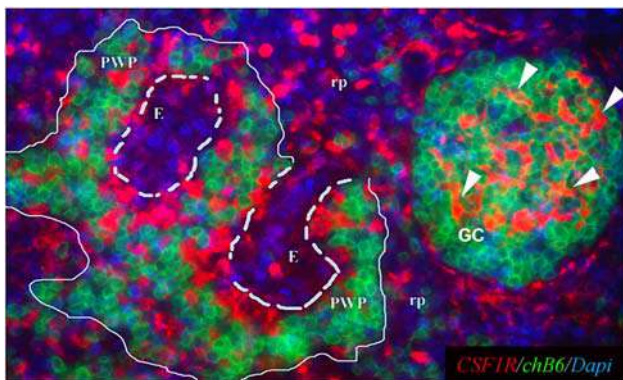


FIGURE 2.11 Distribution of $CSF1R^+$ splenic mononuclear phagocytes (red) and $chB6^+$ B cells (green) in adult chicken spleen. Dashed line outlines the ellipsoid (E). $CSF1R$ -expressing ellipsoid-associated cells are found in close association with the ellipsoid (E). Germinal centers (GC) contain $CSF1R^+$ follicular dendritic cells (FDC) (arrowheads).

MHC-restricted antigen presentation to T cells occurs predominantly in the PALS. Each compartment of the spleen has its own population of nonlymphoid cells: macrophages in the red pulp, IDC in the PALS, and marginal-zone macrophages and FDC in the GC. Being involved in the breakdown of antigens, these nonlymphoid cells can be recognized in situ by their enzyme contents. Almost all nonlymphoid cells contain acid phosphatase, while nonspecific esterase is found only in marginal-zone macrophages.

In contrast, a marginal zone cannot be distinguished in the chicken spleen morphologically. A compartment that is functionally similar, with respect to antigen handling and reaction to mitogenic antigens, has been described by Jeurissen et al. [16]. This compartment is made up of the complex ellipsoidal sheath of reticular cells - a sheath of B cells with a ring of macrophages around it. EAC are responsible for the clearance of antigens from the circulation and consequently become localized in the ellipsoids. This process is independent of the nature of the antigens; that is, they can be alive or dead, soluble or particulate, thymus-dependent or thymus-independent type-1 and -2, with or without an Fc tail. In this respect, the ellipsoid is a region equivalent to the mammalian marginal zone.

The nature of the antigen influences subsequent transport from the ellipsoids to the B cells and the surrounding ring of macrophages. Colloidal carbon has frequently been used to investigate the fate of antigens, but it does not evoke immune responses and so it is not clear that the observations reflect the handling of real antigens. Carbon particles initially localize in the ellipsoid, and the majority is redistributed to the red pulp macrophages. Small amounts are found in the PALS around, but not inside, GC. During the 1960s and early 1970s, White and colleagues [98,99] carried out functional studies which clearly showed the transport of fluorescently labeled antigens or immune complexes from PWP toward the PALS and GC. They named these antigen-carrying cells “dendritic-like cells”: they are probably identical to the EAC later described by Oláh and Glick [80]. Light and electron microscopical studies, using different intravenously injected tracers, suggested that an EAC, after trapping antigen, detaches from the ellipsoid and appears in the PALS around the GC and then in the GC [80,98,99]. This migration pathway led to the hypothesis that the EAC could be the precursor cells of the IDC and FDC [80]. The relationship between the EAC and FDC was recently studied by intravenous injection of bacterial β -galactosidase, which was taken up exclusively by the EAC and later appeared in T and B cell aggregates and in FDC of the existing GC. The mAb E5G12 recognized both EAC and FDC, and double-staining for the E5G12 antigen and β -galactosidase demonstrated that the EAC carries β -galactosidase to the PALS and the GC.

Therefore the EAC could be precursors of the FDC in splenic GC but not precursors of the FDC in GC located elsewhere [67].

A question remains as to whether the population of 68.2^+ and $E5G12^+$ cells may in fact be one or more subpopulations, each with its own antigen specificity. When multiple antigens were intravenously injected, differences in localization were found [94]. Two hours after simultaneous injection of FITC-Ficoll and peroxidase anti-peroxidase (PAP) immune complexes, both antigens colocalized in 68.2^+ cells. In contrast to PAP, FITC-Ficoll continued to be detected on 68.2^+ cells in the ellipsoid at later time points, whereas PAP migrated via the PALS to the GC on cells lacking the 68.2 marker. Again, this suggests either of two things: (1) that multiple subpopulations of EAC exist and, depending on the antigen, these cells either migrate into the PALS or not; (2) that antigens are given to other cells. Cells may lose their markers during migration and differentiation, which makes tracking them complex. For all of these mAbs specific for chicken macrophages and dendritic and reticular cells, the molecules that are recognized remain undefined, which does not facilitate progress in this field. However, it is generally accepted that T-dependent antigens, such as keyhole limpet hemocyanin, and T-independent type-1 antigens, such as lipopolysaccharide (LPS), are mainly absorbed by red pulp macrophages. T-independent type-2 antigens, such as Ficoll, do not relocate after entry into the ellipsoid but remain in the EAC for longer periods. Immune complexes are also first found in the ellipsoid and relocate to the PALS, more specifically on precursor and mature FDC, and they remain present in mature GC. The localization of preformed immune complexes is dependent on the Fc tail because pepsin diminishes localization.

Nonlymphoid cells which handle antigen can be influenced by viruses, bacteria, and bacterial products. After administration of live viruses, such as Marek's disease virus or human adenovirus, the sheath of ellipsoidal cells becomes disrupted and part of the EAC population migrates to the PALS [100,101]. High doses of LPS or bacteria with LPS have strong effects on the 74.2^+ macrophages around the PWP. After intravenous injection of LPS, macrophages around the PWP disappear, as do the B cells in the PWP, but then reappear after 48 hours. A similar relocation of marginal-zone B cells to B cell follicles has been reported for mice stimulated with LPS; LPS downregulated expression of retention signals (S1P₁ and S1P₃) on marginal-zone B cells [97].

The complex of ellipsoids, the B cell sheath, and its surrounding macrophages can be considered a functional homolog of the marginal zone. This may not be general since the formation of ellipsoids and/or marginal sinuses varies between species. Well-organized ellipsoids, such as those in chickens, have also been reported for dogs and

cats but not for rodents, which have marginal zones instead. In contrast to the mouse spleen, the human spleen has both ellipsoids (poorly developed) and an inner and outer marginal zone [89,93,97].

In the spleen, both innate and adaptive immune responses can be efficiently mounted, making it an important organ for immune regulation. Based on the localization of various lymphocyte and nonlymphoid cells in the spleen, PALS seems most involved in adaptive immunity, whereas the ellipsoids and PWP are involved in both innate and adaptive immune responses.

2.5 Gut-associated lymphoid tissue

Chickens lack encapsulated lymph nodes like those of mammals and instead develop diffuse lymphoid tissue. Since mucosae are primary targets for antigens, chickens have extensive mucosa-associated lymphoid tissues. These include gut-associated lymphoid tissue (GALT), which is dealt with here and in Chapter 11.1, respiratory-associated lymphoid tissue, described in Chapter 11.2, and reproductive-associated lymphoid tissue, described in Chapter 11.3.

Along the entire intestinal tract, isolated nodules or lymphoid follicles and small lymphoid accumulations occur in addition to aggregated lymphoid nodules. Scattered along the tract are several defined lymphoid tissues such as the esophageal tonsil (Fig. 2.12A), the pyloric tonsil (Fig. 2.12B), PP (Fig. 2.12C, Color plate 2.2B), Meckel's diverticulum (Fig. 2.12D) the cecal tonsil (CT) (Fig. 2.12E), and the bursa of Fabricius (as described earlier). Defined lymphoid tissues that have been less well studied include lymphoid accumulations in the roof of the pharynx, in the apex of the caeca, and in the cloaca.

During the embryonic and postnatal phases, most organized lymphoid tissues begin to develop independently of antigen stimulation, since they form at predetermined sites before hatching. However, further maturation of the lymphoid tissues is antigen driven, as determined in studies using germ-free chickens and Japanese quail [7]. Compared to conventional birds, there were no consistent differences in weights of the bursa, thymus, or spleen, but there were markedly reduced lymphoid tissues in the GALT and a complete absence of lymphoid follicles in the CT of germ-free birds [7].

The development of GALT starts in the lamina propria of the villus. Each villus consists of a core of connective tissue fibers, smooth muscle fibers, nerves, and blood and lymph vessels. Around this core, lymphocytes intermingle with macrophages in the lamina propria, and the more lymphocytes that arise in the villus, the more they form distinct areas of B and T cells. Generally, T cell areas are located toward the villus core [54]. The next stage in development is the formation of B cell follicles and GC,

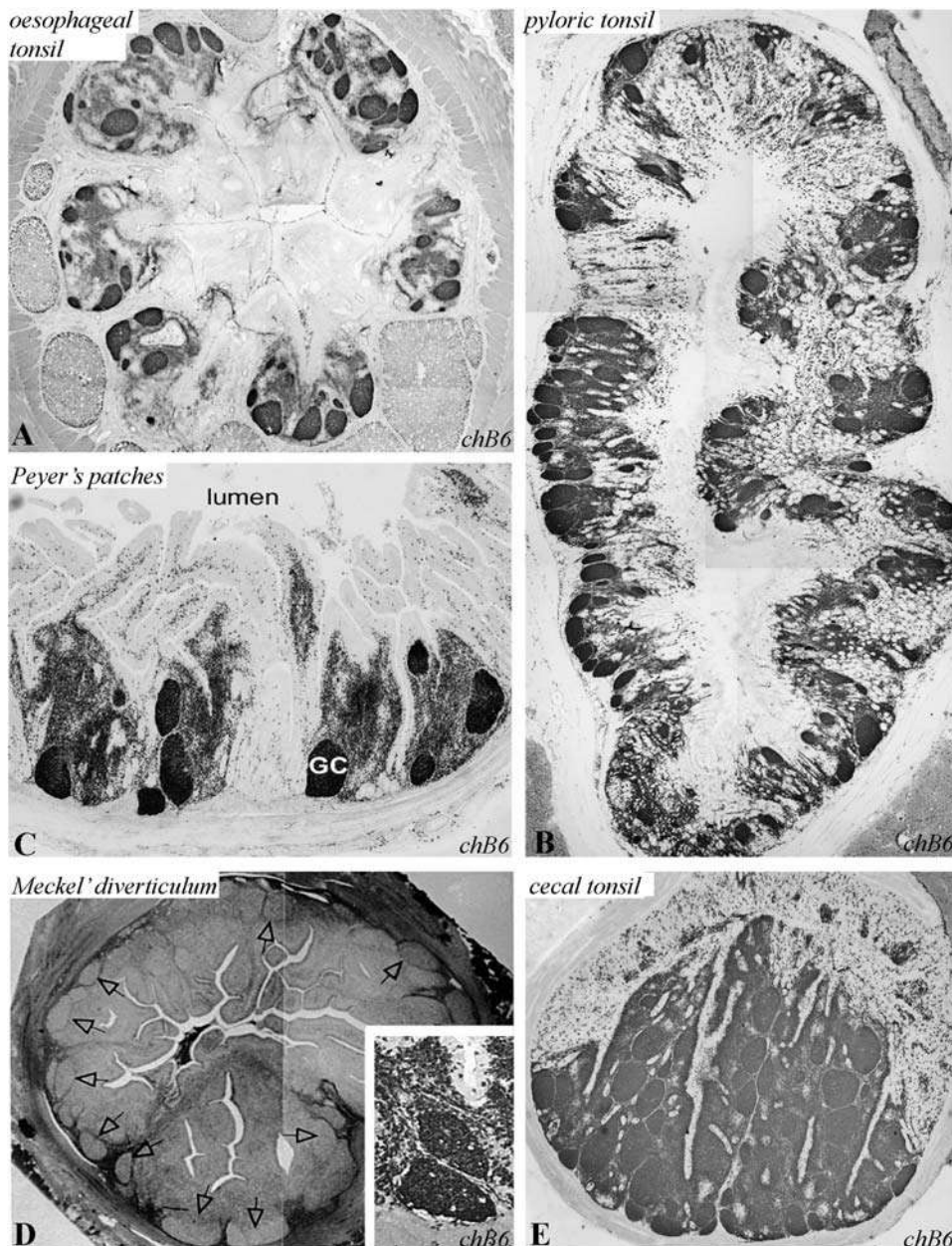


FIGURE 2.12 Detailed structure of the avian GALT (chB6 immunostaining). (A) The esophageal tonsil. The mucosal layer of the esophagus forms six longitudinal folds. Between the folds at the distal end of the grooves, the esophageal tonsils are formed. (B) The pyloric tonsil is located at the border of the stomach and duodenum. The pyloric tonsil forms as a regular ring. (C) Peyer's patches are located at the anti-mesenteric side of the ileum. (D) In Meckel's diverticulum, as in the tonsils, the lymphatic tissue occupies the entire mucosal region. A section is stained with toluidin-blue; the inset shows the chB6-stained germinal center (arrows). (E) The cecal tonsil also forms at the anti-mesenteric side of the caeca.

which appear within the T cell areas of the deep and mid-portion of the lamina propria [102]. The lamina propria is bordered at the luminal side by a thick and continuous basement membrane; situated on this membrane is a layer of columnar epithelial cells. Mucus-producing goblet cells are interspersed between these epithelial cells.

The stroma of the villus consists of a cell-rich ECM. Columnar epithelial cells express a variety of cell determinants, for example, region-specific carbohydrates or lectins [103,104] and B-G-like antigens of the MHC [105]. Mucosal epithelial cells synthesize the polymeric Ig receptor (pIgR) to which polymeric IgA binds. Receptor:Ig complexes are endocytosed and subsequently

transcytosed to the apical surface, where secretory IgA is released into the mucosal lumen by proteolytic cleavage of the receptor ectodomain. Chicken pIgR is expressed in the jejunum [106]. TLR that detect invading pathogens and mount an antimicrobial response are expressed along the chicken intestinal tract [107] (see also Chapter 11.1). Expression of TLR on the gut epithelium remains to be elucidated.

The lymphoid tissues have a well-organized structure with an interfollicular space where T cells accumulate, while B cells form follicles and GC; this is all overlaid by a specialized follicle-associated epithelium. Apart from organized lymphoid tissues, single lymphocytes and

aggregates in the lamina propria and epithelium are found along the intestinal tract. The lamina propria contains both B and T cells. The majority of T cells express CD4 and TCR α V β ₁. Thymectomy and depletion of TCR α V β 1⁺ cells severely compromise IgA production in the gut, serum, bile, and lung lavage fluids [87]. CD8⁺ TCR γ δ ⁺ cells and CD4⁺ TCR α V β 2⁺ cells are scattered throughout the lamina propria [87,108] (see also Chapter 11.1). TCR α V β 2⁺ cells are rarely evident in the small intestine, but their relative frequency is higher in the CT. IgM⁺, IgA⁺, and IgY⁺ plasma cells are found in the villi, with a predominance of IgA-producing cells in the lamina propria. IgM⁺ and IgY⁺ plasma cells are mostly located between the crypts, whereas IgA⁺ plasma cells are often scattered from the crypts to the tips of the villi. Macrophages especially are located in the subepithelial space; they express MHC class II antigen and contain acid phosphatase. Although age does not influence the characteristic distribution pattern of these lymphocytes, the number of cells increases in older birds [109].

A heterogeneous population of intraepithelial leukocytes (IEL) resides among the epithelial cells. Studies on chickens dosed with ³H-thymidine demonstrated that the IEL represent lymphocytes that migrate from the lamina propria into the epithelium [110]. IEL are found in the stratified epithelium of the esophagus and in the columnar epithelium lining the gut, but they are rarely found in the epithelium of the proventriculus. Few IEL are detected at hatching, and their numbers increase greatly with age; however, in old birds the number decreases significantly, returning to the level in young chicks [111]. IEL consist of a heterogeneous population of cells that are located in the basal and apical part of the epithelium [111,112]. CD3⁺ IEL are predominantly CD8 α ⁺ cells, and most express TCR γ δ [108]. However, about 30% of the CD8 α ⁺ IEL lack CD3 and coexpress the 28–4 antigens (found to be the IL-2R) [84]. These cells show NK cell-like activity. Interestingly, chickens harbor a second population of cells with spontaneous cytotoxic reactivity. A novel CD8 α [−] TCR γ δ ⁺ “innate-like” cell population was described in the intestinal epithelium [113]. The physiologic roles of these distinct IEL subsets still need to be elucidated.

Another, or may be the same, ill-defined cell within the epithelium is the globular leukocyte. This cell is slightly larger than the other IEL and contains granules, such as chB6⁺ cells, which have a different absorbance spectral curve than mast cells and are morphologically similar to NK cells [114]. The chB6⁺ cells in the epithelium are recognized by the mAbs HIS-C1, AV20, BoA1, and either L22 or 11G2, specific for chB6a and chB6b alleles, respectively [96,111,115,116]. These cells do not express Ig, and their origin remains unclear because bursectomy does not affect the IEL population [114]. During

the first week after hatching, intestinal lymphocytes already segregate not only anatomically but also functionally into lamina propria B cells, T helper cells, and epithelial cytotoxic/suppressor cells.

2.5.1 Follicle-associated epithelium or lymphoepithelium

The epithelium that covers organized lymphoid tissue, such as PP, CT, the bursa of Fabricius, the pyloric tonsil, Meckel's diverticulum, and the bronchus-associated lymphoid tissue (BALT), is not arranged as in the villi and intestinal glands. The term FAE was introduced by Bockman and Cooper [32] and FAE was shown to be devoid of goblet cells but FAE can be infiltrated by lymphocytes which led to the term lymphoepithelium [117]. The brush border of the FAE is characterized by irregular microvilli. In both mammals and chickens, the FAE contains a specialized cell population that is highly endocytic and transfers particle antigens and microorganisms to the underlying immune cells. These so-called M cells have unique morphological features including reduced glycocalyx and microvilli, and an intraepithelial pocket beneath the basolateral membrane which contains lymphocytes [118]. The FAE can be visualized in Meckel's diverticulum and CT using lectins specific for *N*-acetyl-D-galactosamine, α -L-fucose, and *N*-acetyl-D-glucosamine [119], but to date, no unique markers have been described for the avian M cells. Only a few studies have been carried out on the actual uptake of antigen, and these were limited to the uptake of Indian ink and ferritin [32,119,120]. Although uptake of ferritin by M cells was observed, uptake by other epithelial cells also occurred. More recently, using *CSF1R*-transgenic reporter chickens, antigen sampling cells in the FAE of the bursa were shown to express CSF1R and while annexin A10 and early endosome antigen 1 (EEA-1) were expressed in *CSF1R*-transgene expressing cells in the bursa, neither marker was expressed in the *CSF1R*-transgene expressing cells in FAE overlying the BALT [36]. While mammalian intestinal M cells translocate a broad range size particles [121,122], bursal FAE exhibited efficient uptake of latex beads between 0.02 and 0.1 μ m, while uptake of 0.5 μ m beads was poor [36].

2.5.2 Esophageal and pyloric tonsils

Although the existence of lymphoid tissue at the junction of the esophagus and proventriculus was observed over 30 years ago [9], a detailed description was provided much later [123–125]. Like the CT, the esophageal and pyloric tonsils are permanent lymphoid structures located at the junction of the esophagus and the proventriculus and the junction of the stomach and the duodenum,

respectively. The esophagus forms 6–8 longitudinal folds, with the lymphoid tissue located at the folds' distal ends, where it forms isolated units of tonsils (Fig. 2.12A). The number of units is identical to the number of folds, suggesting they are stable structures [126]. The tonsillar unit consists of a crypt, beginning at the bottom of a fold, and the surrounding lymphoid tissue. These units are restricted to the lamina propria and do not occupy submucosal layers like PP. Lymphoid cells infiltrate the stratified squamous epithelium of the esophagus and the simple columnar epithelium of the pyloric region, transforming both into a lymphoepithelium. In the pyloric tonsil (Fig. 2.12B), the Lieberkühn crypts are transformed into tonsillar crypts, and in the tunica propria around the crypt, well-organized lymphoid tissue is formed [125]. Strongly positive chB6^+ B cells form GC, and many weak chB6^+ cells occur in the interfollicular T cell areas, located between and above the GC beneath the epithelium [124].

The significance of this lymphoid tissue is twofold: first, it is the only substantial lymphoid accumulation located in the digestive tract cranial to the stomach and intestine and is thus exposed to undigested or partially digested environmental antigens; second, like PP, it may participate in B cell development. Oláh et al. [123] speculated that the tonsils function as a “gate” for environmental antigens, through which antigens or allergens continuously stimulate the immune system. The importance of this organ to the development of oral vaccines needs to be investigated.

2.5.3 Peyer's patches

Lymphoid aggregates with characteristics of mammalian PP have been described [120]. PP appear in embryonic development around ED10–13 when clusters of MHC II^+ cells accumulate near the Meckel's diverticulum and the ileocecal junction [14,76,127]. Up to six PP can be detected widely scattered in the intestine, with the exception of one that is consistently found anterior to the ileocecal junction [117] (Fig. 2.12C, Color plate 2.2B). Specific features include widened villi, a follicular structure, a specialized epithelium containing M cells, active antigen uptake development in the embryo, and age-associated involution. GC, subepithelial zone, and interfollicular areas are similar to those in the CT. The subepithelial zone, where macrophages are more prevalent, is B cell dependent, and all B cells express the chB6 antigen [109]. The interfollicular zone is T cell dependent, and almost all T cells express $\text{TCR}\alpha\text{V}\beta_1$, the majority being CD4^+ cells [108]. Few $\text{TCR}\gamma\delta^+$ cells (<5%) are evident in the PP. Mostly IgY^+ and fewer IgA^+ and IgM^+ plasma cells are found in the GC and interfollicular areas. The luminal border of the epithelium is positive for IgA and

IgY but not IgM [120]. Muir et al. [128] showed that, besides the bursa, the PP and CT contain precursor B cells, which, upon transfer into bursectomized chickens, repopulate the intestinal lamina propria with IgA^+ plasma cells. This signifies a role for GALT as a primary lymphoid tissue.

2.5.4 Meckel's diverticulum

The remnant of the yolk stalk is an appendage of the small intestine, halfway along the jejunum; it is called Meckel's diverticulum (Fig. 2.12D). After hatching, significant amounts of yolk pass into the intestine, providing a food supply for the chick. The remnant of the omphalomesenteric, or vitelline, duct, which originally connected the yolk sac and the gut of the embryo, persists for about the first 5 weeks after hatch [129].

The proximal end of Meckel's diverticulum opens as a slit into the small intestine on a flat, elongated papilla. Two longitudinal folds formed by submucosal connective tissue line the opening. These folds are covered by the intestinal epithelium. The wall of Meckel's diverticulum consists of four histologically distinct layers. A serosa covers a thick layer of connective tissue, which may correspond to the subserosa of the intestine. Internal to this layer, bundles of smooth muscles are arranged around Meckel's diverticulum. On the luminal side of the muscle, the connective tissue contains large vessels and ganglions. This layer folds into the lumen of Meckel's diverticulum, and its surface is covered by columnar epithelium with mucus-producing goblet cells. At hatching, no lymphoid tissue is found in Meckel's diverticulum; however, during regression of the yolk sac in the first two weeks after hatching, myelopoietic tissue appears distal to the end of the diverticulum, whereas lymphopoietic tissue appears more proximal to the intestine, in the subepithelial connective tissue. In myelopoietic tissue, three zones are recognized on the basis of their cellular content. The zone closest to the lumen of the yolk sac consists of monocytic cells; next is a zone with large numbers of undifferentiated blast cells; the largest zone consists almost entirely of immature granulocytic cells, which produce two types of granules: one is large, electron-dense and homogenous, while the other is smaller and filled with a finely granulated substance with a visible central crystalline structure. Mature granulocytic cells are rare [130].

After hatching, the epithelium growing into the subepithelial connective tissue forms longitudinal folds, and beneath the epithelium small groups of lymphoid cells accumulate. Single CD45^+ cells infiltrate the epithelium and connective tissue. Single B cells that are IgM^+ , but not IgY^+ or IgA^+ , are located in these infiltrates. Mononuclear phagocytes are found as single cells dispersed over Meckel's diverticulum. Jeurissen et al. [131]

injected colloidal carbon at ED 10 into the yolk and studied absorption during the first 2 weeks after hatching. Carbon particles were first located in the epithelium and subsequently in the subepithelial layer and the lamina propria, showing that yolk-derived antigen can be taken up. Brambell [132], however, found that maternal antibodies in the yolk are transmitted to chicken embryos via the veins in the yolk sac wall and not via the intestine, indicating that a dichotomy between absorption of serum proteins and foreign antigens might exist.

The lymphoid tissue in Meckel's diverticulum gradually increases with age and fills the folds. Concomitantly, the number of goblet cells is reduced, the epithelium is infiltrated by lymphocytes, and inside the folds clusters of lymphoblasts are formed around the DC. After 2–3 months, GC appear, large numbers of which are located close to the muscle layer [133,134]. Dispersed throughout Meckel's diverticulum are IgA and IgY plasma cells and sIgA⁺ and sIgY⁺ cells. At a later age, both B and T cell areas can be distinguished. The T cell areas are adjacent to GC, whereas the B cell areas are generally beneath the epithelium [113]. The anatomical resemblance to CT and PP is clear; however, the development of GC appears to come later, perhaps because of lower exposure to gut antigens. The large number of plasma cells is reminiscent of the HG; however, development of the latter precedes that of Meckel's diverticulum [132,134].

2.5.5 Cecal tonsils

CT are located at the proximal end of each of the cecal pouches (Color plate 2.2A and B). The CT primordium appears at about ED 10, and lymphocytes are present by ED 18 [76]. During the first week after hatching, the number of lymphocytes increases; GC appear in the second week, their number increasing with age. In chickens and quail reared in a germ-free environment, no GC were evident in the CT, and lymphoid tissue was markedly reduced, suggesting that the gut flora is essential to stimulate full development [7]. A suspension of fine particles applied to the cloacal lips can reach the CT by retroperistalsis, similar to that described for the bursa of Fabricius, which suggests that this could be important for antigen sampling [33,135,136].

The general structure of the CT (Figs. 2.12E and 2.13A) is comparable to the structure of the PP (Fig. 2.12C) and includes a specialized lymphoepithelium, a subepithelial zone, GC, and interfollicular areas. The subepithelial zone has a mixed appearance consisting mostly of chB6⁺ (Fig. 2.13A), IgM⁺ B cells, some IgY⁺ B cells and occasionally IgM⁺ and IgA⁺ plasma cells, and few CD4⁺ cells and CD8⁺ T cells expressing TCR $\gamma\delta$ or TCR $\alpha\beta$ 1 (Fig. 2.13B, C, E, and F). Mononuclear phagocytes can be found throughout the

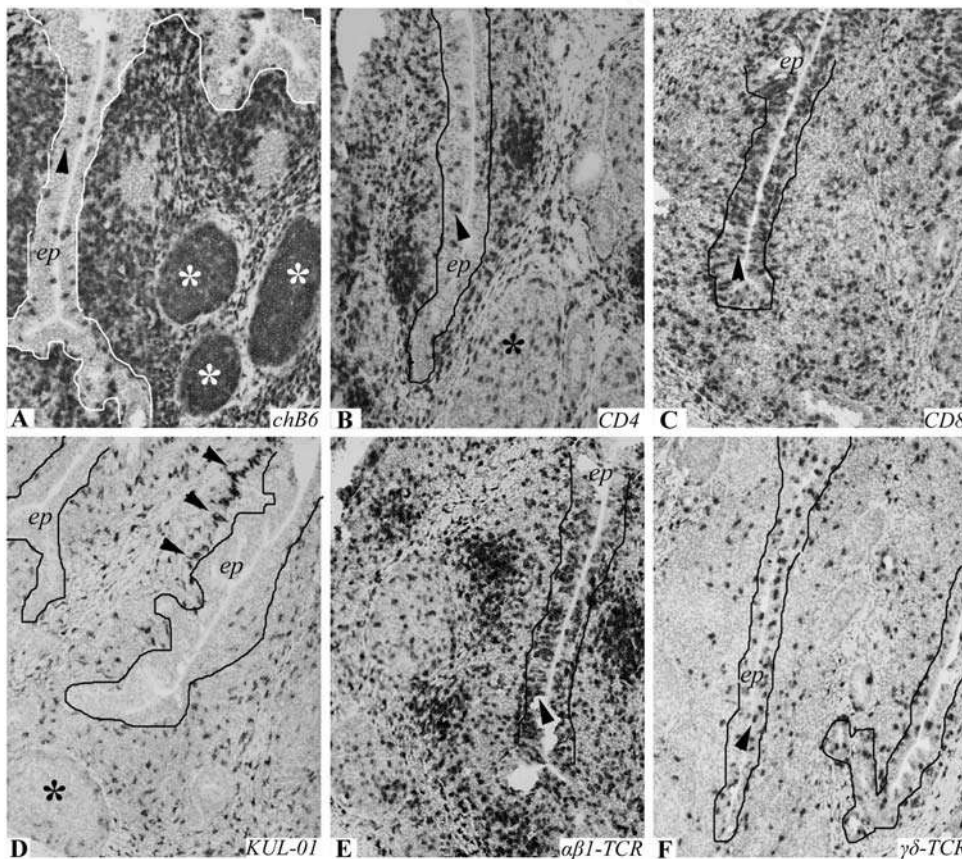


FIGURE 2.13 Detailed structure of the chicken cecal tonsil. The luminal side of the cecal tonsil is located at the top. (A) ChB6⁺ B cells occur in the lamina propria and in germinal centers (asterisk); whereas few ChB6⁺ intraepithelial cells (non-B cells, NK-like cells) also occur in the epithelium (ep; arrowhead). (B) CD4⁺ cells are located in the center of the villi, in the interfollicular areas, with a few in germinal centers. (C) CD8⁺ cells are most prevalent in the epithelium and scattered through the lamina propria. (D) KUL01⁺ macrophages occur scattered through the lamina propria and form a lining under the epithelium. The location of TCR $\alpha\beta$ 1⁺ cells (E) is comparable to that of both CD4⁺ and CD8⁺ cells, whereas TCR $\gamma\delta$ ⁺ cells (F) are located mostly in the epithelium and in the subepithelial zone.

tonsils but are most prevalent directly under the epithelium (Fig. 2.13D). The interfollicular T cell-dependent area consists mainly of $CD4^+$ $TCR\alpha V\beta 1^+$ cells [53,108]. More detailed information on the CT can be found in Chapter 11.1.

2.6 Harderian gland

The major eye-associated lymphoid tissues are located in the HG and the conjunctiva of the lower eyelid (Color plate 2.2D), although scattered lymphocytes and plasma cells can be found in the lacrimal gland and other connective tissues around the eye. In specified-pathogen-free chickens, conjunctiva-associated lymphoid tissue is found only occasionally, but it is constitutively present in conventionally reared poultry, especially turkeys [109,137].

The HG is an ectodermally derived, exocrine tubuloacinar gland located in the orbit behind the eye. It is responsible for lubricating and maintaining the nictitating membrane. Structurally, an excretory duct links the gland to the nictitating membrane, and the gland itself is divided into head and body parts based on differences in the surface epithelium and the underlying lymphoid organization [138]. The excretory duct is invaginated into the head of the gland and shows a classical FAE or lymphoepithelial organization, which continues into the gland's central canal. At the border of the invaginated excretory duct and the central canal, the secretory acini appear; their gradual increase displaces the dense lymphoid tissue. The lymphoid tissue of the HG can be divided into two major histologically different areas: the head, which contains FAE with lymphoid tissue and GC; and the body (Fig. 2.14A and B). These areas form the significantly larger part of the gland containing large numbers of plasma cells in different stages of maturation. Plasma cells are closely associated with the main excretory duct and secretory ducts originating from the acini. Plasma cells cross the BL of the ducts and appear between the epithelial cells, changing the cytoskeletal pattern of the epithelium.

The head of the gland shows the structure of a typical secondary lymphoid organ with B cell-dependent GC (Fig. 2.14C), FAE, and T cell-dependent interfollicular regions with scattered T cells and macrophages. The body of the gland contains many $chB6^+$ B lymphocytes and $EIV-E12^+$ plasma cells [86], and, depending on the level of development, B and T lymphocytes are clustered in separate areas.

Bang and Bang [139] demonstrated lymphocytic infiltrates in the HG of germ-free chickens, indicating that lymphoid tissue within the HG might be induced without microbial stimulation, although the presence of inflammatory substances in the air could not be excluded. Single $CD45^+$ cells and small groups of leukocytes are found in the connective tissue of the glandular lobes of 5-day-old

chicks [109]. These leukocytes comprise $chB6^+$ B cells, macrophages, interdigitating cells, and heterophils [109,139]. As chickens age, leukocytic infiltrates gradually increase in size and, depending on the development of B and T lymphocytes, are clustered in separated areas. The majority of T cells are $CD4^+$ and $TCR\alpha V\beta 1^+$, with a few $CD8^+$ $TCR\gamma\delta^+$ cells scattered around. Leukocytes are mostly absent from the epithelium. The number of plasma cells increases tremendously with age; these cells are found near tubular ducts and interalveolar connective tissue. Uniquely, the HG has a large number of plasma cells which are capable of proliferation in situ [140,141]. This phenomenon is age dependent, and rates of high proliferation are evident in 6–8-week-old chickens.

Discrepancies exist regarding B cell Ig expression. Oláh et al. [138] reported numerous IgM-producing (Fig. 2.14B) and IgA-producing (Fig. 2.14D) plasma cells but rarely IgY^+ plasma cells, whereas Jeurissen et al. [109] described IgY^+ plasma cells (Fig. 2.12E), including IgY, in the overlying epithelium but only in birds older than 6 weeks. Jalkanen et al. [142] reported more cytoplasmic (c) IgY^+ cells than $cIgM^+$ cells in 10-week-old chickens and only some $cIgA^+$ cells. In embryonally bursectomized chickens (at 60 hours of incubation), the number of $cIgY^+$ and $cIgM^+$ cells was halved, but the most striking effect was the significant loss of $cIgA^+$ cells compared to intact controls [142]. It is not clear if the decrease in $cIgA^+$ cells was compensated by an increase in IgM^+ cells. In bursectomized chickens, the number of GC was also significantly reduced. Although Ig-producing cells were still present, none of the bursectomized birds showed any evidence of the production of specific antibodies after four immunizations. The variable findings concerning the presence of different Ig isotypes could be related to age, health status, and environmental stimulatory agents. Although the HG is used as a source for IgA, IgY^+ plasma cells have been found; their activity is reflected in their contributions to tear fluid. The respective concentrations per mL are 0.8 mg IgM, 0.2 mg IgA, and 2.3 mg IgY. Almost all Ig in tears is produced locally because surgical removal of the HG abrogates IgM and IgA levels and reduces IgY levels to 0.5 mg [143].

The route of uptake, processing, and presentation of environmental antigens which leads to humoral antibodies in the tear fluid is still not fully understood. Survashe et al. [144] suggested that the immune response in the HG is initiated in the lymphoid tissue surrounding the opening of the gland duct to the nictitating membrane, whereas others have suggested that, in the turkey at least, uptake of antigen occurs in the lower eyelid and processing in the conjunctiva-associated lymphoid tissue, subsequently leading to plasma cells in the HG [137,145]. More detailed information on antigen uptake, and the effectiveness of antigen administration via various ocular routes, is described in Chapter 11.2.

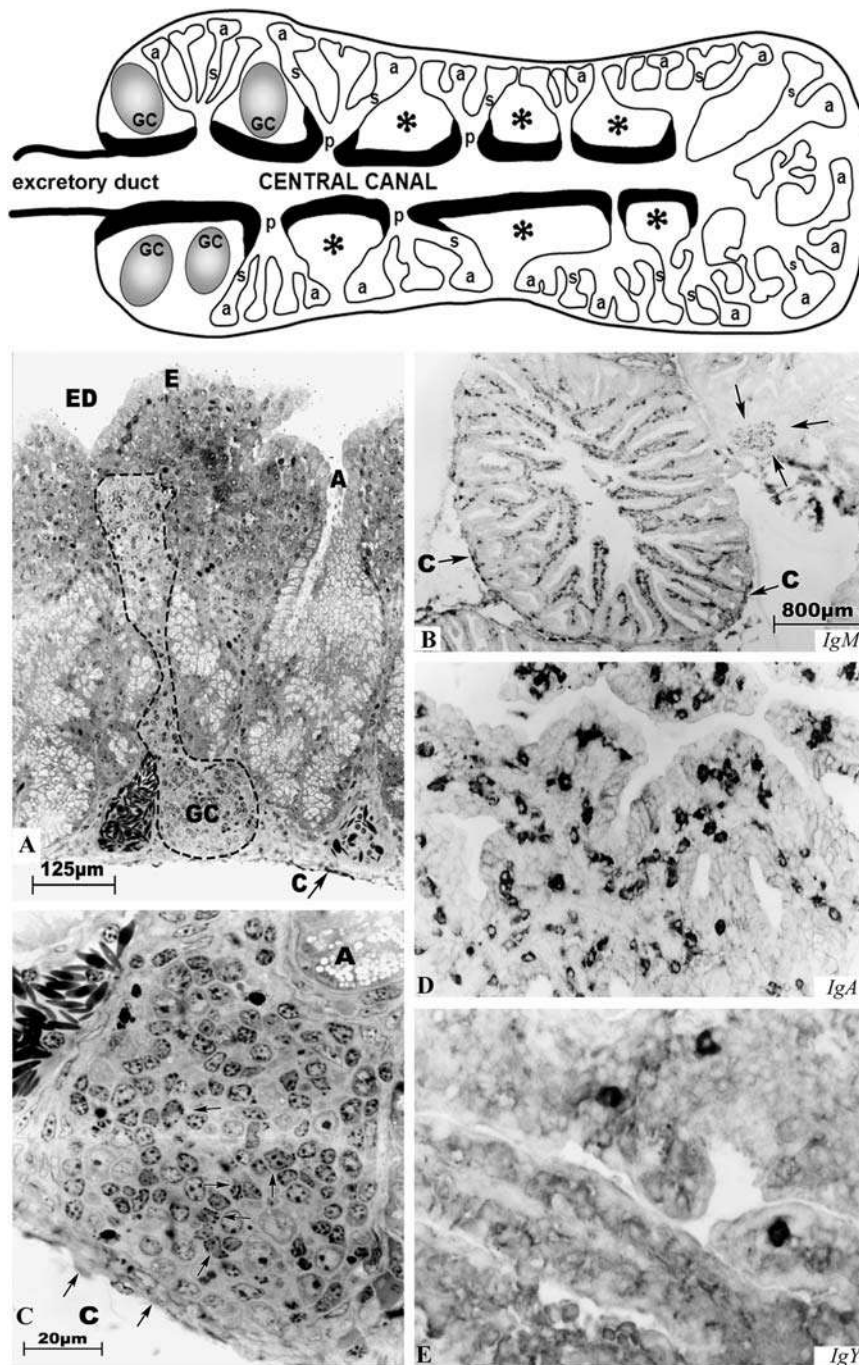


FIGURE 2.14 Detailed structure of the chicken Harderian gland. Schematic illustration of the avian Harderian gland. The lymphoid tissue of the Harderian gland can be divided into two compartments: (1) lympho-epithelial tissue with lymphoid accumulation under the epithelium (lymphocytes, macrophages, FDCs) and germinal centers (GC), which occupies the head region of the gland, and (2) plasmacytic region of the body, which forms the significantly larger compartment of the gland (asterisks indicated the location of plasma cells). The acini (a) are located close to the capsule. (A) The lymphoid region under the epithelium (E) of the main excretory duct (ED), which contains almost exclusively plasmablasts and plasma cells. The head is rich in dense lymphoid tissue (outlined), which includes germinal centers (GC). C, capsule; A, secretory acinus. (B) Anti-IgM recognizes plasma cells in the wall of the secretory duct. No plasma cells are close to the capsule and the secretory acinus. An arrow indicates one GC in which the follicular dendritic cells (FDC) are surface IgM-positive. (C) Detail of Fig. 2.12(A). In the dense lymphoid tissue, a GC is formed close to the C. In the center of the GC, several FDC are shown (arrow). (D) Immunostaining shows that, under the secretory duct epithelium, a large number of IgA-producing cells occur. (E) Scattered IgY-positive cells occur in the Harderian gland.

2.7 Mural lymph node

With regard to the evolution of the lymphoid system, two major phylogenetic events emerged in birds. One was the appearance of a rudimentary lymph node—a major step toward the generalization of the local immune response; the other was the emergence of the GC, in which antigen-specific B cells proliferate, Ig isotype switching takes place and memory B cells are formed. These two

evolutionary steps make the avian and mammalian lymphoid (immune) systems highly comparable, both structurally and functionally.

The first detailed histological description of the avian lymph node was published by Biggs [146]. He proposed that avian mural lymph nodes (MLN) are normal structures and not ectopic lymphoid accumulations, so they must be considered part of the avian lymphoid system. Ectopic lymphoid accumulations would destroy normal

structure, unlike those in the MLN [146]. Generally, the MLN are associated with the deep lymphatics, which run together with the femoral, popliteal, and posterior tibial veins; however, very small lymphoid accumulations also occur along the wing vein. The emergence of the MLN usually takes place after 6 weeks of age and persists in the adult.

There are several anatomical and histological differences between a “true” lymph node (mammal) and the MLN (avian). The former anatomically interrupts the lymph flow, while the MLN is associated with the lateral side of the lymphatic; hence, the lymph flow is not interrupted (Fig. 2.15A). In the largest MLN, lymph sinuses form a bypass in which the lymph flow is possibly very slow. The basic histological distinction is the absence of reticular fibers and macrophages in the lymph sinuses of the MLN. In the “true” lymph node, reticular fibers and macrophages contribute to mechanical and biological filtering of afferent lymph, respectively. The lack of a filtering system in the MLN suggests an evolutionary stage in the development of the lymphoid system. Nevertheless, a foot-pad injection of antigen or phytohaemagglutinin enlarges the MLN located along the posterior tibial vein. This shows that antigens can gain access to the dense lymphatic tissue of the MLN and react with it [147–149]. After injection of sheep red blood cells, plaque-forming cells appear in the spleen, suggesting that activated lymphoid and/or accessory cells leave the MLN and enter the blood circulation [148].

Lymphoid tissue of the large MLN does not differ appreciably from that of a “true” lymph node. GC are embedded in the dense lymphoid tissue occupied by B and T cells. Although the number of GC in the MLN is low, the proportion of B cells is higher than that of T cells, whereas the converse applies in the “true” lymph node. This finding suggests that the interfollicular region, which corresponds to the paracortex of the “true” lymph node, is not well defined in birds and contains a large number of B cells as well as T cells [148]. There is no HEV in the MLN, but lymphocyte migration frequently occurs through the wall of the sinuses [149]. The size of the MLN seems to be related to its histological structures, namely, the sinuses and GC, allowing classification of MLN in three different groups. Possibly these three groups represent different developmental stages rather than different structural identities. Type I is the smallest, consisting of only a lymphoid accumulation on one side of the lymphatic without sinuses or GC (Fig. 2.15A and B). These nodes are not encapsulated, are tightly surrounded by adipocytes, and may be penetrated by blood vessels. The second type is basically an enlarged type I with few GC. The type II node

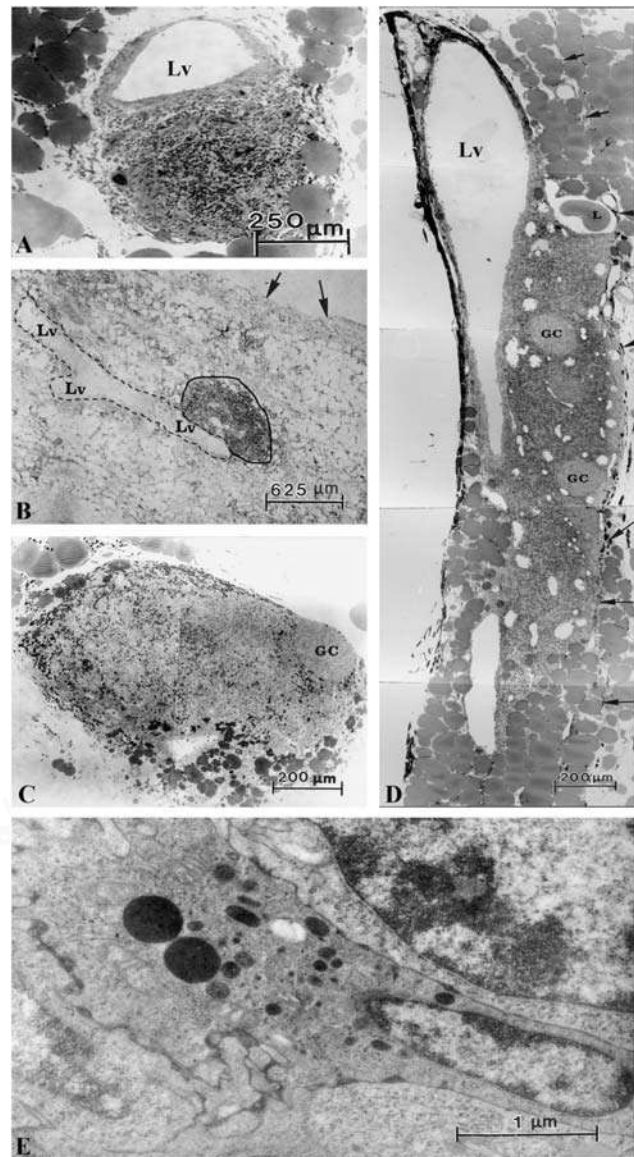


FIGURE 2.15 Details of the structure of the avian lymph node. (A) *Type I*: The mural lymph node (MLN) is associated with the lateral side of the lymphatic. Adipocytes surround the uncapsulated node. (B) The MLN is embedded in adipose tissue. The upper right corner shows the edge of the lipid compartment border (arrow), *Lv*, lymphatic vessel. (C) *Type II*: The MLN with a germinal center (GC). Many red blood cells are located around the node. (D) *Type III*: Lymphoid tissue fills the space between the lymphatic and the loose connective sheath (arrow); hence, the lateral side of the MLN is capsulated while the proximal and distal ends are uncapsulated. GC are embedded in the dense lymphatic tissue. Lymph sinuses do not have traversing reticular fibers and macrophages, but lymphocytes occur in them. In the upper end of the MLN, lipid (*L*) is in the sinus. (E) An electron micrograph of a follicular dendritic cell.

frequently contains many scattered erythrocytes (Fig. 2.13C); also, small, possibly blind-ended, out-pocketing of the lymphatic may occasionally occur. The third and largest MLN produces branching sinuses and

many GC embedded in the dense lymphoid tissue (Fig. 2.15D). The second and third node types are partially encapsulated, located between the lymphatic and a surrounding loose connective tissue sheath. The space between the lymphatic and the sheath is filled with fat and lymphoid tissue.

MLN GC are identical with other GC located elsewhere in the peripheral lymphoid tissue (Fig. 2.15D). GC can be divided into three zones. The peripheral zone does not have FDC, although the lymphoid cells synthesize DNA and are mitotically active. The central zone contains FDC with lymphoid cells that are less mitotically active. The surface of the FDC is highly indented and covered by an electron-dense substance, which possibly consists of immune complexes (Fig. 2.15E). The cytoplasm reveals several electron-dense granules in different shapes and sizes intermingled with well-developed cytoplasmic organelles.

The histology of MLN indicates that their formation takes place in two ways, either outside the lymphatic wall (Fig. 2.15A–D) or inside the lymphatic lumen. The former does not narrow the lumen because the cells migrate out of the lymphatic and settle in the compartmentalized adipose tissue. The latter is formed inside the lymphatic. In the distended lymphatic, the endothelium becomes adhesive for lymphoid cells, which become covered by newly formed endothelial cells or migrate through them [150]. This process is repeated several times, resulting in a lamellated structure inside the lymphatic. The sinuses in

this MLN are formed from the original lumen of the lymphatic, from which the lymphocytes continuously migrate through the newly formed endothelium to enlarge the MLN.

2.8 Ectopic lymphatic tissue and pineal gland

Ectopic lymphoid tissue begins to develop after 3 weeks of age in nonlymphoid organs such as the liver, pancreas, kidney, endocrine glands (thyroid, adrenal, pituitary), gonads, and even the central nervous system brain and spinal cord [9,76]. A major unresolved question is whether ectopic lymphoid tissue represents a burst of lymphomatosis, which is destructive for the nonlymphoid organ, or a normal reaction to external antigens. Biggs [146] considered the appearance of ectopic lymphoid tissue as a normal reaction of the avian immune system. It is transient, disappearing at about 3–4 months of age. The possibility that ectopic lymphatic tissue destroys the normal organ needs to be reconsidered. In the chicken, there is a lymphoid burst after 3 weeks of age, which is manifested by the appearance of lymphoid tissue in nonlymphoid organs. Lymphomyeloid stem cells occur in almost every adult tissue, and it is not surprising that lymphoid foci (more or less organized lymphoid tissue) appear in many nonlymphoid organs without causing any major functional disturbances. One such ectopic lymphoid

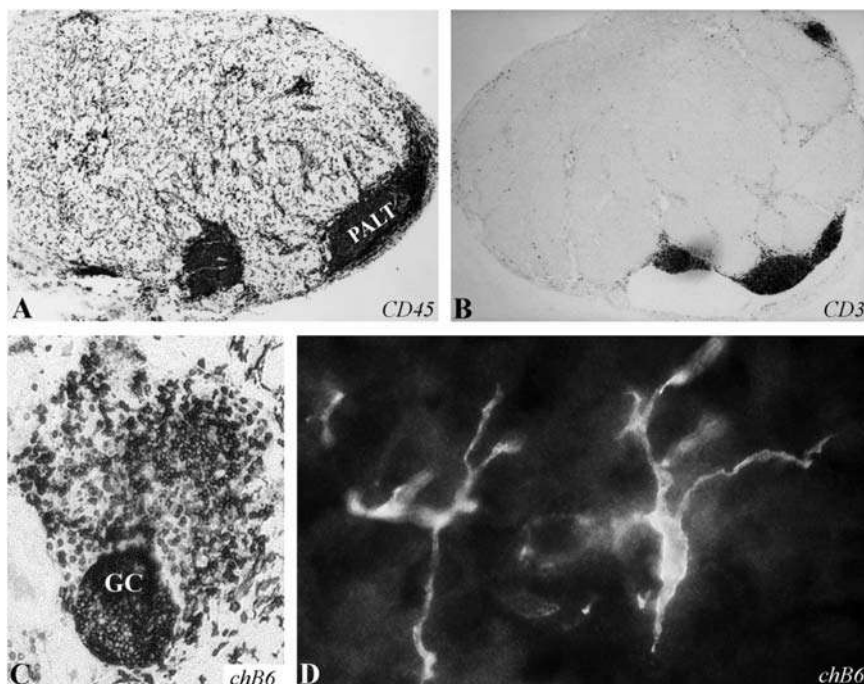


FIGURE 2.16 Details of the pineal lymphoid tissue. (A) CD45 immunocytochemistry shows the hematopoietic cell accumulation in the pineal gland. (B) Among the CD45⁺ cells, many CD3⁺ T cells occur. (C) chB6 immunostaining shows germinal center (GC) formation and scattered ramified chB6⁺ cells. (D) The pineal parenchyma is infiltrated by highly ramified chB6⁺ microglia.

tissue emerges in the pineal gland, an organ usually beyond the scope of avian immunologists.

Lymphoid infiltration in the pineal gland begins around 3 weeks of age and may increase to 50% of the pineal mass [151]. This pineal-associated lymphoid tissue is immunologically active and capable of antibody production [152]. Lymphoid accumulation is closely associated with the vein at the distal end of the gland (Fig. 2.16A and B) and fills up the interfollicular connective tissue septae [152], where GC are formed (Fig. 2.16C). The pineal parenchyma is isolated from the pial connective tissue by a BL of the pinealocytes, through which mobile lymphatic and highly ramified $chB6^+$ myeloid cells (microglia/macrophage) enter the wall of the pineal follicles (Fig. 2.16D), suggesting a novel mechanisms of neuroimmune interactions in this part of the brain [153]. The topographical relationship between the pineal gland cells and the lymphoid cells is identical to that in the lymphoepithelial tissue [149]. From the wall of the pineal follicles, the lymphoid cells can enter the follicle lumen. The invading cells are lymphocytes, plasma cells, and a few myeloid cells, mainly eosinophil and basophil granulocytes. Thymidine uptake by lymphocytes located inside the

pineal parenchyma is about twice as high as that of lymphocytes in the pial connective tissue. In lymphopineal tissue, there is an intimate connection between pinealocytes and lymphoid cells, producing a unique interaction between the immune and central nervous systems. Like its peripheral counterpart, lymphoid tissue in the pineal is organized into dense lymphoid tissue and GC that contain B and T cells, respectively (Fig. 2.16B and C).

2.9 Bone marrow

Histologically, two separate compartments can be distinguished in the bone marrow. The intravascular compartment is possibly responsible for erythro- and thrombopoiesis, while the extravascular compartment is responsible for myelo-, mono-, and lymphopoiesis. Post-hatch, many bone marrow sinuses appear densely packed with immature cells of erythroid lineage. Thrombocytes cannot be distinguished on histological sections. The sinus endothelial cells are flat and frequently seem to be discontinuous, allowing cell migration between the two compartments (Fig. 2.17A). Most immature cells are located close to the sinus endothelium, while

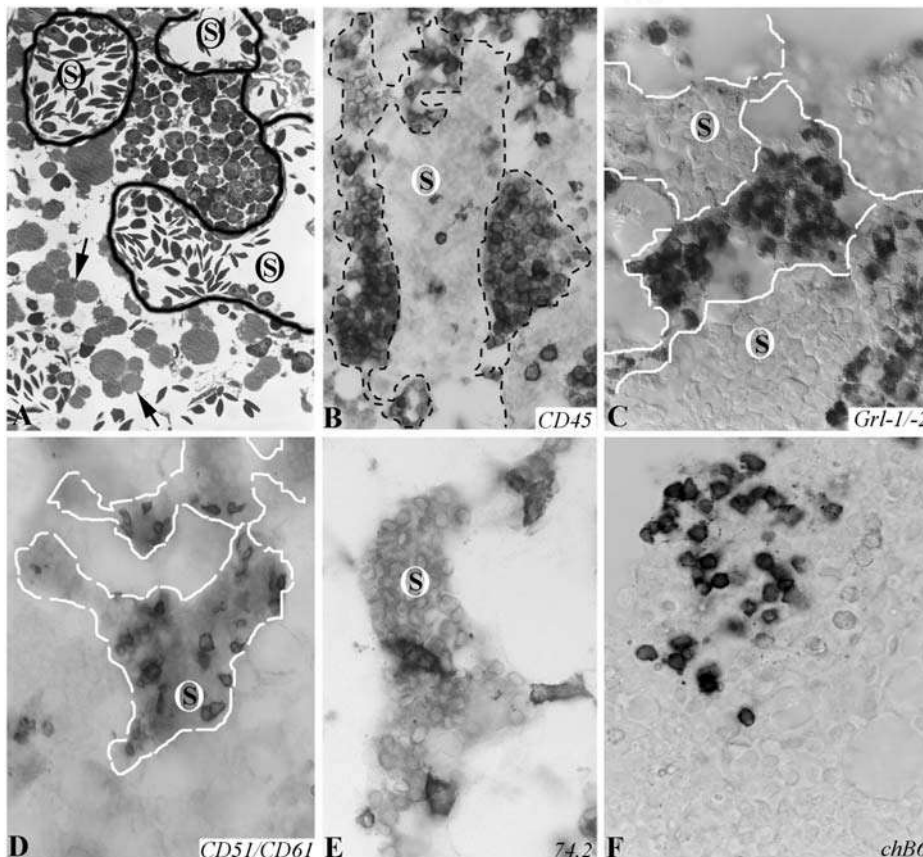


FIGURE 2.17 Details of the avian bone marrow. (A) A 1 μm -thick section stained with toluidin-blue. The sinus wall is outlined. The extravascular region is populated by granulopoietic cells, and many lipid droplets locate there (arrow). The irregular-shaped sinuses (S) contain numerous red blood cells. (B) The $CD45^+$ hematopoietic cells are grouped outside the sinuses (S). (C) The extravascular site of the bone marrow shows a large numbers of $Gr1/2^+$ granulocytes. (D) $CD51/CD61^+$ thrombocytes are grouped inside the sinuses, unlike $Gr1/2^+$ granulocytic and monocytic cells. (E) 74.2^+ monocytic cells are seen in the sinuses. (F) $chB6^+$ B lymphocytes can be found in foci in the extravascular portion of the bone marrow.

the mature red blood cells generally occupy the center of the sinus. Mitosis is common, indicating that the sinus, unlike that in mammals, contains an actively proliferating cell population, which possibly comprises erythroid and/or thrombocyte progenitors. In some cases, the sinus appears so crowded with immature cells that circulation appears to have stopped or to be very slow. This provides a transitory niche and a hematopoietic microenvironment for the maturation of erythroid and/or thrombocytoid cells (Fig. 2.17A–D). Granulocytes can be found only occasionally inside the sinuses.

The extravascular compartment is filled with granulocytes in different stages of maturation as well as a few lipid cells (Fig. 2.17A). With increasing age, granulopoiesis becomes restricted to small areas in the extravascular compartment, while the bulk of this compartment contains only a few scattered cells among the increasing number of adipocytes. The intravascular compartment contains mature erythrocytes. Macrophages (Fig. 2.17E) and occasional immature blast-like cells occur in the sinus lumen. In the extravascular compartment, lymphopoiesis occurs in close proximity to the bone marrow arteries. Unlike granulopoiesis, which is scattered throughout the bone marrow, lymphopoiesis is restricted to small foci (Fig. 2.17F).

The crude supporting system of the bone marrow is formed by 3D branching bony spicules whose surfaces may be covered by giant, multinucleated osteoclasts. The free surface of the spicules is ruffled, and monocyte-like cells frequently occur alongside. Together with the sinus endothelium, bone marrow reticular cells and adipocytes, osteoclasts are the major stromal cells providing the hematopoietic microenvironment for pluripotent hematopoietic stem cells.

Hematopoietic stem cells can differentiate to form common myeloid and lymphoid progenitors by asymmetrical cell division. One of the daughter cells remains in the hematopoietic stem cell pool, while the other differentiates to form one of the progenitor cells. In mammals, erythropoiesis takes place in the extravascular compartment, and it is generally accepted that the common myeloid progenitors provide the common megakaryocyte and erythroid progenitors. In the chicken, thrombopoiesis and erythropoiesis occur in the sinuses; consequently, some sinuses, or parts thereof, may provide a hematopoietic microenvironment by temporarily slowing down, or even stopping, the circulation.

In mammalian species, bone marrow is a major site of antibody formation; in humans and mice, it is a niche for long-living plasma cells [154,155]. The contribution of avian bone marrow to antibody formation is not fully

understood and contradictory data exist. Using a reverse hemolytic plaque assay, Lawrence et al. [156] detected large numbers of Ig-secreting cells in bone marrow, whereas Jeurissen et al. [70], using immunohistochemistry, detected low numbers of bone marrow cells with surface IgM and none with surface IgY or IgA. More recently, de Geus et al. [157] used Elispot assays to demonstrate IgM⁺, IgY⁺, and IgA⁺-producing cells in chicken bone marrow. Russell and Koch [158] immunized chickens with Newcastle disease virus (NDV) by the oculonasal or intravenous route. This resulted in a primary and secondary Ig-secreting cell response in the bone marrow, which was lower than the response in the HG but of similar magnitude to that in the spleen. After infection with avian influenza virus (AIV), the total number of virus-specific Ig-secreting cells in the bone marrow significantly increased after primary and secondary infection [157] comparable to NDV infection. The number of Ig-secreting cells in the bone marrow of AIV-infected birds correlated with the number of Ig-secreting cells in the lung, but not with Ig-secreting cells in the spleen [157]. Both studies [158] emphasized that Ig-secreting cell responses in the chicken are relatively less spleen-centered than in the mouse because the HG and bone marrow can make comparable responses to the spleen.

2.10 Blood

Blood can be considered a special type of connective tissue, in which the ECM is a fluid—the plasma in which blood cells are suspended. There is a continuous exchange of products and cells between the plasma and the extravascular tissue fluid. Plasma serves as a vehicle for gases, nutrients, hormones, proteins (such as albumin and Ig), fibrinogen, and metabolic waste products. The cellular content, on the basis of function, consists of three major types: erythrocytes (oxygen and carbon dioxide transport), leukocytes (immune and inflammatory reactions and phagocytosis), and thrombocytes (control of bleeding and phagocytosis). Leukocytes are present in the blood only transiently (generally for 12–20 hours). After this relatively short period, they leave the circulation and migrate into the tissues, where they perform their specialist functions. Detailed descriptions of blood cells and plasma are beyond the scope of this chapter; however, leukocytes are such important components of the immune system that the basic cytological structure of the key players is shown in Fig. 2.18. More detailed discussions on individual leukocytes are provided in the respective chapters.

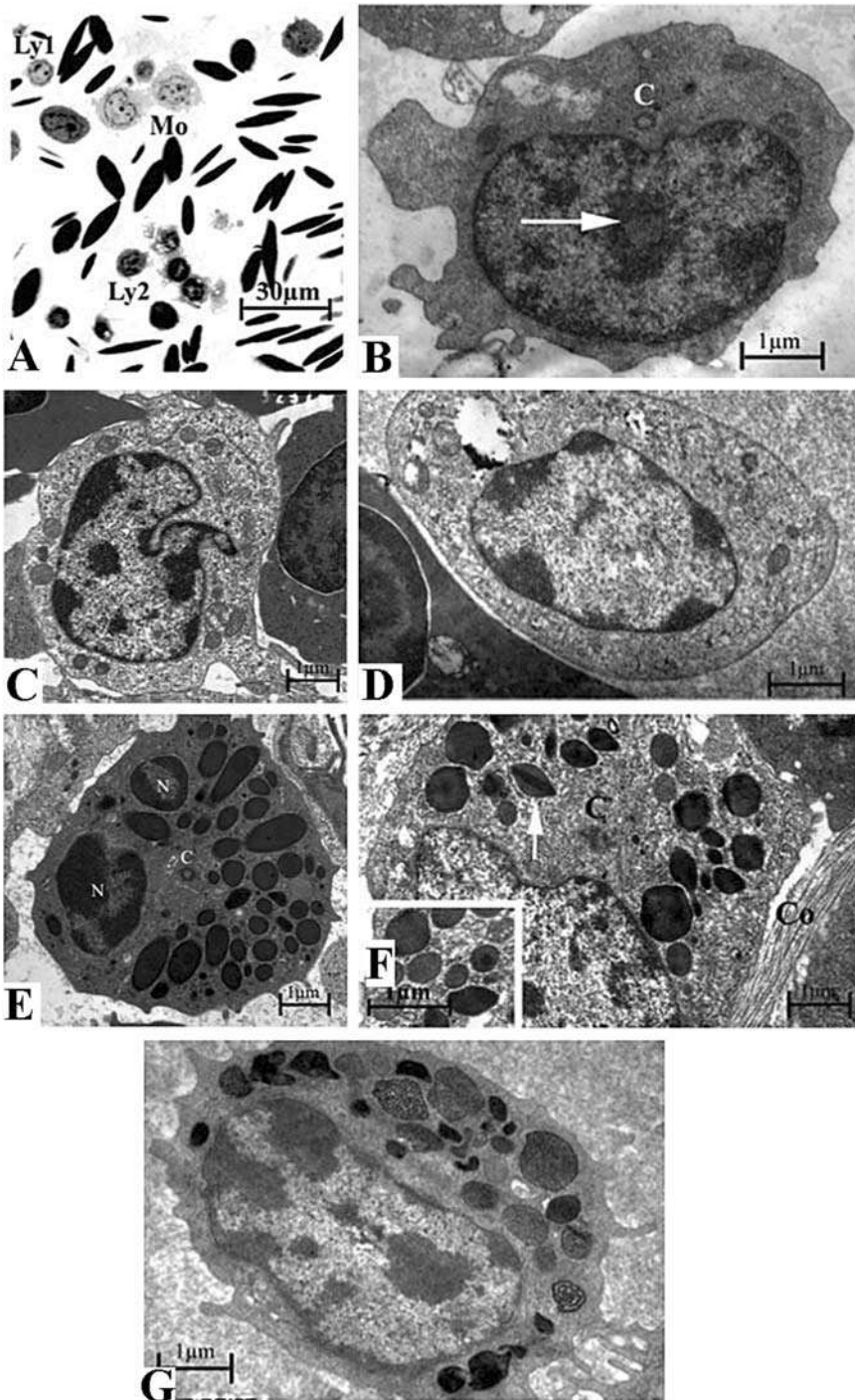


FIGURE 2.18 Details of avian blood. (A) A light micrograph shows two monocytes (Mo) and medium-sized (Ly1) and small (Ly2) lymphocytes with highly ruffled surface membranes. Ovoid-shaped erythrocytes are shown in different planes. (B) Lymphocyte. In the center of the nucleus, the heterochromatic substance is associated with the nucleolus (arrow). The centriole (C) is located close to the moderate indentation of the nucleus. The cytoplasm contains few mitochondria. (C) Monocyte the nuclear membrane is highly invaginated, and the cytoplasm is rich in organelles: mitochondria, free ribosomes, smooth- and rough-surfaced endoplasmic reticulum, and a few granules. (D) Thrombocyte the ovoid-shaped cell reveals a thick layer of cytoplasm with few mitochondria and vesicles. (E) Heterophil granulocyte. Two lobes of the nucleus (N) are evident in this section. The centriole (C) is surrounded by round and ovoid variably sized granules. (F) Eosinophil granulocyte in the connective tissue. The eosinophil-specific granule contains a crystal in its center (arrow), and a centriole (C). Collagen fibers (Co) are deposited around the granulocytes. Inset: two of the specific granules reveal crystals, while the content of the azurophilic granules is more heavily electron-dense and homogeneous. (G) Basophil granulocyte. Around the kidney-shaped nucleus, irregularly shaped and sized, highly textured granules fill the cytoplasm.

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Development of the avian hematopoietic and immune systems

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3.1 Introduction

The avian embryo provides several advantages for studying the development of the hematopoietic and immune systems. These include the existence of a clear demarcation between B and T cells, with each population differentiating in a specialized primary lymphoid organ—T cells in the thymus and B cells in the bursa of Fabricius. In addition, avian embryos can be obtained in large numbers at precise stages of development. Because of their importance for the poultry industry, research on birds has used the domestic chicken and developed different congenic and inbred lines, genetic markers, and monoclonal antibodies (mAb). Nowadays, the availability of several transgenic bird lines developed by the Roslin Institute made the avian model entering the era of genetic models. These tools are essential for studying the development of the immune system. The quail–chicken and now the green fluorescent protein GFP⁺ chicken–chicken chimeras have also proved to be strong developmental models, particularly for studying the emergence of hematopoietic stem cells (HSCs), their migration to the primary lymphoid organs during ontogeny, and the establishment of the adult hematopoietic system.

3.2 Origins and migration routes of hematopoietic cells using quail/chicken complementary chimeras

3.2.1 Looking for the source of hematopoietic cells during development

Based on pioneering avian studies made almost a century ago, it was proposed that the yolk sac (YS) was the site

giving rise to all hematopoietic cells (HCs). This was proposed because the first blood cells, that is, the primitive erythrocytes, appear in this embryonic appendage [1,2]. Moore and Owen [3–5] expanded this view by using parabiosed avian embryo. They proposed that definitive HSCs are generated only once in the YS during early embryonic development prior to migrate and seed the hematopoietic organ rudiments; the bone marrow (BM) being the site of life-long hematopoiesis. Early experiments using mouse embryos supported this view [6,7]. Soon afterward, Dieterlen-Lièvre and coworkers challenged the notion of the YS origin for the definitive HSCs by taking advantage of a newly discovered quail/chicken marker [8] and by performing a series of elegant grafting experiments. They used a unique type of quail/chicken chimera, initially devised by Martin [9], which consisted of homotypic grafting of a quail embryo onto a chicken YS at a very early stage of development, before the onset of heart beating. Such “complementary chimeras” revealed that the cells colonizing the hematopoietic organs were derived from the embryo and not from the YS [10–12]. The YS did generate definitive blood cells, but their numbers decreased rapidly. Circulating blood cells were of chicken origin (derived from YS progenitors) until the embryonic day (E) 5 when they were progressively replaced by quail erythrocytes [10]. This replacement was confirmed when chimeras were constructed between congenic strains of chickens differing in their immunoglobulin (Ig) allotypes or major histocompatibility (MHC) antigens [13,14]. Therefore in contrast to the earlier hypothesis, these results led to the undisputable conclusion that YS-derived progenitors contribute to a single transient wave of hematopoiesis,

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whereas the embryo proper contributes to the definitive HSCs that maintain life-long hematopoiesis.

3.2.2 Macrophage production by the yolk sac

Another function of the YS besides producing erythrocytes is to give rise to the first macrophages. Experiments using reversed YS chimeras (chicken embryos onto a quail YS) combined with cell type- and species-specific mAb, such as QH1/MB1 [15,16] that recognize quail HSCs and endothelial cells (ECs), respectively, were performed to demonstrate that a small HC population of YS origin differentiates into primitive macrophages. These are located in zones of apoptosis, notably in neural derivatives, and may represent the first microglial cells [17,18]. Such a contribution from the YS to the early macrophage population has also been demonstrated for the mouse embryo [19,20,21], confirming that mechanisms giving rise to the different hematopoietic lineages are strongly conserved among species.

3.2.3 The aortic region produces HSCs

Once it has become abundantly clear that another source must provide definitive HSCs, the aortic region became the prime candidate for their origin. At the beginning of the 20th century, the aorta was shown to contain clusters of putative HC protruding from the ventral endothelium [22–24]. Cells in these clusters exhibited a high nuclear-to-cytoplasmic ratio and an affinity for basophilic stains, both indicative of a hematopoietic nature [25], and were later shown to express hematopoietic markers [15,26]. Subsequent *in vitro* and *in vivo* functional analyses demonstrated that the definitive hematopoietic system originates in this aortic region [27–30]. Whether these hematopoietic clusters were the source of HSCs was however not clear from these experiments. Indeed, the avian embryonic aorta region also contains paraaortic foci that are large groups of cells located underneath the aorta, emerging after the cluster formation [25,31,32], and including HSCs [33] (Fig. 3.1).

3.3 Aortic clusters as the intraembryonic source of definitive hematopoiesis

3.3.1 Cellular and molecular identification of the clusters

The most prominent feature of aortic hematopoiesis is the presence of small groups of HCs protruding in the aortic lumen, the intraaortic hematopoietic clusters (IAHCs). IAHCs are invariably restricted to the ventral aspect or floor of the aortic endothelium (Fig. 3.2) but they extend to the vitelline arteries where, importantly, they display

the same ventral polarization. The distribution is similar to that described for mouse and human embryos [34–39] with the exception that the mouse is also harboring small dorsal clusters [40]. IAHCs appear soon after the point at which the two distinct, original aortic anlagen fuse to form the dorsal aorta. The hematopoietic nature of IAHCs is confirmed by the expression of the pan-leukocytic antigen CD45 [26,31]. Furthermore, these cells exhibit the CD41 antigen or $\alpha 2b$ integrin subunit [41]. This integrin, which is the fibrinogen receptor, was first recognized for its important functional role in megakaryocytes and platelets [42]. It was later found on the surface of myeloid progenitors and finally adopted as a tool for enriching IAHCs-derived HSCs, on the basis of its marked expression by IAHC cells in the chicken [43] and in the mouse [44,45]. CD41 is now used as the hallmark of HSCs in the fetus [46,47]. Interestingly, the endothelial-specific αV -integrin subunit (CD51) and the hematopoietic-specific CD41 display complementary patterns in the dorsal aorta (Fig. 3.2A–C).

IAHCs share most of the surface proteins with ECs and express many common transcription factors. However, several molecular features other than CD41 make it possible to distinguish IAHCs from the endothelium in particular by the expression of transcription factors c-MYB and RUNX1, the pan-leukocytic CD45 antigen, and the progressive disappearance of VEGF-R2 and VE-CADHERIN [26,48,49].

3.3.2 The paraaortic foci

A later aspect of aorta-associated hematopoiesis is the presence of large groups of HCs located in the loose mesenchyme located in the ventral part of the aorta and prominent between E6 and E9. Due to their position, these structures have been designated as the paraaortic foci. These cells were shown to be responsible for the first wave of thymus colonization [50,51] and, at the same time, for the seeding of the BM [52]. While a few cells expressing HC characteristics have been reported as present in a similar location in the mouse aorta, the existence of a homologous site has not yet been confirmed in mammals.

3.3.3 Tracing the origins and fates of the aortic clusters

The long-proposed hypothesis that ECs from the aorta give rise to HCs was investigated in the avian model *in vivo*. The fate of aortic ECs was followed by tagging the aortic endothelium with two vital tracers, either the acetylated low-density lipoprotein (AcLDL) coupled with a fluorescent lipophilic marker (DiI) [26] or a nonreplicative retroviral vector carrying a reporter gene [31]. The

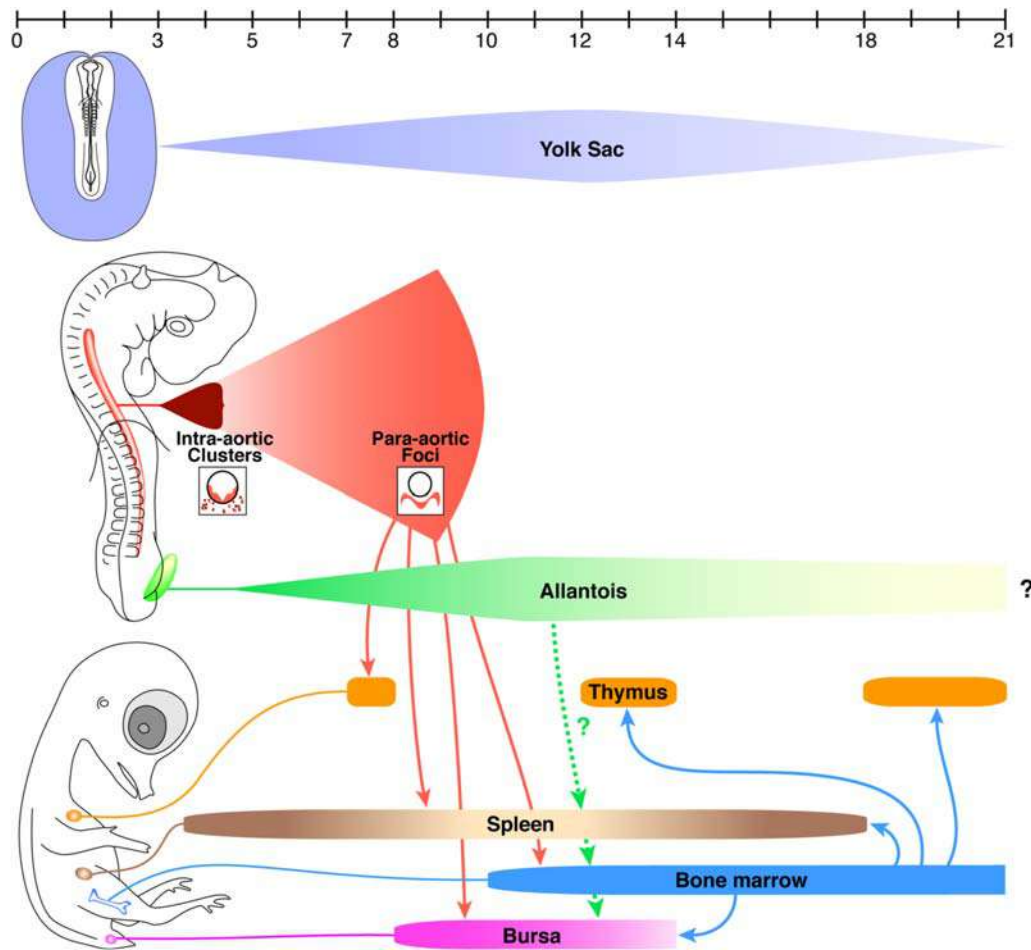


FIGURE 3.1 Schematic description of the developmental pathways at hematopoietic sites, hematopoietic rudiments and the migration routes of HCs in the avian embryo. Timeline: Embryonic days (E) of development. The anatomical locations of the hematopoietic sites and organs are indicated in the left hand drawings. The YS (blue) produces two distinct generations of erythroid and monocyte progenitors. One, occurring between E1 and E5, derives from *in situ* committed progenitors. The other one, beginning at E5 and ending at hatching, derives from both YS and embryo-committed progenitors. The circulating blood cells derived from YS progenitors disappear around E10. Intraaortic hematopoietic clusters and paraaortic foci (red) are secondary sites of progenitor commitment. Paraaortic foci cells derive from intraaortic progenitor cells (see text for details). The allantois (green) is a site of HC emergence (see text for details). The precise contribution of the allantois to the colonization of hematopoietic organs is not known. The thymus (orange) is committed to three distinct waves of colonization, the first from the paraaortic foci, and the second and third from BM-derived cells. The spleen (brown) is colonized very early on, which continues during the remainder of embryonic life. The BM (blue) becomes open to colonization from paraaortic progenitors around E10–11. The bursa (pink) is committed to a unique phase of colonization by progenitors likely to be derived from the paraaortic foci and (when functional) the BM. BM, Bone marrow; HC, hematopoietic cell; YS, yolk sac.

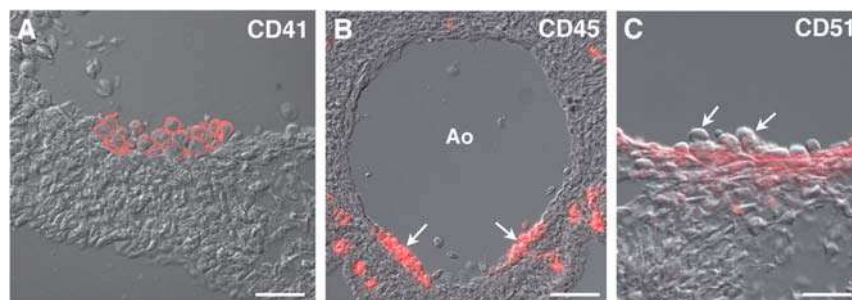


FIGURE 3.2 Intraaortic hematopoietic clusters (IAHCs) in the chicken embryo at E3. Immunofluorescent immunohistology and transverse confocal sections. (A) and (C): Bar = 40 μm ; (B): Bar = 100 μm . (A) CD41⁺ IAHC cells: confocal section (0.2 μm thick) merged using Nomarski optics. Because the section is so thin, only the periphery of the cells appears labeled. (B) Merged CD45⁺ clusters (arrows), Nomarski images. (C) Merged CD51⁺ immunohistology, Nomarski images. CD51⁺ endothelial cells (ECs), CD51⁻ hematopoietic cells (HCs, arrows).

first labeling method has two major advantages: (1) the tag is endocytosed *via* a specific receptor on ECs and macrophages (the latter being absent at the stage of inoculation) and (2) it has a short half-life and quickly disappears from the circulation, if not immediately endocytosed. The second method allows stable expression of the vector's reporter gene for an extended period of time and allows the identification of clones since the integration occurs in sparse cell populations. Both tracers are inoculated into the heart, ensuring direct contact with the endothelium lining the vessels, early on E2, one day before the emergence of the aorta-associated HCs. Cell identification can be achieved using the anti-VEGF-R2 [53] and anti-CD45 antibodies [54] as EC- and HC-specific markers, respectively.

As early as 2 hours after AcLDL-DiI inoculation, the whole vascular tree is labeled. The aortae, still paired at this time, are entirely lined by AcLDL-DiI⁺ ECs, while no CD45⁺ cells are present in the mesenchyme around the aorta nor in the circulation yet. One day after inoculation, when IAHCs emerge, all cells therein are AcLDL-DiI⁺/CD45⁺. On the other hand, retrovirus-labeled clones, on the other hand, comprise ECs or HCs but never both [33].

AcLDL-DiI⁺/CD45⁺ double-positive cells are also found in the mesenchyme ventral underneath the dorsal aorta suggesting that a subset of HCs have ingressed into this region from the ventral endothelium [26]. Moreover, after retroviral labeling, the hematopoietic foci, which develop in the mesenchyme ventral to the aorta [33], contain numerous lacZ⁺ cells. Thus these foci are seeded by progenitors derived from the aortic floor at E3. It was therefore concluded that during the first week of avian embryo development, intraembryonic progenitors are derived from the aortic floor through a unique process that switches the fate of cells that previously contributed to the endothelial lining of the aorta. To elucidate the origin and peculiar cell disposition of the clusters, it is necessary to trace back the developmental history of the aorta.

3.4 Formation of the aorta: a dorsal angioblastic lineage and a ventral hemangioblasts lineage

3.4.1 Two endothelial lineages form the vascular network of the embryo

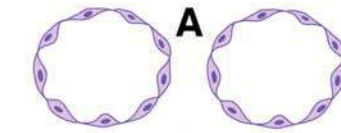
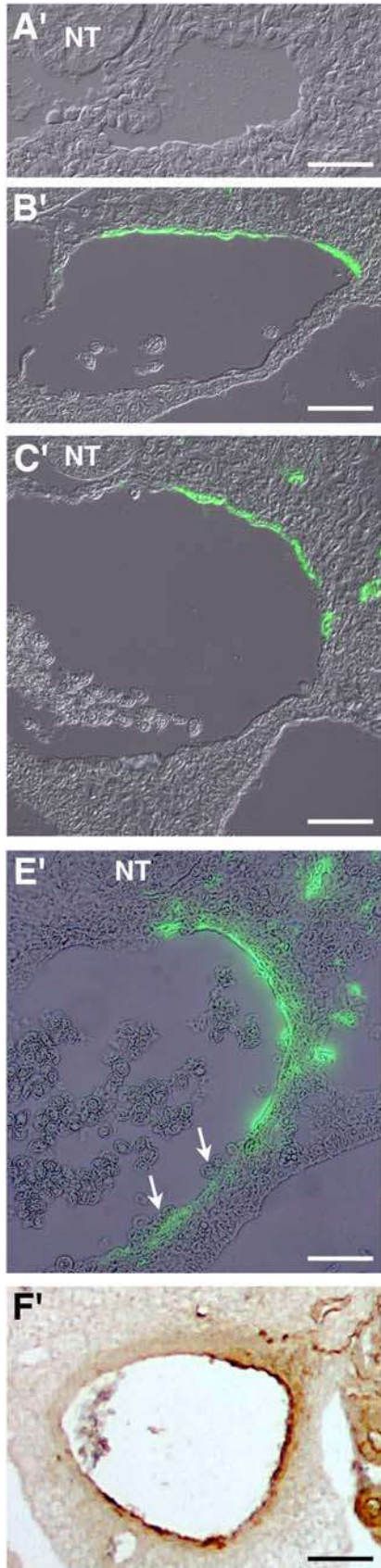
The embryonic mesoderm, that is, the germ layer which gives rise—together with other derivatives—to the blood system, is laid down during gastrulation as a single sheet. It then divides into several mediolateral components, whose respective contributions to the blood/vascular system were established in a series of experiments employing

the quail/chicken chimera. The conclusions were as follows: the somatopleural derivatives (limb buds and body wall) are vascularized by exogenous angioblasts [55], by a colonization process known as angiogenesis [56], while viscera (splanchnopleural derivatives) form their vascular network from their own mesoderm, a process designated as vasculogenesis [55]. The source for ECs colonizing the somatopleural derivative was later shown to be the somite [57]. This dual mode of blood vessel development has been confirmed recently using the mouse model [58].

3.4.2 Chimeric origin of the aortic endothelial cells

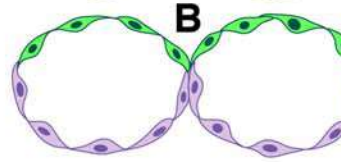
Interestingly, the experiments of Pardanaud et al. [57] have also led to the conclusion that somite-derived and splanchnopleura-derived angioblasts have distinct homing potentials in the aorta. The somite-derived angioblasts integrate the roof and sides of the aortic endothelium. Importantly, they never penetrate visceral organs or integrate the aortic floor. In contrast, angioblasts derived from the splanchnopleural mesoderm invade the floor of the aorta and visceral organs. Furthermore, when integrated in the aortic floor—and only there—cells of splanchnopleural origin proliferate into IAHCs. Thus the homing site is a second requirement for the emergence of clusters. To summarize, the body wall (somatopleura), unable by itself to make blood vessels, becomes vascularized by angioblasts emigrating from the somites, while the splanchnopleural mesoderm gives rise to both angioblasts and HSCs.

The mosaic appearance of the aortic floor at the peak of cluster formation (at E3, 24 hours after grafting) was described in studies conducted by Pardanaud et al. [57]. Jaffredo laboratory obtained a dynamic image of the aortic evolution using a modified version of the same experimental approach though differing in two crucial aspects [59]. Firstly, the grafted material involved the whole segmental plate (approximately 10 future somites) plus the last segmented somite; secondly, the host embryos were examined at different time points (Fig. 3.3). Quail somitic cells invaded the roof of the chicken aorta around 15 hours after grafting. At 24 hours, only the aortic floor, as well as the IAHCs, were from the chicken host (i.e., splanchnopleural-derived). As IAHCs disappeared, the floor became quail (i.e., derived from the grafted somites). In other words, the cluster-forming splanchnopleural cells disappear after HC production. As a result, the endothelium of the definitive aorta is entirely composed of somite-derived cells at E4–4.5. The inability of somitic angioblasts to penetrate the viscera was confirmed using this modified method, despite the replacement of much longer strips of somitic material.

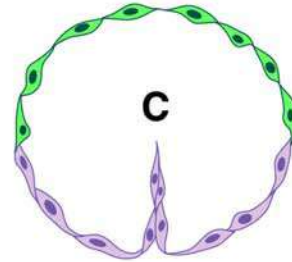


A Two aortae of splanchnopleural origin.

Uniform expression of endothelial markers.

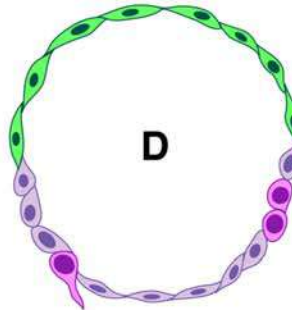


B Cells in the initial roof are replaced by somitic EC.



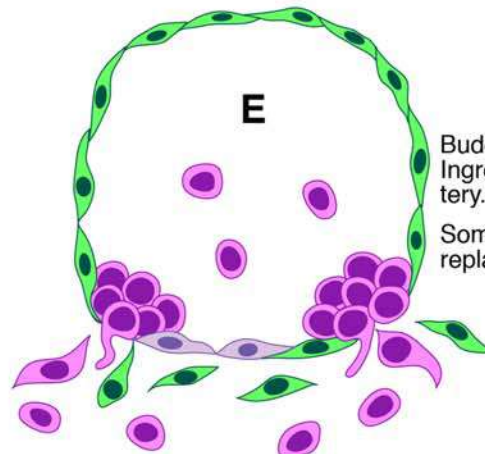
C Complete replacement of the roof.

The aorta is now a chimeric vessel with the roof of somitic origin and the floor of splanchnopleural origin.



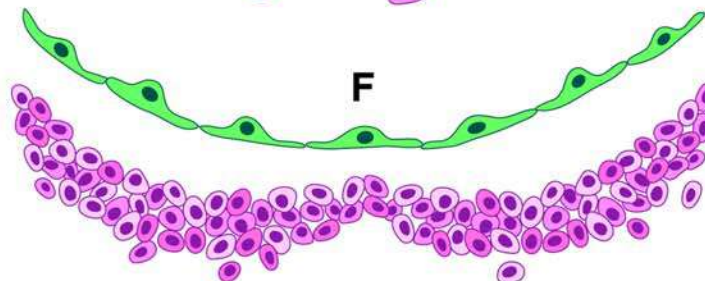
D Downgrading of EC-specific markers.

Upgrading of HC-specific markers.



E Budding into the lumen. Ingression into the mesentery.

Somitic EC progressively replace the aortic floor.



F The floor is now of somitic origin.

Para-aortic foci derive from the clusters.

(Continued)

These experiments shed an interesting light on the problem of developmental relationships between endothelium and HSCs, showing that the aortic floor endothelium is a transient structure that becomes spent and replaced as hematopoiesis terminates. In addition, they explain the brevity of IAHC production, a feature observed in all species studied so far; hematopoietic production in this site depends on a limited, nonrenewable pool of hemogenic ECs that further give rise to the paraaortic foci (Fig. 3.3, right panel).

3.5 Developing an *in vitro* model of hemogenic endothelium commitment and endothelial-to-hematopoietic transition

The first HSCs and HCs are generated early during embryonic life at the level of the aorta and associated arteries from specialized ECs designated as hematogenic. Due to the relative inaccessibility and the low number of hemogenic ECs within the embryo, capturing their identity and the mechanisms driving their commitment remain challenging. Despite efforts to generate robust models, little is known about the mechanisms that drive ECs to the hemogenic fate and about the subsequent molecular control of endothelial-to-hematopoietic transition (EHT).

To circumvent these problems, *in vitro* methods have been developed to recapitulate EC commitment into hemogenic ECs and the EHT using pluripotent stem cells. This was useful to show the existence of the hemangioblast, that is, a bipotent cell that differentiates into hematopoietic and ECs [60,61] or to isolate key regulators of the hemogenic endothelium [62]. Direct reprogramming approaches introducing a set of transcription factors into fibroblasts were shown to be successful in generating ECs that were able to undergo EHT [63,64]. However, the number of hemogenic ECs generated in these cultures was reported to be low (1/3000) [61,65], hampering the establishment of a versatile and simple system that could

be employed to address questions concerning hemogenic endothelium commitment and EHT.

The presomitic mesoderm (PSM) is a noncommitted tissue that gives rise to the somites that produce a cohort of nonhemogenic ECs that, ultimately, vascularize the body wall and limbs [57,59]. These nonhemogenic ECs can be turned into hemogenic following treatment with growth factors or with contact with the endoderm [66]. Yverneau and coworkers, therefore, reasoned that PSM could be used to generate an *in vitro* model to trigger the differentiation of a large number of ECs and hemogenic ECs able to recapitulate the cellular and molecular differentiation events occurring in the embryonic aorta. We thus developed a feeder-free culture system that allows the commitment of PSM cells into ECs and subsequently into hemogenic ECs that undergo EHT and eventually produce multilineage hematopoiesis over a period of 12 days. The approach uses pieces of PSM that are submitted to a cocktail of growth factors to drive their differentiation toward ECs. In such conditions, about 80% of the culture cells differentiate into ECs. Hemogenic ECs, characterized by the expression of the transcription factor RUNX1, are specified from cells already expressing endothelial markers. Single-cell tracking of hemogenic ECs allows capturing the ephemeral flat-to-round transition and revealed unexpected traits of EHT (Fig. 3.4A).

PSMs are isolated microsurgically as described in Pouget et al. [59], free of contaminating tissues. We chose the quail embryo because this species produces much more HCs than the chicken in similar culture conditions. RT-PCR analysis using mesoderm-, endothelial-, and hematopoietic-specific genes indicate that the PSM expresses mesoderm-specific genes such as BACHYURY or MEOX1 but no endothelial- and hematopoietic-specific genes. We tested different culture media and extracellular matrices. Collagen I gave better results over collagen IV. Opti-MEM (Gibco) was supplemented with a cocktail of growth factors known to promote the growth of primary ECs and to be efficient on avian cells (our unpublished work): Opti-MEM/fetal calf

FIGURE 3.3 Aortic remodeling (E2–5). The double aortic rudiments (A) fuse (B and C) and splanchnopleural cells (light pink) become replaced by somite-derived cells (green, B–F). A scheme describing the derivation and fate of the cells is shown on the right. The left column describes aortic remodeling as demonstrated experimentally. Left column: (A) quail segmental plate + last somite was grafted orthotopically in chicken hosts. QH1 immunofluorescence is shown in A', E', and QH1 diaminobenzidine staining is shown in F'. Before fusion, all cells in the paired aortae (A) are of chicken (unlabeled cells, A') splanchnopleural origin. One of the two rudiments is shown in A'. Bar = 50 μ m. Immediately before fusion, cells in the initial aortic roof have been replaced by quail cells of somite origin (B'). The central pillar between the two aortic rudiments is regressing. The origin of the floor remains unchanged with uniform expression of EC-specific genes. Bar = 70 μ m. After fusion, aortic sides become replaced by somite-derived quail cells (C and C'). There are no signs of hematopoiesis. Bar = 100 μ m. At 30–36 somite pairs (not illustrated experimentally in the left column). Floor EC thicken (D) and switch gene expression (downregulating endothelial-specific genes, upregulating hematopoietic-specific genes). At E3–4, there are prominent hematopoietic clusters. Cells of the clusters leave the aortic floor through the circulation. A subpopulation ingresses into the mesentery ventral to the vessel. At the same time, somite-derived ECs insert beneath hematopoietic clusters (arrows) and progressively replace the initial floor (E'). Bar = 150 μ m. At E5 after hematopoiesis, intraaortic cluster cells that have ingressed give rise to the paraaortic foci. Splanchnopleura-derived hemogenic ECs disappear from the aortic floor and are replaced by somite-derived ECs (F'). There is uniform expression of EC-specific genes. Note that somite-derived cells (brown) have invaded most of the aortic endothelium despite unilateral grafting. Bar = 230 μ m. ECs, endothelial cells; NT, notochord.

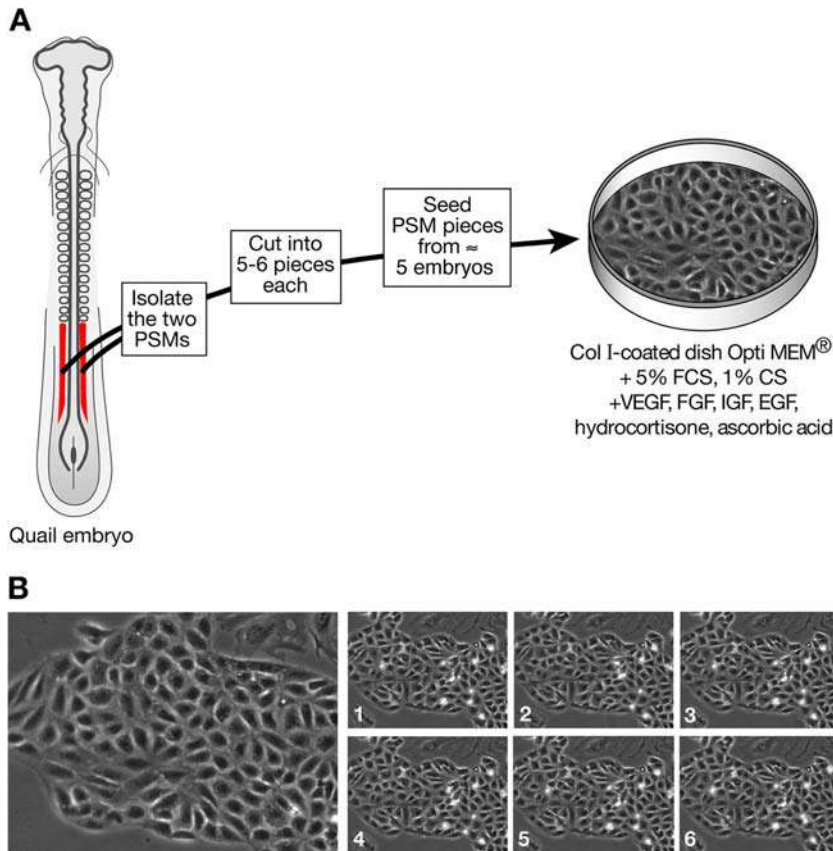


FIGURE 3.4 Culture system and molecular identification of the presomitic mesoderm (PSM). (A) The two quail PSMs (red) encompassing a length of about 10 somites were isolated, cut into five or six pieces each, and placed in 35-mm collagen I-coated culture dishes. Ten PSMs were plated per dish in Opti-MEM in the presence of 5% fetal calf serum, 1% chicken serum, VEGF, FGF, IGF, EGF, hydrocortisone and ascorbic acid. Cultures were followed over a period of 12 days and time-lapse imaged at different time points. (B) Phase contrast. Flat, endothelial-specific phenotype. 1–6 progressive flat-to-round changes typical of EHT. EHT, Endothelial-to-hematopoietic transition.

serum (5%)/chicken serum (1%) supplemented with vascular endothelial growth factor (VEGF 2 ng/mL), fibroblast growth factor (FGF 4 ng/mL), insulin growth factor (IGF 3 ng/mL), epidermal growth factor (EGF 10 ng/mL), hydrocortisone (200 ng/mL), and ascorbic acid (75 ng/mL). Five PSMs, each cut into 4–6 pieces, were seeded on 35 mm collagen I-coated dishes. Retrospectively, the culture period could be divided into three phases: (1) spreading and EC differentiation from day (D) D0 to D3–4; (2) HC emergence and production from D4–5 to D8–9; (3) the persistence of HC production and multiplication from D8–9 to D12. Gene expression analyses using a gene set representative of uncommitted mesoderm, ECs, hemogenic ECs and HCs indicated that mesoderm-specific genes were expressed until D2. FLK1 expression appeared at D0.5–D1 and coexpression of BRACHYURY and FLK1, reported to be associated with the hemangioblast stage [67,68], was detected at the same time. The transcription factor SCL (also known as TAL1), which is crucial for the emergence of the hematopoietic and endothelial lineages [69], was expressed from D0.5, as was GATA2, known to be expressed by mesoderm and nascent ECs [70,71]. Expression of genes encoding the endothelial and hematopoietic progenitor cell antigen CD34 [35,37] and, to a less extent, the endothelial-specific calcium-dependent cell adhesion molecule CD144 (also known

as cadherin 5) was activated at the same time, indicating that endothelial commitment has occurred. RUNX1, the hallmark of the hemogenic endothelium, was expressed at low levels from D0.5 and upregulated from D3. PU1 (a myeloid-specific transcription factor) and CD45 (a pan-hematopoietic marker) shortly follow RUNX1 expression indicating that hematopoietic differentiation has occurred. The hematopoietic production within this system is massive. From D4 to D12, a mean of $2\text{--}3 \times 10^4$ cells were produced daily. Multilineage production was detected by both RT-PCR and May-Grünwald-Giemsa staining. Moreover, it is possible to preclude EC commitment, accelerate EHT, or promote smooth muscle cell versus EC differentiation by modulating the combination of growth factors [72].

Besides, time-lapse culture imaging showed that the EHT was rapid and left a cell-free area on the adherent cell layer, indicating that the fate change occurred at the single-cell level (Fig. 3.4B). The passage from a flat, adherent cell, to a nonadherent cell took between 15 and 30 minutes. In general, no cell division was observed prior to the passage from flat to round, ruling out asymmetric cell division as a prerequisite for EHT. Round cell production thus caused a progressive exhaustion of the layer of flat cells. Upon detachment, the cell underwent dynamic movements, emitting cellular processes during a

period of 30–45 minutes. Following these movements, cells became round and left the site of production. In rare instances, round cells immediately initiated cell division, whereas the vast majority of round cells that were produced underwent cell division some hours after EHT [72].

The design of this robust and highly reproducible *in vitro* model will improve our understanding of the precise cellular and molecular changes associated with EHT and will pave the way for the future *ex vivo* production of blood cells and potentiate the discovery of blood cell modulators.

3.6 Spatiotemporal emergence and organization of the chicken IAHCs

In the aorta of the developing embryo, hemogenic ECs generate IAHCs endowed with HSC potential through an EHT (see Sections 3.5 above). However, the number of IAHC cells that are generated during development was unknown until Yokomizo et al. [73] used a whole-mount fluorescent immunostaining protocol to stain the endothelium (using a CD31/PECAM antibody) and the IAHCs (using a CD117/CKIT antibody) of a whole mouse embryo. This protocol adapted from Gard DL [74] allows, after clarification of the embryo using chemical reagents (benzyl alcohol/benzyl benzoate), to get access and to image deep inside the tissue. With this approach, they could visualize the organization of the blood vessels and distribution of IAHCs in an entire mouse embryo. Yokomizo and Dzierzak [39] performed immunostaining along the developmental course of the mouse embryo and calculated the number of IAHC cells present for every developmental stage. They showed that IAHCs start to emerge in the aorta at E9.5 to reach a peak of ≈ 700 IAHC cells at E10.5. Then, the IAHC number progressively decreases until E14.5 [39].

Such quantification and cartography were missing in the avian system until Yvernogeu and Robin applied a similar approach on the chicken embryo [75]. For this purpose, they used a MEP21 antibody [76] to visualize all blood vessels, a CD45 antibody for HCs [54], and a RUNX1 antibody to stain both IAHCs and hemogenic ECs [77]. This antibody combination allowed the authors to identify the aortic endothelium ($\text{MEP21}^+\text{Runx1}^-\text{CD45}^-$), the hemogenic ECs ($\text{MEP21}^+\text{Runx1}^+\text{CD45}^-$) and emerging HCs ($\text{MEP21}^+\text{Runx1}^+\text{CD45}^+$) (see above for the role of Runx1 in the EHT process). They performed whole-mount fluorescent immunostaining of the chicken embryo and showed that IAHC cells start to emerge by E2.25. This number quickly increases to reach a peak at E3 with around 1500

IAHC cells present within the chicken aorta. From E3, the number of IAHCs decreases and vanishes around E5. [75] (Fig. 3.5A and B). Not only the authors precisely determined the number of IAHC cells but they also estimated the amount of paraaortic foci cells present for several developmental time points. They calculated that at E2.5, 300 paraaortic cells are present and that this number exponentially increases with the development of the embryo to reach 6000 cells at E4. Then, the number of paraaortic foci cells became too high to be accurately counted (Fig. 3.5C). This emphasizes the potential role of the paraaortic foci in the amplification of the newly formed HSCs and HCs of the aorta (see Chapter 3.3.2 above for the role of the paraaortic foci). Finally, the authors could demonstrate that the IAHC emergence is occurring as a “wave” along the aorta during development. Indeed, when IAHC cells start to emerge, they are concentrated to the most anterior part of the aorta. As development proceeds, they are found in the middle part of the aorta. Finally, they become more present close to the vitelline-aorta connection but are never found more caudal to this point after (Fig. 3.5D).

Furthermore, Yvernogeu et al. [75] adapted the “ancient” grafting technic referred to as the chorioallantoic membrane grafting technic developed by Moore and Owen [4,5] to unravel the unique role of the aortic region. They transplanted either the aortic region (equivalent of the AGM region), the YS, the allantois, or the embryonic head before vascularization, isolated from a GFP^+ chicken embryo into the CAM of a wild-type (WT) recipient embryo. Then, they evaluated the hematopoietic contribution of the different grafted GFP^+ tissues to the hematopoietic system of the adult recipient (> 5 months posttransplantation). They obtained long-term multipotential hematopoietic reconstitution only when they transplanted the aortic region, reinforcing the unique role of the AGM in producing HSCs [75].

Besides the precise quantification of the amount of IAHC and paraaortic cells present within the aorta of the developing chicken embryo, the whole-mount staining protocol authorizes performing 3D reconstruction of the whole embryo/aorta for all developmental stages. This allowed us to conclude that chicken hematopoiesis is not only restricted to the ventral side of the aorta, as previously observed [26,57,78] but also restricted to the anterior part of the embryo, that is, from the brachial arches to the vitelline-aorta connection [75] (Fig. 3.5A and D). These two specific features of the avian system are interesting and of importance from a developmental point of view. Why is there is such a spatiotemporal “restriction” in the emergence of the IAHC cells within the aorta? Why is this process only occurring in the anterior portion and only in

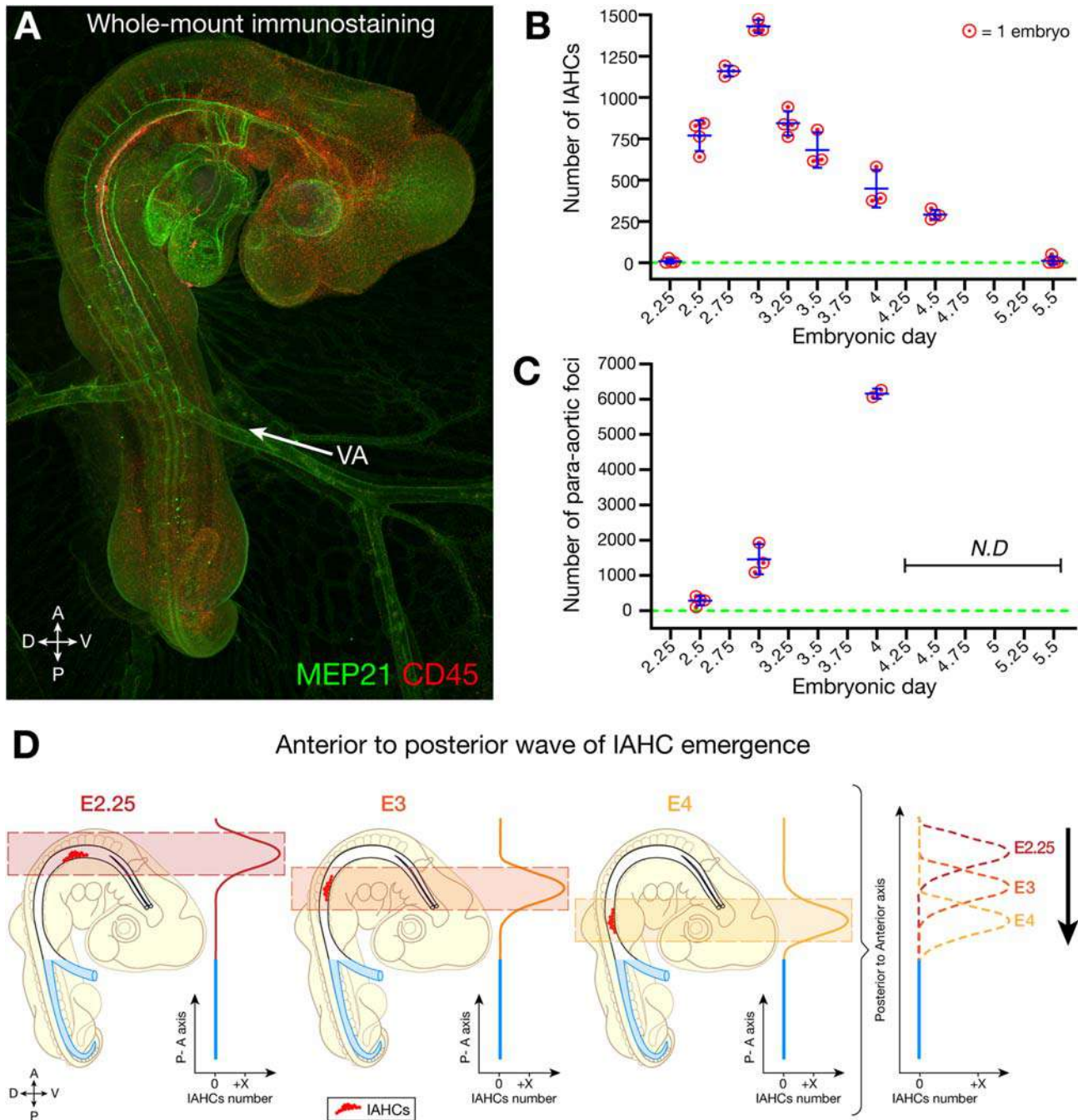


FIGURE 3.5 Quantification and spatiotemporal emergence of IAHCs. (A) Maximum projection of an E3 chicken whole-mount immunostaining. The blood vessels are visualized in green with an anti-MEP21 antibody, while HCs are visualized in red using an anti-CD45 antibody. (B) Quantification of the number of IAHC cells present within the aorta along the development of the chicken embryo, using the whole-mount immunostaining procedure. (C) Quantification of the number of paraaortic foci cells present underneath the aorta along the development of the chicken embryo, using the whole-mount immunostaining procedure. (D) Schematic representation of the chicken IAHC emergence occurring as a wave along the anterior to posterior axis of the embryo. IAHCs are first present more concentrated in the anterior portion of the aorta (E2.25), then are more present in the middle portion (E3) and finally are more located close to the aorta-vitelline artery connection (E4). Chicken IAHCs are never found posterior to the aorta-vitelline artery connection. *IAHCs*, Intraaortic hematopoietic clusters. Adapted from Yvernogeau L., Robin C. Restricted intra-embryonic origin of bona fide hematopoietic stem cells in the chicken. *Development*. 2017;144(13):2352–2363.

the ventral part of the aorta? The only reasonable explanation could solely come from the adjacent tissues present in these specific areas. The microenvironment in these specific locations must provide information/molecular cues driving this peculiar EHT to occur. What are those crucial signals? This will be the matter of Chapter 3.8.

3.7 Ecs of the late fetus/young adult bone marrow harbor hemogenic potential and generate multilineage hematopoiesis

Pouget et al. [59] demonstrated the pivotal role of the somite in vascularizing the body wall and limbs. Yvernogeu et al. [79] identified the somite as the source of BM vascularization by tracing the BM-forming ECs through experimental embryology, genetic labeling, and live imaging approaches and subsequently by analyzing their hematopoietic progeny during late embryogenesis and in adults. We demonstrated that the late fetal/young adult BM contains hemogenic ECs capable of producing HSCs and HCs that are released into the blood circulation before colonizing the secondary hematopoietic organs. Phenotypic and functional analyses showed that BM hemogenic endothelium-derived HCs are mainly multipotent progenitors (MPPs) and a few HSCs. These cells harbor a specific molecular signature close to cells

undergoing EHT or recently emerged HSCs with a prominent signature for the Notch pathway, endothelial-specific genes, and transcription factors involved in EHT [79].

To determine the developmental origin of the BM hemogenic ECs, we used a combination of experimental embryology, genetic and transcriptomic approaches, and functional analyses on the chicken and mouse models. To trace the endothelial derivatives of the somite in the chicken, we performed isotopic and isochronic grafts of GFP⁺ chicken PSM into WT chicken embryos at E2 (15 somite pairs). GFP⁺ somite-derived cells were followed in individual grafted chickens from the time of grafting (E2) to the adult stage by performing immunostaining and flow cytometry analyses on circulating blood, embryo sections, or whole-mount organs. A collection of endothelial/hematopoietic antibodies (MEP21) or hematopoietic-specific antibodies against CD45 (PTPRC, pan-hematopoietic marker), CD41 (ITGA2B, HSPC marker), c-KIT (CD117, HSPC marker), KUL1 (macrophage/monocyte marker), and CD3 (T-cell marker) were used. The contribution of GFP⁺ cells to the BM was extensively analyzed at E16/E17, a developmental stage when sufficient BM cells could be observed or collected for flow cytometry analyses. Whole BM mounts showed the colonization of the grafted limb by GFP⁺ cells and the presence of a dense GFP⁺ vascular network in the forming BM (Fig. 3.6A–E). However, a subpopulation of

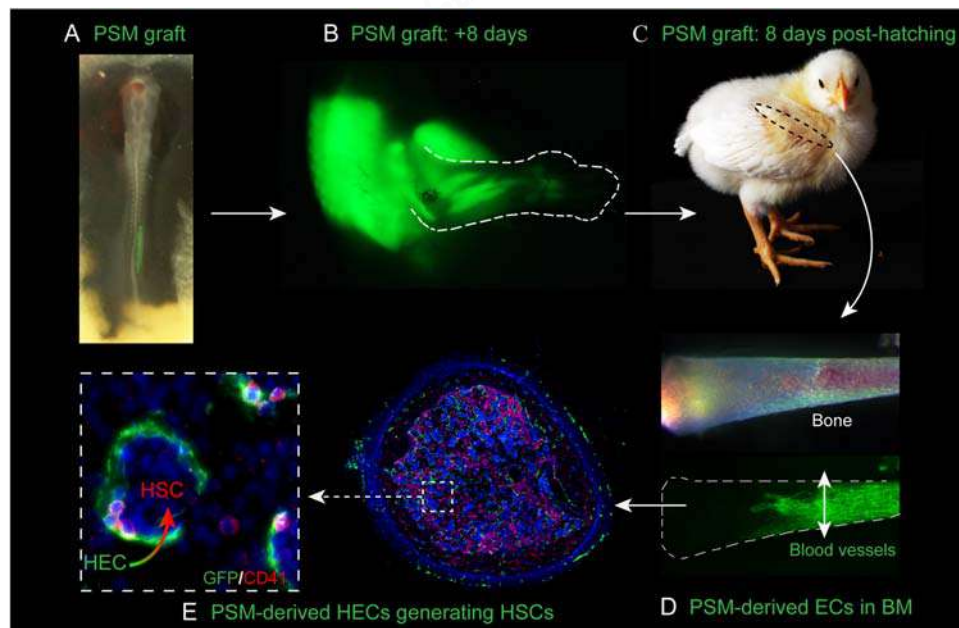


FIGURE 3.6 ECs of the bone marrow (BM) generate a cohort of HSPCs. (A) GFP⁺ PSM is grafted into a WT chicken embryo at the presumptive wing bud level. (B) GFP⁺ PSM-derived cells colonized the limbs (GFP⁺ muscle masses are visible). (C) Eight days after hatching, a slightly color pigmentation difference is visible at the wing level reflecting of the graft surgery. (D) The BM of the grafted wing is colonized by GFP⁺ PSM-derived cells that had formed the vascularization. (E) The whole vascularization of the grafted wing is made of GFP⁺ derived cells that also contributed to the generation of new HSCs through an EHT process. *BM*, Bone marrow; *EHT*, endothelial-to-hematopoietic transition; *EC*, endothelial cell; *GFP*, green fluorescent protein; *WT*, wild-type. Adapted from Yvernogeu L., Gautier R., Petit L., Khoury H., Relaix F., Ribes V., et al. *In vivo* generation of haematopoietic stem/progenitor cells from bone marrow-derived haemogenic endothelium. *Nat Cell Biol.* 2019;21(11):1334–1345.

GFP⁺CD41⁺, and to a lesser extent of GFP⁺CD45⁺ cells, was tightly associated with the GFP⁺ vascular sinusoids (Fig. 3.6E). The coexpression of blood cell markers and GFP (as shown on confocal sections) clearly demonstrated that a fraction of CD45⁺ and CD41⁺ cells arose from GFP⁺ somite-derived ECs in the BM.

To ascertain the hematopoietic production *in situ*, we adapted the previously described time-lapse live confocal imaging [80,81] to thick slices of BM from E16/E17 grafted chicken embryos that were stained with anti-CD41 antibodies. We witnessed flat GFP⁺CD41⁺ ECs rounding up while expressing CD41⁺ (as shown by CD41 staining after overnight imaging), therefore demonstrating the EHT [79].

In the grafted limbs, GFP⁺ cells represented an average of $1.10\% \pm 0.53\%$ of the total BM mononucleated cells that were found unevenly distributed among the endothelial and hematopoietic lineages. Strikingly, the GFP⁺ cells significantly contributed to the BM hematopoietic production, representing $13.7\% \pm 7.3\%$ of the CD41⁺ cells, $3.0\% \pm 1.3\%$ of c-Kit⁺ cells, $7.4\% \pm 2.9\%$ of CD3⁺ cells, to $14.2\% \pm 5.1\%$ of the MEP21⁺ population, and $0.97\% \pm 0.52\%$ of CD45⁺ cells. On the other hand, $42.9\% \pm 5.4\%$ of the GFP⁺ cells were MEP21⁺ ECs/HCs, whereas $32.4\% \pm 10.4\%$, $28.4\% \pm 8.7\%$, $17.2\% \pm 4.7\%$, and $5.0\% \pm 2.8\%$ of the GFP⁺ cells were CD45⁺, CD41⁺, c-Kit⁺, or CD3⁺, respectively. Moreover, the MEP21⁺CD45⁺ population, known to be enriched in multipotent progenitors and representing 2.4% of the mononucleated cells, harbored 15.2% of GFP⁺ cells. Of note, no obvious difference was found in the hematopoietic composition of the BM between grafted and WT embryos. Long-term analysis of the grafted chicken embryos revealed that secondary hematopoietic organs were colonized by GFP⁺ cells. For example, GFP⁺ T cells were visible in the thymic lobules of E16/E17 grafted recipients. A rather small but clear GFP⁺ B cell population was also present in the spleen and the bursa of Fabricius, therefore supporting the multilineage contribution by the BM endothelium-derived HC population.

GFP chimeras were also examined at different time points posthatching and analyzed for the presence of GFP⁺ cells associated with endothelial and hematopoietic lineages in the BM for up to 41 weeks. We observed a continued presence of GFP⁺ cells in the BM and a rather similar distribution of GFP among the endothelial and hematopoietic fractions compared to E16/E17 embryos. Therefore the data demonstrate that chicken BM hemogenic ECs produce multilineage HCs/HSCs capable of colonizing secondary hematopoietic tissues in the long term. Moreover, phenotypic and functional analysis in the mouse showed that this production is conserved between species and that the hematopoietic production mainly contains multipotent progenitors of MPP3 type and a few HSCs.

Overall, our study has proved that HSCs and multilineage HC progenitors can be produced in the BM through a hemogenic endothelium intermediate past the YS and embryonic aorta stages. This production is conserved between chicken and mouse species. This important knowledge should help in the design of therapies for hematopoietic disorders.

3.8 Spatial transcriptomics in the chicken embryo reveals regulators of hematopoiesis

Since HSCs are found in specific locations in the adult BM, it was suggested that cells surrounding HSCs might be crucial for their fate. It is now four decades ago that Schofield [82] proposed the concept of a niche, that is, cells forming a microenvironment for HSCs that regulate their quiescence, self-renewal, or differentiation/proliferation. The concept of a niche within the BM is now accepted and well documented and was proposed to exist also within the embryo. Indeed, if HSC emergence occurs during a specific time window of embryonic development and in specific locations, a microenvironment allowing this emergence to occur must exist. In the mouse embryo, several signaling pathways are critical for HSC emergence. To name a few shown to be critical for HSC emergence in the aorta: interleukin 3 (IL3), the stem cell factor, BMP4, the catecholamines produced by the sympathetic nervous system, the retinoic acid signaling pathways [83–87], for a review see Ref. [88]. In the avian model, experimental block in the migration of the subaortic mesenchyme demonstrates the critical role of this tissue for RUNX1 expression and aortic hematopoiesis [89].

Obtaining a global picture of all the molecular players expressed by the surroundings of the aorta during HSC emergence would be the “holy grail.” For this purpose, the chicken appears as a model of choice for two specific features: (1) IAHC/HSC emergence is restricted to the most anterior part of the embryo and the ventral side of the aorta [26,57,78]. (2) The number of IAHCs generated for each developmental time point is now quantified [75], and see Chapter 3.6 above). Therefore the anterior and ventral mesenchyme of the aorta must provide molecular signals allowing IAHC/HSC emergence to occur, while the posterior and dorsal mesenchyme should provide inhibitory or null signals. Based on these criteria, the Robin Laboratory recently adapted a genome-wide RNA tomography approach, also called tomo-sequencing [90], which allows to perform RNA sequencing of an entire tissue/embryo section keeping the spatial information, on the chicken embryo. Tomo-sequencing consists in doing cryosections of an unfixed embryo/tissue/slice according to a defined axis (Fig. 3.7A). Every cryosection is collected into single Eppendorf tubes;

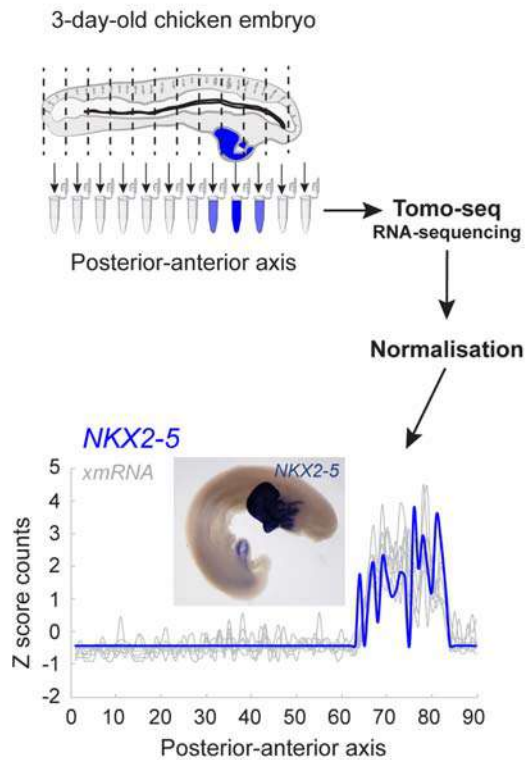
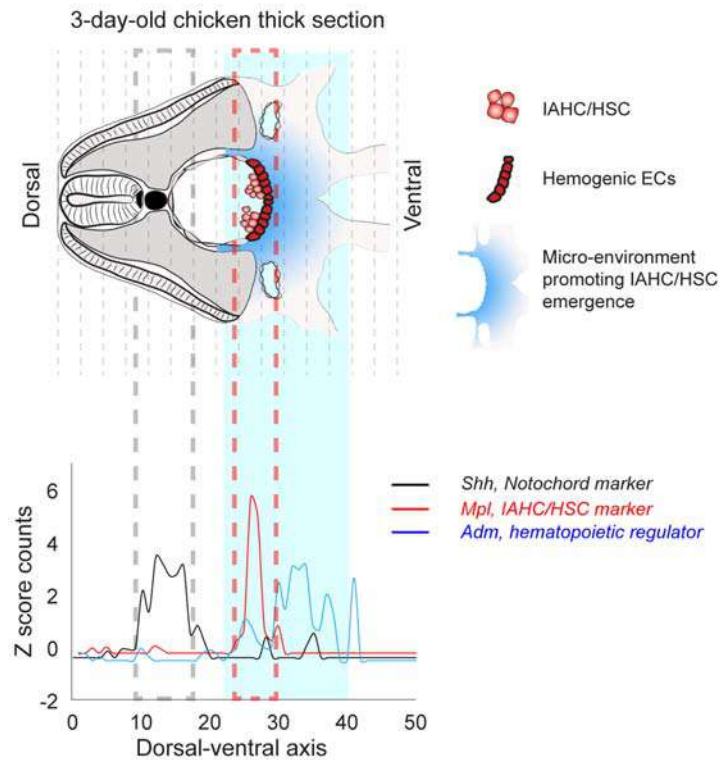
A/ Tomo-sequencing principle**B/ Tomo-sequencing on transverse thick slice**

FIGURE 3.7 Tomo-sequencing on chicken embryos identifies new regulators of hematopoiesis. (A) Principle of the tomo-sequencing technique applied to the chicken embryo. An E3 chicken embryo (without the head) is cryo-sectioned along the posterior to the anterior axis. Every section is collected into Eppendorf tubes. RNA is extracted for each tube and sequenced. After normalization, RNA expression of a gene of interest can be visualized along the axis that was chosen for the cryo-sectioning, as shown as an example for *Nkx2.5*. (B) Tomo-seq is applied on thick transverse slices of chicken embryos to unravel genes more expressed in the ventral mesenchyme (located underneath the aorta) and to identify new hematopoietic regulators. Adapted from Yvernogeu L., Klaus A., Maas J., Morin-Poulard I., Weijts B., Schulte-Merker S., et al. *Multispecies RNA tomography reveals regulators of hematopoietic stem cell birth in the embryonic aorta*. *Blood*. 2020;136(7):831–844.

RNA is extracted for all tubes and sequenced (Fig. 3.7A). After normalization, it is then possible to visualize RNA expression profiles along the axis that was chosen during the cryo-sectioning, as shown for *Nkx2.5* expression on a whole chicken embryo (Fig. 3.7A). Yvernogeu and coworkers applied this technique on whole chicken embryo and thick transversal slices at crucial developmental stages for hematopoiesis [91]. The authors could easily identify known anatomical structures such as the notochord (based on the expression of *SHH*) and also the region containing IAHCs (based on *MPL* expression). They could therefore determine regions of interest, that is, the ventral mesenchyme located underneath the aorta, to look for genes that were specifically expressed in this area (blue area in Fig. 3.7B). Using this approach and by applying the same technique on human, mouse, and zebrafish embryos, they unraveled the common molecular landscape of the ventral mesenchyme of the aorta that supports IAHC/HSC emergence *in vivo* [91]. Notably, they identified ADM (Adrenomedullin, a hypotensive and vasodilator agent) (as

shown as an example in Fig. 3.7B) but also SVEP1 (Sushi, Von Willebrand Factor Type A, EGF, and Pentraxin Domain Containing 1, a secreted extracellular factor critical for proper lymphangiogenesis) as new and conserved regulators of IAHC/HSC emergence in the aorta.

Because the authors applied the tomo-sequencing on four different species for several developmental points and in different axes of the tissues, they also provided a useful website where all samples that were processed can be found and where one can visualize how any genes of interest are expressed along the axis of the tissues processed ([91]; <http://multi-species.embryos.tomoseq.genomes.nl>).

Validation of potentially more HSC regulatory genes will be the next step of this unique study. Also, it remains to be determined how to integrate all these RNA-seq datasets obtained in different species for different time developmental time points to analyze in-depth and identify the key molecular players and the important signaling pathways involved in IAHC/HSC emergence [92,93].

3.9 The avian thymus and T-cell development

3.9.1 Thymic development

Avian T-cell development is remarkably similar to that of mammals, although it has some unique features. Using quail–chicken chimeras, it was shown that the thymus becomes populated with cells originating in the three germ layers (i.e., ectoderm, mesoderm, and endoderm). Stromal epithelial cells form a network, which contains abundant lymphoid cells in both the cortex and medulla. These stromal cells originate from endodermal buds arising from the third and fourth branchial pouches (for further details, see Chapter 2). The connective tissue, which separates the thymic lobes and lobules, and the pericytes and smooth muscle cells associated with intrathymic blood vessels are derived from the ectoderm via the neural crest. The hematopoietic component, which differentiates in the thymus into T lymphocytes, macrophages, and medullary dendritic cells, is derived from the mesoderm. The endothelial network originates from the mesoderm [94,95]. The mesenchymal cells, which participate in thymic histogenesis, are critical for the differentiation of the endodermal thymic stroma. As shown using quail–chicken chimeras, in the head of the vertebrate embryo there are supporting tissues (bone cartilage, connective cells but not striated muscles), which originate from a unique type of mesenchymal cells, that originate in the neural crest, the so-called mesectoderm.

Mesenchymal cells are closely associated with the endodermal thymic rudiment which, in chicken and quail embryos, first appear on E3 on either side of the pharynx as two buds from the third and fourth pharyngeal pouches (in the mouse, a unique thymic bud arises from the third pharyngeal pouch). One day later, the thymic rudiments have detached from the pharynx and form two compact cords of epithelial cells which, eventually, fuse and become surrounded by blood vessels. Further development of the thymic stroma depends on signals arising from the surrounding mesenchymal cells. These signals are responsible for the rapid transformation of the thymic epithelia into a lymphoid organ. The well-defined cortex and medulla are established by E12 [96].

3.9.2 Colonization of the thymus

That all lymphocytes developing in the thymus are of extrinsic origin was demonstrated by experiments in which the thymic rudiment was removed at regular intervals from E3 to E7 quail or chicken embryos and grafted into the somatopleura of the other species. It was shown that the thymic epithelium becomes attractive to blood-borne HC at precise stages in development. T-cell

progenitors colonize the epithelial thymus in three successive waves, the first beginning at E6, the second at E12, and the third occurring around E18 until just after hatching [97] (Fig. 3.1). Each wave of thymic colonization lasts 1 or 2 days and is separated from later waves of progenitor cell influx by refractory periods. Although no information about the internal clock governing thymic influx is available, it has been demonstrated that each wave of colonization is correlated with a peak in the number of progenitor T cells present in the blood circulation [50]. Progenitor cells are almost completely absent from the blood during the refractory periods. Experiments in which T-cell progenitors have been injected intravenously have clearly shown that these cells are able to home to the thymus without delay, even during the refractory periods [50]. Adoptive transfers have shown that T-cell progenitors in the first wave originate from paraortic foci, whereas progenitors in the second and third waves originate from the BM [27,50,98]. Some molecules expressed on the surface of the BM T-cell progenitor have been identified, these include c-kit, HEMCAM, MHC class II antigens, and $\alpha\text{II}\beta\text{3}$ integrin [99]. Interestingly, expression of the terminal deoxynucleotidyl transferase (TdT) appears on thymocytes at E12, that is, after the first wave, and increases linearly thereafter suggesting that the second generation of lymphocytes becomes TdT positive [100,101]. TdT is the crucial enzyme involved in the non-germline nucleotide addition (N-region) during V(D)J T-cell receptor (TCR) gene segment recombination and can be regarded as an early T-cell marker [102]. Expression of TdT appears at a critical developmental stage for the thymic lymphocytes; namely, when they begin to be immunologically competent.

3.9.3 T-cell differentiation

Progenitors in each wave give rise to $\gamma\delta\text{T}$ cells about 3 days earlier than the appearance of $\alpha\beta\text{T}$ cells. The development of each T-cell lineage can be traced using mAb recognizing different chains of TCR. At E12, about 5 days after the initial influx of thymocyte precursors, a subpopulation of thymocytes begins to express the $\gamma\delta\text{TCR-CD3}$ complex on the surface (recognized by the TCR1 mAb) [103]. Numbers of TCR1⁺ cells increase reaching a peak by E15 when about 30% of the thymocytes express the $\gamma\delta\text{TCR}$. Cells that express the $\gamma\delta\text{TCR-CD3}$ complex fail to express either CD4 or CD8 accessory molecules. $\alpha\beta\text{TCR}$ -bearing T cells which express the V β 1 variable domain (recognized by the TCR2 mAb) are first detected in the thymocyte population on E15, and these become the predominant type of thymocytes by E17–18 [96]. $\alpha\beta\text{TCR}$ -bearing cells expressing the V β 2 variable domain (recognized by the mAb TCR3) emerge around E18. The developmental

patterns of TCR2⁺ cells and TCR3⁺ cells appear to be very similar to those described for mammalian $\alpha\beta$ T cells, where there is dual expression of the CD4 and CD8 accessory molecules on the surface of both TCR2⁺ and TCR3⁺ cortical thymocytes [97]. After expansion in the cortex, these CD4⁺CD8⁺ double-positive cells undergo clonal selection and maturation to become either single positive CD4⁺ or CD8⁺ T cells [104].

Recently, using in silico and gene synteny-based approaches, the pre-TCR α chain (pT α) gene (*PTCRA*) was identified in four sauropsids (three birds and one reptile) [105]. In humans and mice, the pT α is covalently associated with the TCR β chain at the immature thymocyte surface to form the pre-TCR. Pre-TCR controls thymocyte development in a ligand-independent manner through self-oligomerization mediated by pT α . *PTCRA* organization appears highly similar in sauropsids and mammals with four different exons. However, comparative analyses of the pT α functional domains indicate that sauropsids display a short pT α cytoplasmic tail and lack most residues shown to be critical for human and murine pre-TCR oligomerization. These findings suggest that the ancestral function of pT α was exclusively to enable expression of the TCR β chain at the thymocyte surface and to allow binding of pre-TCR to the CD3 complex.

3.9.4 TCR rearrangement

Genes encoding the α , β , γ and δ chains of the chicken TCR have been cloned [106]. The TCR β locus contains two V β gene families, 14 D β gene segments, 4 J β elements, and a single C β gene. The two V β families contain six and four V β elements, respectively. TCR β rearrangements involving gene segments from the V β 1 gene family can be detected beginning on E12, while rearrangements involving the V β 2 family are first detected on E14 [107]. As with mammals, the chicken TCR δ gene locus is nested within the TCR α locus. Several V α and multiple J α elements are associated with a single C α region. The large number of J α elements could favor successive rearrangements of the TCR α locus and therefore enhance the production of in-frame rearranged TCR α chains. One V δ gene family (20–30 V δ elements), two D δ gene segments, two J δ gene segments, and one C δ gene have been identified. V α and V δ gene segments rearrange with one, both or neither of the D δ segments and with either of the two J δ segments. Three different V γ families, each with about 10 members, three J γ elements, and a single C γ region have been identified in the chicken [108]. Random V, (D), J rearrangements, exonuclease activity, template (P), and nontemplate (N) nucleotide addition contribute to the diversification of the TCR repertoire during avian T-cell differentiation. This process is described in detail in Chapter 5.

3.10 The bursa of Fabricius, B-cell ontogeny, and immunoglobulins

3.10.1 Bursal development

The bursa of Fabricius is a primary lymphoid organ in birds that is responsible for the amplification and differentiation of B lymphoid progenitors within its follicular microenvironment. As described in Chapter 3, the bursa has been important in the discovery of the dual function of the immunologic response with a thymus-dependent and a humoral (bursal-dependent) arm [109,110]. Surgical removal of the bursa during the early embryonic period results in impairment of the humoral immune system. Bursectomy of late-stage embryos or neonate chicks causes a marked reduction in the number of circulating B lymphocytes and an inability to produce specific antibodies in response to antigenic challenge. This demonstrates that the bursa provides a unique microenvironment essential for the proliferation and differentiation of B cells [111].

In the chicken, the bursa of Fabricius is a chestnut-size, sac-like organ located dorsal to the rectum, anterior to the sacrum communicating with the posterior portion of the cloaca by a short duct. Its inner surface contains 12–20 longitudinal folds tightly packed with lymphoid follicles and is covered by interfollicular and follicle-associated epithelium (FAE). The chicken bursa of Fabricius consists of around 12,000 follicles, [111] composed of an outer cortical part of mesodermal origin and a medullary part of ectodermal origin. Individual follicles are separated from one another by a collagen type I positive connective tissue layer. Each follicle contains about 2 to 4 $\times 10^5$ cells (immunoglobulin expressing B lymphocytes, dendritic cells, macrophages, and epithelial cells). During ontogeny of the bursa of Fabricius, extensive interactions between two embryological different compartments (ectodermal epithelium of the anal invagination and mesenchyme of the tail bud) lead to the formation of the bursal follicles into which, as a third player, dendritic cell and B-cell precursors immigrate.

This complex developmental process is regulated in time and space. Morphologically, several well-delineated stages can be identified: (1) formation of a vesicle-like bursal epithelial anlage in the tail-bud mesenchyme; (2) HC colonization of bursal mesenchyme and immigration of blood-borne cells into bursal epithelium to initiate follicle formation; and (3) formation of the follicular cortex around hatching (Fig. 3.8).

3.10.2 Formation of the bursal epithelial anlage

The bursa of Fabricius, similar to the thymus, develops by an epithelial–mesenchymal interaction. The bursal

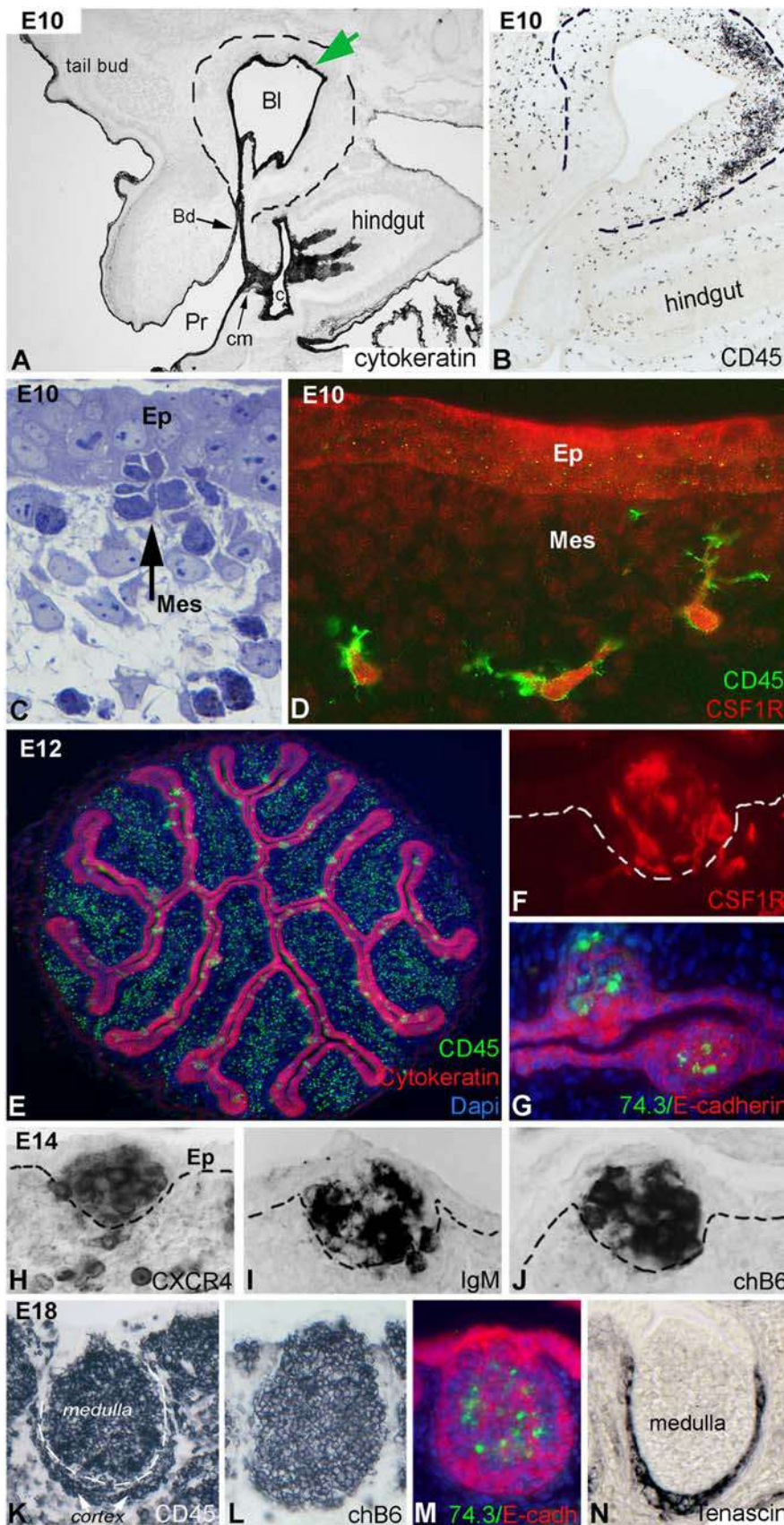


FIGURE 3.8 Bursal development. *Formation of bursal anlage.* (A–D) Sagittal sections of E10 chicken embryo. (A) Cytokeratin immunostaining indicates connection of the proctodeum (Pr) to bursal duct (Bd) and bursal lumen (Bl). (B) At this stage, the CD45⁺ hematopoietic cells are scattered over the bursal mesenchyme and migrate toward the epithelium. (C) Semithin section; darkly stained basophilic cells (BSDC precursors) accumulate under the epithelium and enter the surface epithelium (arrow). (D) CSF1R⁺ cells (darkly stained basophilic cells) with dendritic morphology accumulate under the epithelium. *Formation of the medullary region of follicles.* (E) Cross-section of E12 chicken bursa of Fabricius, showing CD45⁺ (green) HCs colonizing the mesenchyme and entering the cytotokeratin⁺ (red) surface epithelium. (F,G) Follicle buds begin to form when blood-borne CSF1R⁺/74.3⁺ precursors of bursal DCs (green) cross the basement membrane and form clusters among E-cadherin⁺ epithelial cells (red). (H–J) At E14, the developing follicle contains phenotypically functional, CXCR4⁺/chB6⁺/IgM-producing B lymphocytes. The dashed line shows the basement membrane. *Development of the follicular cortex.* (K, L) E18 chicken bursa of Fabricius; CD45⁺ cells fill up the bursal follicles, and most of the HCs express the chB6 chick-B cell-specific antigen. (M) 74.3 mAb (green) labels the DCs inside the E-cadherin⁺ (red) epithelial cells of the follicular medulla. (N) Strong tenascin⁺ extracellular matrix (ECM) appears in the follicular cortex, which subsequently receives chB6⁺ B cells. Mesenchymal reticular cells are the cortical supporting cells, which produce ECM proteins. No histologically or immunocytochemically identifiable ECM are present in the follicular medulla.

rudiment is colonized later by HCs of extrinsic origin. In the chicken embryo, the epithelial anlage of the BF emerges on E5 as a vesicle-like diverticulum of the proctodeal region of the cloaca [112,113]. The proctodeum is the third, distal-most chamber of the avian cloaca; it develops from the ectoderm-derived anal invagination. The ectodermal epithelium is juxtaposed with endoderm-derived uroanal epithelium (the middle cloacal chamber, receiving the ureters and allantois), which forms the cloacal membrane.

At E5–7, the dorsal epithelial wall of the cloacal membrane shows extensive vacuolization, and fusion of these vacuolized epithelial compartments results in the formation of bursal lumen. Twenty-four hours later, the bursal lumen becomes continuous with the proctodeum through a short bursal duct, thereby permitting communication with the amniotic cavity. Sonic hedgehog growth factor is universally secreted by the whole hindgut and cloacal endoderm, but it is not expressed by ectoderm-derived proctodeum and bursal epithelium [114]. This expression difference raises the possibility that the epithelial anlage of the bursa of Fabricius is of ectodermal origin.

Quail–quail chimeric bursa generated by recombination of quail tail-bud ectoderm and chicken tail-bud mesenchyme support the central role of the ectoderm in the formation of the bursal epithelial anlage [114]. By E8, the bursa consists of the E-cadherin⁺/cytokeratin⁺ pseudostratified cuboidal epithelium surrounded by a loose network of undifferentiated vimentin⁺ mesenchyme. At about E9–10, the BF looks like a hollow organ connected to the cloaca (Fig. 3.8A). After 24 hours, the increased proliferation of mesenchymal cells initiates the formation of 11–13 longitudinal folds covered by epithelium; each fold contains a central artery and vein.

3.10.3 Hematopoietic colonization of the bursal rudiment and follicle bud formation

Colonization of the bursal epitheliomesenchymal rudiment by HCs takes place in two steps [112,115–117]. It is a unique event restricted to a window occurring at E8–15. At first, incoming CD45⁺ HCs enter the bursal rudiment and seed into the richly vascularized bursal mesenchyme (Fig. 3.8B). Among these CD45⁺ HCs, highly basophilic “dark cells” (“dark” because they are heavily stained by toluidin blue on semithin sections) appear (Fig. 3.8C). By E10, the highly basophilic CD45⁺ cells with ramified morphology express CSF1R (colony-stimulating factor 1 receptor; Fig. 3.8D) and MHC class II antigens and migrate close to the surface epithelium, locating in small niches under the basement membrane. From the niches, the cells penetrate the basement membrane and

appear in the surface epithelium. Intensive migration of CD45⁺/MHC II⁺/CSF1R⁺ cells into the epithelium is preceded by the appearance and increased production of CXCL12 (SDF-1, stromal cell-derived factor) chemokine [118], plasminogen activator [119], and alkaline phosphatase [120] enzymes in the subepithelial mesenchyme. CXCL12 signaling regulates mesenchyme differentiation by promoting alkaline phosphatase activity.

It has been suggested that increased alkaline phosphatase activity and high protease secretion modify the mesenchymal environment, allowing the ramified CD45⁺/MHC II⁺/CSF1R⁺ cells to migrate through the basement membrane (Fig. 3.8E and F). During the clustering and invasion of the cells into surface epithelium of the bursal folds, the CSF1R⁺ cells start to produce vimentin-intermediate filament, express NIC2 and 8F2 antigens (putative CD11c) and elaborate small granules that are positive for the dendritic cell-specific 74.3 monoclonal antibody (Fig. 3.8G) [114,121]. Interaction of CD45⁺/MHC II⁺/74.3⁺/vimentin⁺ cells with the surface epithelium stimulates local proliferation of epithelial cells, which consequently initiates epithelial follicle bud formation (Fig. 3.8F–J). Follicle buds are formed in asynchronous manner because new buds emerge at any time E11–15.

Transmission electron microscopic studies and quail–chicken chimeras demonstrated that the CD45⁺/MHC II⁺/74.3⁺/CSF1R⁺ cells are the inducer cells for follicle bud formation and represent the precursors of bursal secretory dendritic cells (BSDC) [116,117]. Furthermore, recent experiments using GFP (green fluorescent protein)-YS chimeras demonstrated that the hematopoietic precursors of CD45⁺/MHC II⁺/74.3⁺/CSF1R⁺ BSDC originate from CD45⁺ ramified YS-derived cells [122]. Epithelial cells of the follicle buds and the precursors of BSDC form a transitory dendroepithelial tissue [112,115,123] that creates the bursa-specific microenvironment for B-cell differentiation (Fig. 3.9). This environment receives B-cell precursors to establish lymphoepithelial tissue resulting in B-cell maturation.

Formation of dendroepithelial tissue is highly sensitive for testosterone treatment. Testosterone-treated and control embryos develop normally up to the follicle formation stage (E10), but further development of the bursa is severely inhibited in testosterone-treated birds: The mesenchymal cells become elongated and densely packed under the vacuolated epithelium, inhibiting the migration of B cell precursors from the mesenchyme into the surface epithelium [112,124]. In the testosterone-treated embryos, alkaline phosphatase activity is almost completely inhibited, which eliminates follicle bud formation [120]. Testosterone treatment does not prevent the entry of HCs into the bursal mesenchyme because granulocytes and macrophages appear in it, but the follicle bud

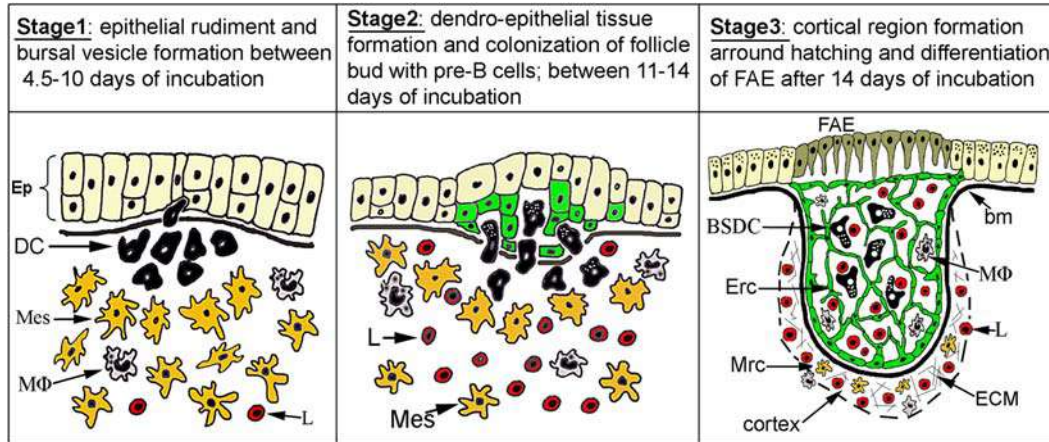


FIGURE 3.9 Schematic of development of the bursal follicles. Stage 1: Before follicle bud formation, blood-borne dendritic precursor cells (DC) accumulate under the surface epithelium (Ep) of the mesenchymal (Mes) rudiment of the bursa of Fabricius, while other immigrating HCs are the macrophages ($M\Phi$) and B-lymphocyte precursors (L). Stage 2: Follicle bud formation: DCs migrate into the epithelium, inducing the follicle bud. Dendritic and epithelial cells create “dendroepithelial tissue” to receive pre-B cells. Stage 3: The uppermost epithelial cells of the follicle buds become the follicle-associated epithelium (FAE). Lymphocytes (L) differentiate inside the follicles and migrate out to of the epithelial cells to establish the cortex. BSDC, bursa secretory dendritic cells; *bm*, basement membrane; *ECM*, extracellular matrix; *Ep*, epithelium; *Erc*, epithelial reticular cell; *Mes*, mesenchyme; $M\Phi$, macrophages; *Mrc*, mesenchymal reticular cell.

inducer cells, the precursors of BSDC, are absent [124]. Thus testosterone treatment influences the differentiation of BSDC precursors. Few of the $CD45^+$ HCs that first colonized the bursa of Fabricius do not reach the surface epithelium and become engaged in $Gr1-1^+/Gr1-2^+$ granulocyte and $KUL01^+/74.2^+/68.2^+$ macrophage differentiation pathways [117,125,126]. The next phase of bursal development is pre-B cell colonization. Significant numbers of pre-B cells enter the BF mesenchyme during a restricted window of time (E10–15) [116,117]. They emerge in the bursal mesenchyme before follicle formation, providing evidence for extrabursal B-cell differentiation [111,127].

The first committed B-cell precursors, the prebursal stem cells that colonize the bursa, are characterized by the expression of CD45, the chicken B cell marker chB6 (formerly Bu-1 antigen), CD1, the sialyl-Lewis^x carbohydrate epitope and CXCR4 [115,117,128]. Experiments using quail–chicken chimera, parabiosis and species-specific antibodies proved that both the dendritic cell and B-cell precursor cells migrate into the bursa primordium through the blood [129]. This is also supported by the expression of sCD15 (sialyl-Lewis^x carbohydrate), which suggests a selectin-mediated migration from the circulation. Starting on E10, the $chB6^+$ pre-B cells leave the blood vessels and migrate toward the dendroepithelial follicle buds (Fig. 3.8H–J). Immigration of B-cell precursors into follicle buds transforms the dendroepithelial tissue into lymphoepithelial tissue, establishing the bursal follicular medulla. Based on parabiotic experiments, each follicle bud is initially colonized by 2–3 pre-B cells and continues to develop by extensive cell proliferation [130].

The first surface IgM^+ B cells are detected from E12 onward only in the follicle bud; at hatch, more than 90% of bursal cells are mature B cells. These cells have already undergone Ig gene rearrangement at sites of hematopoiesis—namely, the paraaortic foci, spleen, and BM [131,132]. These Ig gene rearrangements occur in the absence of TdT, generating only minimal antibody diversity [133]. Cells that fail to express surface Ig are eliminated by apoptosis; only B-cell precursors that productively rearrange the Ig gene express cell surface Ig and are selected for expansion in bursal follicles. Subsequently, the rearranged variable region, which generates Ig specificity, undergoes somatic diversification by a process of intrachromosomal gene conversion in which blocks of nucleotide sequence are transferred from the pseudo V genes into the unique functional rearranged VH and VL genes (Fig. 3.10). The process of Ig gene conversion initiates in the proliferating pool of developing B cells at E15–17 and continues until bursal involution (for details see Chapter 4).

B cells undergoing Ig gene conversion switch from sialyl-Lewis^x to Lewis^x antigen and express the chB1 gene, which is a proapoptotic receptor [128,134,135]. Diversification by segmental gene conversion can result in the creation of an Ig repertoire of at least 10^{11} distinct antibody molecules [136]. Since gene conversion occurs only in the bursal follicles, this process might be regulated by the stromal cells of the follicles (BSDCs, reticular epithelial cells). The molecular nature of the stromal microenvironment and the regulatory processes are not known, but it is thought that the secretion product of dendritic cells may have a crucial role [137]. BSDCs contain large numbers of secretory granules in their cytoplasm,

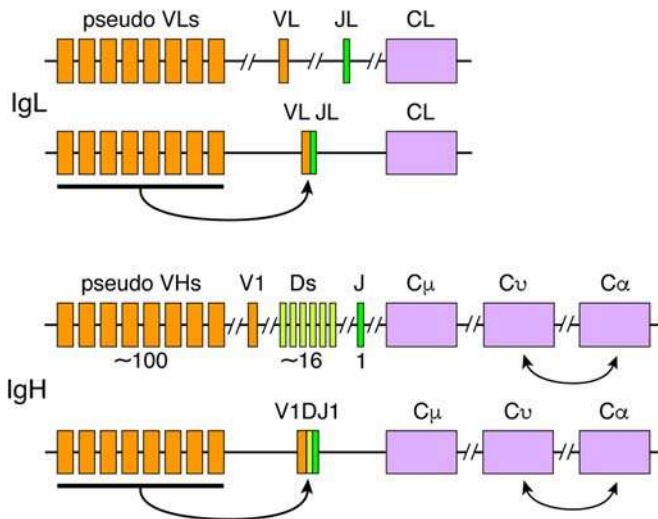


FIGURE 3.10 Development of the avian B-lymphocyte repertoire. Generation of chicken antibody repertoire. The chicken Ig light (L) and heavy (H) chain loci possess only a single functional variable gene (V_L and V_H) downstream of clusters of V pseudogenes. Between the two loci, there is a unique J (J_L and J_H) segment. About 15 functional D_H elements are present in the IgH chain locus. The 3' ends of the two loci contain genes encoding the constant (C) region of the Ig chains. In developing B cells, the only functional V_L gene rearranges with the only J_L segment. Similarly, the only functional V_H gene rearranges with one D segment and the only J_H segment. Diversity is, thence introduced into the rearranged V(D)J segments by somatic gene conversion using DNA sequence donated from V pseudogenes, as described schematically by an arrow.

and the release of biologically active molecules from the granules provides survival and differentiation signals to B cells [125].

The effects of the mesenchymal environment in which pre-B cells migrate, proliferate, and differentiate may be mediated by various mechanisms: via molecules expressed on their surface or via secreted molecules that signal to them. During follicle development, pre-B cells migrate through the mesenchyme, an environment rich in extracellular matrix. Probably, fibronectin is necessary for this colonization process, since B-cell adhesion and migration can be partially blocked when the synthetic CS-1 peptide or functional blocking antibodies against fibronectin receptor are applied [138]. There is some evidence that surface IgM may be involved in colonization because treatment of B cells with antichickan IgM monoclonal antibody can prevent the recolonization of the cyclophosphamide-treated bursa [138]. Protein profile analysis of the developing bursa of Fabricius revealed several proteins associated with Ephrin receptor, fibroblast growth factor receptor and retinoic acid receptor-mediated signal transduction, suggesting a possible role for these proteins in developing bursal B cells and the associated stromal microenvironment [123,139]. Recent experiments demonstrate that chicken CXCL12 chemokine produced by the bursa mesenchyme and its cognate receptor CXCR4 expressed by the pre-B cells are simultaneously expressed during bursal development [126]. *In vivo* inhibition of CXCR4 using its antagonist called AMD3100 at the time of B-cell precursor colonization strongly inhibited follicle development [126]. Similar to this, generation of B cell-specific CXCR4 knockout chicken using the CRISPR/Cas9 system led to chemotaxis inhibition toward CXCL12 source [140]. These results strongly support the idea that CXCL12 chemokine

attracts $chB6^+/CXCR4^+$ prebursal B cells into the bursal anlage. Later during bursa ontogeny, CXCR4 is expressed on $chB6^+$ B cells in follicle buds and the developing follicular cortex following the presence of its chemotactic ligand CXCL12.

3.10.4 Development of the follicle-associated epithelium and the follicular cortex

At E14–15 a new type of epithelium, known as the FAE, develops above the lymphoepithelial compartment of the growing follicle. The FAE does not possess a basement membrane because, as the follicle buds develop, they push the surface epithelial cells toward the mesenchyme, and the basement membrane of the epithelium continues under the corticomedullary border and interfollicular epithelium (Fig. 3.9) [129]. FAE cells are responsible for the transport of antigen (YS proteins and antigens derived from the cloaca flora) between the bursal lumen and the medulla of the follicles; functionally, they are closely related to M cells in cecal tonsils and Peyer's patches [141]. Balic et al., recently demonstrated that CSF1R is expressed by a subset of cells in the FAE and these cells show the putative M cells markers, like annexin 10 and CD44 [142].

Around hatch, a structural rearrangement takes place: B cells start to migrate out from the follicles to establish the cortical area and the original follicle bud, which contains reticular epithelial cells and vimentin⁺/CSF1R⁺74.3⁺ BSDCs, becomes the medullary area (Fig. 3.8K–M). The chalice-shaped cortex of the lymphoepithelial follicles starts to develop around hatch as well. It is separated from the medulla by the basement membrane of the corticomedullary border (Fig. 3.9). Also, around hatch, the first cortical cells appear. Unlike the medulla, the cortex develops

exclusively from the mesoderm and is fully developed by 2 weeks of age. Supporting cells of the cortex are mesenchymal reticular cells, which produce extracellular matrix proteins such as collagens type I and III, fibronectin, and tenascin (Fig. 3.8N); they express CXCL12, vimentin- and desmin-intermediate filaments [115,123,126,143]. The outer surface of the cortex is covered by fine collagen-rich capsules. At about 3 months of age, and well before sexual maturation, the BF undergoes age-dependent involution, and by 6–7 months of age, it is heavily involuted.

3.10.5 Immunoglobulins

Three main classes of antibodies have been described for birds: IgM, IgA, and IgY, the latter is the homolog of mammalian IgG (see Chapter 5). Immunofluorescence staining using mAb to the different Ig- μ and light (L) chain detected IgM synthesis by bursal cells as soon as recognizable lymphocytes appear in the bursa—at about E14 [144]. IgY-producing lymphocytes appeared in bursal follicles later, at around hatch (equivalent to E21). Cells containing IgM were observed outside of the bursa from E17, whereas cells containing IgY chains were not detected at extrabursal (spleen, cecal tonsils, and thymus) sites until 4 days posthatch [145]. Rapid expansion of IgM- and IgY-containing cells begins in the spleen on days 3 and 8 posthatch, respectively. Experiments using bursectomized chickens treated with antibodies against Ig α and Ig- μ indicated that during ontogeny IgM-forming cells are the direct precursors for IgY- and IgA-forming cells by a genetic switchover mechanism [133,145].

The architecture of the chicken heavy (H) and light (L) chain loci is unique in that only single functional V and J segments are present in both loci. Upstream of the functional V gene segment, 80–100 pseudo V_H genes are present in the IgH locus, and 26 pseudo V_L in the IgL locus [146]. About 16 D_H segments are present in the IgH locus [136] (Fig. 3.10).

The Ig chain gene rearrangements are not restricted to the bursa of Fabricius. The site for earliest detection of D_HJ_H rearrangement is the YS, at E5–6. D_HJ_H rearrangements are first detected in the spleen at E6 or 7, in the blood at E8, in the bursa at E9 or 10, in the thymus at E9, in the BM at E10 but in the paraaortic region only at E9 [147]. A systematic lag for the other rearrangements (V_H to DJ_H , V_L to J_L) has been observed: at E9 in the blood and the YS, at E8 or 9 in the spleen, at E10 or 11 in the bursa, and at E11 in the BM and thymus. In the chicken, $V_HD_HJ_H$ and V_LJ_L rearrangements are observed simultaneously, indicating that there is no sequential rearrangement of IgH- and IgL-chain genes. This suggests, in the chicken, the lack of a pre-B cell stage containing a functionally rearranged $V_HD_HJ_H$ sequence prior to IgL gene rearrangement.

3.11 Lymphocyte-differentiating hormones

Several studies have demonstrated the role of polypeptide hormones in T- and B-cell differentiation. Thymus extracts have been shown to induce selective T-cell differentiation of precursor cells from the BM *in vitro*. The active extract has been identified as a thymic hormone, designated thymopoietin, which has been shown to be synthesized by nonlymphoid, epithelial and dendritic cells [148].

Bursal extracts have also been shown to induce both B- and T-cell differentiation; however, their effect on chicken B cells is dominant because, as the extract is diluted, the effects on B cell differentiation remain after the effects on T cells are lost [118]. The B cell-differentiating hormone isolated from the bursa of Fabricius has been called bursin. It has a tripeptide structure (lysyl-histidyl-glycyl-amide) and induces phenotypic differentiation of B-cell precursors [149]. Using a B cell line, bursin was shown to increase the cyclic adenosine and guanosine monophosphates. Immunohistochemical staining with an antibody specific against bursin showed that expression is essentially restricted to follicular and dendritic reticular epithelial cells and to the basal layer of interfollicular epithelium in the mature bursa. If antibodies against bursin are inoculated intravenously at E13, they suppress the appearance of bursal IgM cells at E17, suggesting that bursin may act on the emergence of IgM⁺ cells early in bursal development [150].

A role for activin A and B in B cell development has also been suggested. Expression of the two activin subunits increases at E18 until day 1 posthatch. Activin A is predominantly localized in the medullary epithelia, whereas activin B is found in the FAE, which suggests that the two subunits have distinct roles in modulating the bursal microenvironment during B-lymphocyte differentiation [151].

Neurotrophin receptor-like proteins also have been identified in both avian primary lymphoid organs. Using immunohistochemical techniques, a specific immunoreactivity has been detected for three of these tyrosine kinase receptors: TrkA-, B-, and C-like. In the thymus, TrkA-like receptors are present in the medullary epithelial cells and in a subpopulation of cortical epithelial cells; TrkB-like receptors are present in the medullary dendritic cells and cortical macrophages; and Trk C-like receptors are found in the cortical and medullary epithelial cells. In the bursa, Trk A- and C-like receptors are found exclusively in the epithelial cells associated with follicle and the interfollicular epithelia. Trk C-like receptors are found in some medullary reticular epithelial cells. The selective localization of these receptors is consistent with the differential role of neurotrophin in the microenvironment of

the primary lymphoid organs and, indirectly, with its effects on B- and T-lymphocyte differentiation [152].

Glucocorticoid hormones have an important role in mammalian T cell selection. The recent identification of the enzymes and cofactors required for glucocorticoid production, in both the bursa and thymus, suggests that these hormones could have an important role in development and selection of avian B and T lymphocytes. Moreover, the observation that glucocorticoid production occurs in the organ responsible for the development of B lymphocytes is a further step to elucidating the yet poorly defined mechanism of B cell selection in birds [153].

3.12 Development of the immune responses

3.12.1 Early immune responses

Developing avian embryos and neonatal chickens are transiently protected against bacterial toxins, bacteria, parasites, and viruses by maternal Ig transferred via the yolk (see Chapter 5). Maternal antibody can persist for up to a month posthatch [154]. The chicken begins to develop its own defense mechanisms during embryonic life, but immunocompetence only appears a few days posthatch [155]. From the immunological point of view, the posthatch period is crucial, since the chicken is abruptly exposed to a wide range of environmental antigens and is not supplemented with further maternal immunity, such as that provided by mammalian colostrum. Immunization day 1 posthatch does not activate antibody production, probably because of incomplete structural organization of the secondary lymphoid tissues in neonates. However, 1 week later, immunization with the thymus-dependent antigen bovine serum albumin generated an effective humoral response with specific antibody production [155].

3.12.2 Antibody isotype switching and hypersensitivity reaction

As in mammals, after antigenic challenges the initial synthesis of specific IgM in birds is followed by the production of increasing amounts of lower-molecular-weight antibodies (IgY). Isotype switching during the course of an immune response occurs in the germinal centers, where B cell memory develops [156]. The germinal centers are also the site of hypermutation, a process necessary for the affinity maturation of the antibodies involved in the secondary immune response [157]. Immediate hypersensitivity (IH) and delayed hypersensitivity (DH) reactions have been described for chickens following immunization with soluble antigenic proteins, and these reactions seem to be age dependent [158].

Indeed, when injected with human γ -globulin, 6–12-week-old chickens exhibited a significantly greater DH reaction than 3-week-old birds. Consistent with this, IH can be lethal in adults but appears less severe in young chickens. IH reactions can be blocked by antihistamines and are probably induced by degranulation of mast cells [159]. In chickens, gut-associated lymphoid tissue, which provides important enteric protection in the absence of oral maternal antibodies, is functionally mature as early as 4 days posthatch; however, the secretory IgA response against enteric antigens develops only gradually, maturing toward the end of the second week [160].

3.12.3 Allograft rejection

Birds show acute rejection of allografted tissue, with a rapid primary response and a clear-cut secondary response with memory. Chickens become fully competent in their alloimmune responses within a few days posthatch. Acute rejection is controlled by the MHC [161]. Skin grafts between incompatible chickens show the onset of rejection at about 7 days after the first graft but only 3–4 days after the second graft in a series. Graft-versus-host (GVH) reactions were first discovered using the avian system, as described in Chapter 3. These occur when cells from an immunologically competent bird are implanted in an immunologically immature embryo or an immunosuppressed individual; the alloimmune reaction of the lymphoid cells of the donor against the host is detectable about 4 days after the cells are inoculated. In the chicken embryo, this is manifested by lesions on the chorioallantoic membrane and by the enlargement of the host's spleen (splenomegaly). Blood cells from a late embryo (E20) are capable of causing GVH reactions when inoculated into a young embryo. The host chicken becomes able to resist to GVH within a few days of hatching once it has become fully immunocompetent [162,163].

3.13 Conclusion

Aves are a highly evolved and successful class of vertebrates and share a number of common features with that other highly evolved class, the mammals. Some of these characteristics are inherited from a common ancestor, while others are due to convergent evolution. Birds have a number of specialized adaptations, one being the development of the bursa of Fabricius as a primary lymphoid organ. Although functional delineation of the B and T cell lineages and specialized microenvironments for their differentiation occur in other vertebrate classes, it is only in bird that the site for primary differentiation of B lineage cells is restricted to an anatomically distinct organ. This clear-cut separation of B and T cell lineages provides an

extremely useful model, for immunological studies. For example, a “T cell individual” in which the B cell population is depleted (bursectomy), while T cell system remains intact can be produced by a number of different means. Hence, studies on the diversification of antibody repertoires using the chicken has revealed some interesting and unusual features—such as somatic gene conversion—which achieves an impressive immunoglobulin repertoire, the equal of that in mammals. Bursectomy is relatively straightforward and has been employed successfully to investigate the relative contributions of antibodies and cell-mediated immune responses in immune protection. Unfortunately, creating an experimental “B cell individual” by ablating T cells using thymectomy is a much more difficult proposition, and has been achieved on relatively few occasions. With ease of access to large numbers of inbred lines and precisely timed stages in embryonic development, birds provide a tremendously valuable model for studying the mechanisms involved in immunological development as well as contribution of the different responses in immune resistance.

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B cells, the bursa of Fabricius, and the generation of antibody repertoires

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4.1 Introduction

The bursa of Fabricius is critical to the normal development of B lymphocytes and the antibodies they go on to produce. While antibody production and B cells are common to all vertebrates, the bursa as an organ of primary B-cell lymphopoiesis is unique to birds and was first implicated in the development or generation of antibody responses nearly 60 years ago. At that time, Glick and colleagues demonstrated that surgical removal of the bursa from neonatal chicks impaired subsequent antibody responses to *Salmonella typhimurium* type O antigen [1]. Since then, it has become clear that the bursa is indeed the primary site of B-cell lymphopoiesis in birds and that avian B-cell lymphopoiesis has several characteristics that distinguish it from pathways of B-cell development in human or rodent models [2–6]. In this chapter, we will discuss the development of avian B lymphocytes and the function of the bursa in B-cell development.

The bursa itself is colonized during embryonic development by lymphoid precursors that expand and mature in the bursa before migrating from the bursa to the periphery, where they have the potential to take part in immune responses. As a consequence, we can consider B-cell development as occurring in three distinct stages, prebursal, bursal, and postbursal, and as we will see, each of these stages plays a fundamentally different role in B-cell development.

4.2 The generation of avian antibody repertoires

Prior to discussing the development of B cells at the cellular level, it is important to consider the function of the B lymphocyte lineage, which is to generate antibodies

following exposure to foreign pathogens. For this reason, B-cell development also requires the generation of a repertoire of antigen-specific B cells that can provide such protective antibodies. The specificity of B cells is a function of the immunoglobulin genes that encode antibody molecules, and so in the first part of this chapter we will discuss the means by which immunoglobulin genes in birds generate a diverse repertoire of B-cell specificities.

In rodents and primates, antibody diversity is generated by the random assortment of genetic elements that encode the variable region domains of immunoglobulin (Ig) heavy and light chains in a process called Ig gene rearrangement [7]. It has become clear over the past 30 years, however, that although immunoglobulin genes in birds undergo gene rearrangement, antibody diversity is generated by somatic gene conversion (reviewed in Ref. [8]). This process, as will be discussed in more detail later, is fundamentally different from immunoglobulin gene rearrangement. However, based on the number of different variable regions that can be generated, gene conversion appears to be at least as efficient as gene rearrangement as a mechanism for generating antibody diversity. Still, the question begs to be answered: Why has gene conversion evolved in birds?

4.2.1 Immunoglobulin light chains

Immunoglobulin light chains form an essential component of antibody molecules in the great majority of jawed vertebrates. Elasmobranchs such as the shark contain four light-chain isotypes [9], and at least some teleost species contain three light-chain isotypes [10]. Similarly, three light-chain isotypes are present in *Xenopus tropicalis*, a member of the amphibian [11] and two are present in the

reptile *Anolis carolinensis* [12] as well as in both placental mammals and marsupials [13].

In contrast, the chicken contains only one light-chain gene [14], a finding that at this stage appears to be generalized to a wide range of birds, including duck [15], goose [16], zebra finch [17], and ostrich [18]. This locus is more closely related to the mammalian λ light-chain locus than to the κ light-chain locus; this is a relationship based not just on protein or DNA sequence homology but on the structure and organization of the recombination signal sequences (RSSs) required for the generation of functional Ig light chains. It is therefore likely that early in their evolution birds deleted the locus encoding a κ -type light chain as well as any other extant light-chain loci.

Most of our current understanding of avian antibody diversity comes from studies in the chicken. Recently, these studies have been extended to other avian species, and at this point, it seems most likely that the rules that apply to the diversification of chicken immunoglobulin genes will apply to all avian species.

The light-chain V_L protein domain is encoded by the rearrangement of a V_L gene with a J_L segment. In chickens, there is only one functional V_L gene and only one J_L segment, so all chicken B cells undergo essentially the same gene rearrangement event [14,19,20]. This is in striking contrast to V_L - J_L rearrangement in rodents and primates, where large families of V_L genes can undergo rearrangement to one of several distinct J_L segments. While many birds, such as quail, mallard duck, pigeon, turkey, cormorant, and hawk, appear to undergo unique V_L - J_L rearrangements, the Muscovy duck contains at least two [21] and as many as four functional V_L genes [22] that undergo rearrangement to a unique J_L . Nonetheless, at a functional level, while rodents and primates can generate substantial amounts of VJ_L diversity by rearrangement itself, in birds, Ig gene rearrangement at the light-chain locus generates minimal light-chain diversity (Fig. 4.1).

The mechanism of Ig gene rearrangement appears to be highly conserved between birds and mammals. The products of the RAG-1 and RAG-2 genes generate a recombinase complex that is responsible for recognizing the RSS directly flanking gene segments that undergo recombination. RSSs comprise a 7-nucleotide conserved heptamer sequence that is typically palindromic and a conserved 9-nucleotide AT-rich nonamer. The conserved heptamer and nonamer are separated by so-called spacer sequences that can be either 12 or 23 base pairs long. As a general rule, the sequence of the spacer is much less important than its length, suggesting that the relative orientation of the heptamer and nonamer is important for the recombination process. In this regard, the 12- and 23-base-pair spacer lengths approximately correspond to one and two turns of the DNA helix, respectively. Rearrangement takes place between a sequence flanked

by an RSS with a 12-base-pair spacer (RSS_{12}) and a sequence flanked by an RSS with a 23-base-pair spacer (RSS_{23}). Thus, at the chicken light-chain locus, the unique functional V_L sequence is flanked by an RSS_{23} and the unique J_L segment is flanked by an RSS_{12} as is the case in mammalian λ as distinct from κ light-chain gene segments. In chicken B-cell precursors, the coexpression of RAG-1 and RAG-2 therefore coincides with cells undergoing rearrangement of immunoglobulin genes [23].

At a molecular level, V_L - J_L rearrangement in chickens appears indistinguishable from the V(D)J recombination seen in other species [24,25]. Indeed, the chicken immunoglobulin light-chain locus undergoes rearrangement in murine B cells when present as a transgenic locus [26]. RAG-1 and RAG-2 together cause a single-stranded nick at the junction between the RSS and the coding sequence. The nicked end displaces the other strand in a transesterification reaction that results in the coding sequence forming a hairpin structure and leaves the RSS as blunt open-ended DNA (reviewed in Ref. [24]). Opening of the hairpin structure frequently occurs asymmetrically, resulting in the deletion of coding nucleotides or the addition of palindromic (P) nucleotides to the VJ_L junction, prior to the formation of the VJ junction [25]. In contrast to rodent or human VJ_L junctions, where the enzyme terminal deoxyribonucleotidyl transferase adds random nontemplated (N) nucleotides to coding ends prior to their joining, there is no evidence for the presence of N-nucleotide additions in chicken VJ_L junctions. Indeed, it was the presence of P nucleotides in the absence of N nucleotides that provided crucial insights into the definition of the hairpin structure intermediate in V(D)J recombination [27]. The absence of N nucleotides appears to be the only mechanistic difference between V(D)J recombination in chickens and mammals. As far as is known at this point, homologous enzymes are responsible for cleavage, orientation of the recombinationally active ends, and ligation of the VJ_L junction in mammals and birds.

4.2.2 Immunoglobulin heavy chains

Chickens express three Ig heavy-chain isotypes: IgM, IgA, and IgY. From a functional perspective, avian IgM and IgA share many features in common with their mammalian counterparts. Thus avian IgM is the predominant B-cell surface Ig and is the first antigen-specific antibody observed following exposure to antigen. Similarly, avian IgA is the predominant Ig isotype in secretions. In mammals, IgG is the predominant isotype in secondary antibody responses. Chickens express an equivalent isotype which has variously been called IgG or IgY. The chicken IgY heavy chain comprises one V_H domain with four C_H domains, unlike mammalian IgG, which contains only three C_H domains. It is clear from genomic sequencing that there are no equivalents to mammalian IgD or IgE in the chicken.

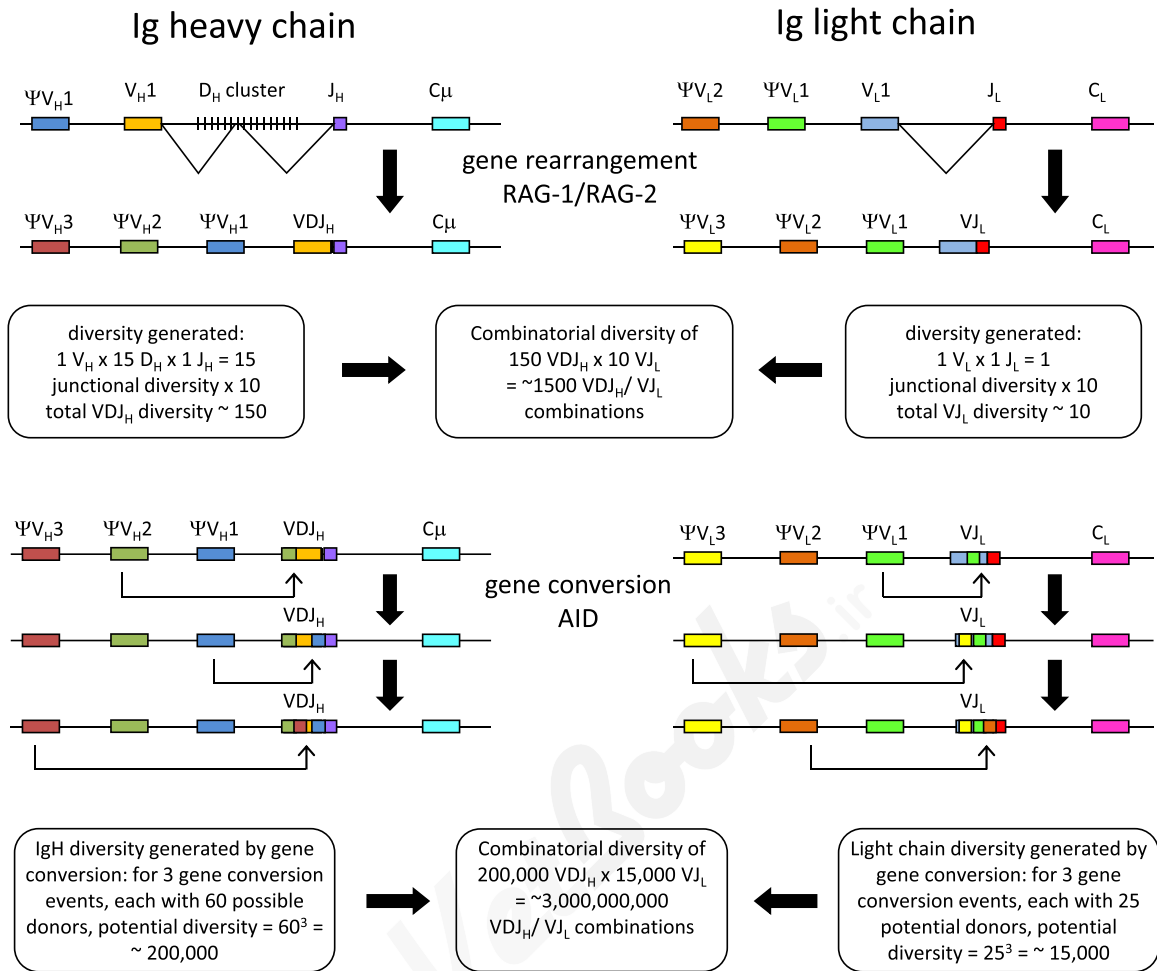


FIGURE 4.1 The generation of antibody diversity in the chicken. Chicken antibody diversity is generated by gene rearrangement followed by gene conversion. At each stage of the process, an estimate is given of the amount of potential diversity among the resulting VDJ_H and VJ_L genes and in the B-cell population overall. Estimates due to rearrangement may be high because of sequence redundancy among the D_H cluster. However, estimates of diversity as a consequence of gene conversion may be low because individual gene conversion events may affect variable numbers of amino acids and somatic point mutations arising in the V regions may not have been taken into consideration.

While some avian species are similar to chickens in that they express single subclasses of IgM, IgA and IgY [16,28–33], it has become clear that this is not the rule for all avian species. Some avian species, including the ostrich and the emu, express an IgD homologue. Similarly, the ostrich and emu express two subclasses of IgM and two subclasses of IgY [34]. Clearly, the constant region of the IgH locus continues to evolve in avian species.

The chicken Ig heavy-chain locus encodes three Ig constant-region genes that encode the μ , α , and ν Ig heavy chains of IgM, IgA, and IgY, respectively [28–30]. This observation has been extended to other avian species such as pheasant (*Phasianus colchicus*), turkey (*Meleagris gallopavo*), and quail (*Coturnix japonica*) [31] as well as duck (*Anas platyrhynchos*) [32,33]. While there is a clear evolutionary relationship between the μ and α genes of birds and mammals, the situation with respect to the chicken ν

gene is more complex. Based on structural and sequence homology, the chicken ν gene is equally closely related to the mammalian γ and ϵ constant-region genes, which suggests that the chicken ν gene and the IgY encoded by it are descended from the common evolutionary ancestor of mammalian γ and ϵ constant regions [30]. In mammals, the constant-region genes all are arranged in the same transcriptional orientation as the rearranged V gene segment. Surprisingly, however, the chicken C_α gene lies between C_μ and C_γ in the opposing orientation [35], an organization also seen in the duck [36].

As with the chicken light chain, gene rearrangement contributes little to the generation of chicken Ig heavy-chain diversity. In common with other species, the Ig heavy-chain V_H domain is encoded by three gene segments: variable (V_H), diversity (D_H), and joining (J_H). However, in contrast to other, especially mammalian,

species where there are large families of V_H , D_H , and J_H segments, the chicken V_H domain is generated by recombination of unique V_H and J_H gene segments with one of a family of D_H elements [37]. Although there are approximately 15 D_H segments in the chicken, these show much lower levels of diversity than equivalent segments in the mouse or the human; indeed, many chicken D_H elements are identical at the protein level [38].

The chicken V_H , D_H , and J_H sequences are also flanked by the appropriate RSSs to guide the recombination process. At the molecular level, chicken Ig heavy-chain rearrangement is mechanistically indistinguishable from light-chain rearrangement. As with rearrangement at the chicken Ig light-chain locus, there appears to be no Tdt-mediated N-nucleotide additions, but there are significant nucleotide deletions and/or P-nucleotide additions [37,38].

The assembly of the chicken heavy-chain VDJ_H exon is ordered, as it is in mammals, with a D_H -to- J_H rearrangement preceding the rearrangement of the unique V_H1 gene into the preformed DJ_H complex. Nonetheless, in contrast to mammalian VDJ_H rearrangement, chicken D_H -to- DJ_H rearrangement occurs at a high frequency. Thus, during embryo development, many examples of DDJ_H , and even DDD_H , rearrangement have been isolated from the chicken bursa. This is further exemplified by the observation that in mature B cells, on chromosomes that have undergone DJ_H rearrangement without undergoing V_H - DJ_H rearrangement, that is, on the nonexpressed allele, between 10% and 20% of loci include a DDJ_H , as opposed to the DJ_H , rearrangement (Agard, Dervovic, Lewis, and Ratcliffe, unpublished data).

DDJ_H and DDD_H rearrangements retain their capacity to rearrange to the unique V_H1 gene, suggesting that they may contribute to the diversity generated by recombination. However, in sequences from hatched chicks, $VDDJ_H$ and $VDDD_H$ sequences are not observed, and so at this point their biological relevance is unclear [37,38]. Indeed, it is quite likely, based on the exclusion of such $VDDJ_H$ and $VDDD_H$ sequences from the functional repertoire, that such rearrangements are not included among the functional rearrangements that form the so-called prediversified Ig repertoire.

The generation of DDJ_H sequences requires D_H -to- D_H rearrangement, and since chicken D_H elements are flanked on both sides by RSS_{12} sequences, such rearrangement should be in contravention of the 12/23 rule of recombination. Recent evidence suggests, however, that such 12/12 recombination is indeed possible at a significant frequency because of the sequence of the 12-base-pair spacers typically found in chicken D_H RSSs. This provides evidence that the spacer sequence in RSS may be more important in regulating the efficiency of V(D)J recombination than was first appreciated (Agard, Dervovic, Lewis and Ratcliffe, unpublished data).

As result of the molecular mechanism of the recombination process, rearrangement is characteristically inefficient in the sense that two thirds of all rearrangements generate out-of-frame products. This is also true in the chicken despite the lack of N-nucleotide insertions at V(D)J junctions. In turn, this means that only one third of light-chain gene rearrangements generate a functional light-chain product. At the chicken heavy-chain locus, the D_H element has to be used in a specific reading frame (as discussed in Ref. [3]) and so only one in nine VDJ_H sequences generated will maintain the correct reading frame of both D_H and J_H relative to V_H .

4.2.3 Generation of Ig molecules by V(D)J recombination

In mammals, VDJ_H rearrangement typically occurs prior to, and is required for, VJ_L rearrangement (reviewed in Ref. [39]). In chickens, however, it has become clear that, following initial DJ_H rearrangement, the rearrangement of V_H1 to DJ_H (or DDJ_H) and that of V_L1 to J_L occurs stochastically during the same window of time. Thus, while cells have been isolated by retroviral transformations that contain VDJ_H rearrangements in the absence of VJ_L rearrangements, other retrovirally transformed cells contained VJ_L rearrangements in the absence of VDJ_H rearrangements [40]. This is consistent with the pattern of rearrangements seen in the developing chick embryo [23], and it means that although the molecular mechanism of rearrangement is indistinguishable between mammals and birds, the way in which recombination is regulated is clearly different. Why this difference has evolved remains unclear. However, since gene rearrangement in mammals is the mechanism by which the primary repertoire is generated, amplification of successful heavy-chain rearrangements by cellular proliferation prior to light-chain rearrangement increases the potential for generating maximal diversity. In contrast, because gene rearrangement in the chicken generates minimal diversity, amplification of productive heavy-chain rearrangement prior to light-chain rearrangement might increase the number of cells containing productive rearrangements but would not increase the diversity of the repertoire in this population. A similar argument can be made for the lack of a second light-chain isotype in birds. In mammals, then, the presence of both κ and λ light-chain loci increases the likelihood that a pre-B cell will form a functional light chain and contribute to the expressed repertoire, whereas birds do not rely on rearrangement to generate diversity.

The coincident rearrangement of chicken V_H and V_L genes represents an important difference between avian and mammalian B-cell development. In mammals, productive heavy-chain VDJ_H rearrangement leads to the

generation of full-length μ heavy chains that associate with the surrogate light-chain molecules VpreB and $\lambda 5$. Together, VpreB and $\lambda 5$ associate with the μ heavy chain to form the multimeric pre-B receptor ($(\mu/\text{VpreB}/\lambda 5)_2$). Expression of this receptor, complexed to the signaling heterodimer Ig α /Ig β , inhibits further heavy-chain rearrangement and is required to support the progression of mammalian B cells to the point where they begin to rearrange at the light-chain locus (reviewed in Ref. [41,42]). In chickens, there is no evidence of a homologous complex to the mammalian pre-B-cell receptor, and chicken homologues to VpreB and $\lambda 5$ have not been identified despite the availability of extensive chicken genome sequence data. Since the expression of the pre-B receptor defines the pre-B cell in the mammalian bone marrow or fetal liver, it is clear that chickens and, likely by extension, other birds, do not contain pre-B cells.

The functionally rearranged chicken light chain includes a leader sequence that encodes the leader peptide required for translocation of the nascent polypeptide chain into the endoplasmic reticulum, the VJ_L sequence that encodes the V_L protein domain, and the unique C_L exon that encodes the C_L protein domain. The posttranscriptional and posttranslational processing required to generate the mature light chain that contains the V_L and C_L domains appears to be indistinguishable from that in mammals.

Similarly, the functionally rearranged heavy chain includes the leader sequence, the VDJ_H sequence that encodes the V_H protein domain, and the C μ exons that encode the constant region of the IgM heavy chain. As in other species, the C μ constant-region gene is found immediately downstream of the VDJ_H region, and IgM is the first Ig molecule expressed by developing B cells. In species, such as the ostrich and emu, that contain two IgM subclasses, it is likely the VDJ_H proximal C μ gene that is used to form the first cell surface IgM molecules.

4.2.4 Generation of Ig diversity by somatic gene conversion

While chicken Ig gene rearrangement generates minimal V region diversity, the diversity of antibodies produced in the chicken appears to be no less than that in mammals. The resolution of this paradox came from the characterization of the chicken Ig light-chain locus. Upstream of the functional V_{L1} gene is a family of sequences that are highly homologous to V_{L1} but show several key differences that render them nonfunctional. As such, these genes have been described as pseudo-V_L genes (ΨV_L), although it has become clear that they do indeed contribute to the development of avian antibody repertoires [19,20]. Nonetheless, the ΨV_L genes lack 5' promoter and leader sequences and functional RSSs. This means that the ΨV_L sequences

cannot themselves undergo recombination to the J_L segment. In addition, many of them contain 5' and/or 3' truncations as compared to the functional V_{L1} gene.

At the light-chain locus, there are approximately 25 ΨV_L genes, and sequence comparisons show that the major blocks of diversity among them occur at positions that correspond to the hypervariable or complementarity-determining regions of the Ig light-chain V region gene, corresponding to the antigen-binding site of the Ig molecule. Thus the diversity of the Ig repertoire is maintained at the germline level among the ΨV_L gene family rather than among functional V gene families, as is the case in mammals.

The result of an individual gene conversion event at the chicken Ig light-chain locus is that a stretch of sequence in the rearranged V_{L1} gene is replaced by a homologous sequence derived from one of the upstream pseudogenes. This is a more variable process than simply a replacement of one V gene with another since the length of the exchanged sequence can vary extensively from one gene conversion event to another. Gene conversion events have been identified that span anywhere from a minimum of about 10 nucleotides up to more than 300 nucleotides, covering most of the V_L sequence. Thus even under circumstances where a single gene conversion event involving a particular rearranged V_L sequence and a specific donor ΨV_L gene, gene conversion can generate multiple different products. This translates into extensive light-chain repertoire diversity.

At the chicken light-chain locus, the 25 ΨV_L genes are distributed in about 20 kb immediately upstream of the functional V_{L1} gene. This spacing is far more compressed than is seen in rodent or primate V gene clusters, where V genes are typically spaced about 10 kb apart. It is widely regarded that the reason for this wide spacing in rodents and primates is to minimize the activation of upstream V gene(s) following VJ_L rearrangement. Closer spacing would put the promoters of upstream V regions—V genes that have not themselves undergone rearrangement—within range of enhancer sequences in the J-C intron, leading to transcript production. Since chicken ΨV_L genes do not themselves include promoters, close spacing would not result in ΨV_L transcription. In addition to being closely spaced, many of these genes are arranged in a head-to-head or tail-to-tail orientation, placing them in the opposing orientation as compared to the V_{L1} gene.

Analysis of the use of different pseudogenes as sequence donors in gene conversion events has revealed several properties that underlie the process of gene conversion. These analyses were based on defining ΨV_L use in B cells typically from the embryo or neonate that contain relatively few gene conversions, which makes it easier to define individual conversion events [43,44].

The first general rule is that ΨV_L genes closest to the functional V_{L1} gene are used more frequently as donor

genes. This has been reasonably interpreted as indicating a requirement for alignment of donor and recipient sequences during gene conversion. It has also become clear that sequences showing the greatest homology to the recipient sequence are used as sequence donors more frequently than are others. Again, this is consistent with a requirement for sequence alignment during gene conversion. Finally, those ΨV_L genes that are in the opposing orientation to the V_L1 gene tend to be used as sequence donors more frequently than those in the same orientation, which has been interpreted as evidence that alignment between the ΨV_L and the functional gene can frequently occur by the folding back of the DNA strand to align the donor and recipient sequences. This is a reasonable interpretation since gene conversion has been shown to occur in cis. In other words, the donor and recipient sequences must be on the same DNA strand, and the ΨV_L sequences on the allelic locus do not contribute to gene conversion events [45]. Although gene conversion at the heavy-chain locus is not as clearly defined as it is at the light-chain locus, it is clear that extensive heavy-chain repertoire generation occurs by gene conversion [37,38]. Again, donor sequences are derived from upstream pseudogenes, although in the case of heavy-chain gene conversion, the donor pseudogenes include both V_H and D_H homologous sequences. As a consequence, the junctional diversity generated by rearrangement at the VD_H junction is frequently overwritten by gene conversion [38]. This may help explain the disappearance of heavy-chain $VDDJ_H$ and $VDDDJ_H$ sequences after hatch.

A major breakthrough in understanding the molecular mechanism of gene conversion came with the identification of the enzyme activation-induced cytidine deaminase (AID) as essential for gene conversion [46]. AID was initially cloned from murine germinal center (GC) B cells, and its targeted deletion in mice resulted in a complete absence of somatic hypermutation and class switch recombination [47]. A subset of human immunodeficiencies that are also characterized by an absence of class switching and point mutations were also determined to be a consequence of deficiencies in functional AID expression [48]. Targeted deletion of AID in a chicken B-cell line, DT40, which normally continues to undergo gene conversion in vitro, resulted in a complete inhibition of gene conversion events, and it has now become clear that AID is indispensable for gene conversion in chicken B cells [46]. As a consequence, expression of genes required for AID expression, such as Bach2 [49], or the translocation of AID to the nucleus, such as SRSF1–3 [50], are required for efficient gene conversion.

AID is homologous to APOBEC1, an enzyme responsible for editing RNA by deaminating cytosine residues to form uracil, which results in changes in translated protein sequences. While it was initially unclear whether AID

initiates V gene mutation by targeting the Ig V region directly or by modifying the RNA encoding a putative “mutator” protein, it has become clear that AID can directly modify single-stranded DNA in the V gene itself [51,52]. Deamination of C residues to form U results in the modification of a dC-dG base pair to a dU-dG base-pair mismatch which is then subject to error repair. It is likely that the nature of the error repair defines the resulting modification, gene conversion, or somatic hypermutation in the V gene [53] or class switch recombination [54], as discussed in Ref. [55].

Since AID targets single-stranded DNA, the question arises as to how such DNA is generated at the Ig locus and why AID-induced mutations are preferentially targeted to Ig loci. One possibility has been widely considered: as transcription of the functional Ig V gene proceeds, the transcription bubble causes unwinding of the DNA and strand separation to allow the formation of the nascent RNA chain. The nontranscribed DNA strand is therefore single-stranded and thus might act as a substrate for AID-induced deamination of C residues. This model could clearly explain why gene conversion is exclusively targeted to the rearranged V region gene: although the ΨV_L genes show considerable homology to the functional gene, they are not transcribed because of their lack of promoters. Similarly, in B cells containing one rearranged V_L1 gene with the other V_L1 gene in germline configuration, gene conversion is restricted to the rearranged allele [20].

Nonetheless, many genes are being transcribed at high levels in B cells undergoing gene conversion, so the question remains as to how AID is preferentially targeted to the Ig loci. The identification of a cis-acting sequence downstream of the light-chain promoter that confers mutability when introduced into other loci suggests that there are sequence-specific elements in the chicken Ig loci that confer susceptibility to AID-mediated V gene diversification [56–58].

While AID targets C residues in the V gene sequence, not all C residues are equally susceptible to AID-induced modification. It has long been appreciated that somatic hypermutation, also initiated by AID, is targeted to the C residue in hotspots defined by W(A/G)R (G/T)C motifs. At this point, it appears that the initial AID binding to single-stranded DNA is independent of sequence, with hotspot specificity coming from a WRC preference for targeted deamination [59]. In any case, the overall consequence is preferential dC to dU mutations in WRC motifs. Comparison of the sequence of Ig V genes with C region sequences reveals that there are much higher frequencies of WRC motifs in V gene sequences, typified by a frequent use of the AGC codon to encode serine. It therefore appears that the V gene sequences have a codon use that enriches for codons susceptible to AID-induced modification.

Following AID-catalyzed dC to dU modification, the resulting dU-dG base pair represents a mismatch in the DNA duplex. Such mismatches are typically repaired by eukaryotic cells to minimize the accumulation of mutations. There is now considerable evidence that the error repair processes invoked can determine the modification induced at the Ig locus. Thus XRCC2, XRCC3, and RAD51B, all of which are paralogues of the RAD-51 gene in yeast, as well as RAD-54 and uracil-DNA glycosylase, all play a role, as their targeted deletion in the DT40 cell line inhibits gene conversion [60,61]. In contrast, several of the molecules involved in gene rearrangement, including RAG-2 [62], Ku70, and DNAPKcs, as well as the RAD-52 gene product, do not play a role.

While deletion of AID precludes both gene conversion and somatic hypermutation, deletion in DT40 cells of several of the molecules selectively involved in gene conversion, particularly XRCC2, XRCC3, or RAD51B, results in DT40 lines that undergo somatic hypermutation, again supporting the contention that gene conversion and somatic hypermutation represent distinct mechanisms by which a common AID-induced lesion is repaired.

4.2.5 Implications of gene conversion for allelic exclusion

In rodents and primates, a high proportion of B cells contain one functionally rearranged heavy chain and one heavy-chain allele in a nonproductively rearranged conformation. The same situation is true at the light-chain locus. The nonproductively rearranged alleles never participate in the repertoire of B-cell specificities since the somatic hypermutation mechanisms that diversify rodent and primate V regions following their rearrangement do not result in changes in the length of the V(D)J junctions and so cannot restore a productive reading frame to a rearrangement that is out of frame.

Gene conversion, on the other hand, results in the replacement of V gene-encoded sequences with sequences derived from upstream Ψ V genes, and it is clear that such gene conversion events are not simply a base-for-base substitution since there are many examples of gene conversion changing the length of the resulting V_L sequence. This is due to the presence of different numbers of codons, typically in the hypervariable regions, of different pseudogenes. As a consequence, it is reasonable that gene conversion might have the potential to “overwrite” a nonproductive gene rearrangement with a gene conversion event spanning the VJ_L junction that could restore the correct reading frame for a productive VJ_L sequence. The net result would be the potential for individual B cells that initially contained one productively and one nonproductively rearranged allele to undergo gene conversions that would result

in both alleles being productively expressed [44]. This in turn would lead to the generation of individual B cells with more than one specificity—a situation that the developing immune system appears to take great pains to prevent.

Chicken B cells, however, contain one VJ_L rearrangement, with the other allele remaining in germline configuration. Similarly, at the heavy-chain locus, each chicken B cell contains one heavy-chain VDJ_H rearrangement, with the other allele typically either containing a DJ_H (or DDJ_H) rearrangement or remaining in germline configuration. Thus gene conversion cannot convert a nonproductive rearrangement to one that is productive, and so the product of a single allele is expressed by the B cell. The expression of a single allele is known as allelic exclusion. The allelically excluded nature of Ig gene expression is maintained in birds at the level of gene rearrangement, whereas in rodents and primates, it is maintained at the level of functional protein expression. At a molecular level, it remains unclear as to how allelic exclusion of Ig gene rearrangement in avians is regulated.

4.3 The development of avian B cells

4.3.1 Prebursal B cell development

In mice, the commitment of hematopoietic precursors to the B-cell lineage occurs by the sequential induction of transcription factors, culminating in the expression of the Pax5. Pax5 activates the expression of a range of B cell-specific proteins and, at the same time, downregulates the expression of protein characteristic of alternative hematopoietic fates [63,64]. Chicken B cells also express Pax5 [65,66] suggesting that similar processes underlie the commitment of avian hematopoietic precursors to the B-cell lineage.

The development of avian hematopoietic lineages is discussed in detail in Chapter 3; however, there are aspects of early B-cell development that should be reemphasized at this time. Early studies based on grafting bursal rudiments between chicken and quail embryos clearly demonstrated that the B-cell progenitors responsible for colonizing the bursa and forming the B-cell lineage are derived from extrabursal tissue and colonize the bursa from about day EID (embryonic incubation day) 8 to EID 14 [67,68].

Cell transfer studies in which embryo spleen cells were transferred into immunocompromized recipient embryos demonstrated the presence of precursors with the potential to colonize both the thymus and the bursa. Importantly, these studies showed that prebursal, but not prethymic, cells express the B-cell surface antigen chB6, demonstrating that the fate determination of the lymphocyte lineage to T- and B-cell potential occurs prior to the migration of prebursal cells to the bursa [69] (Fig. 4.2).

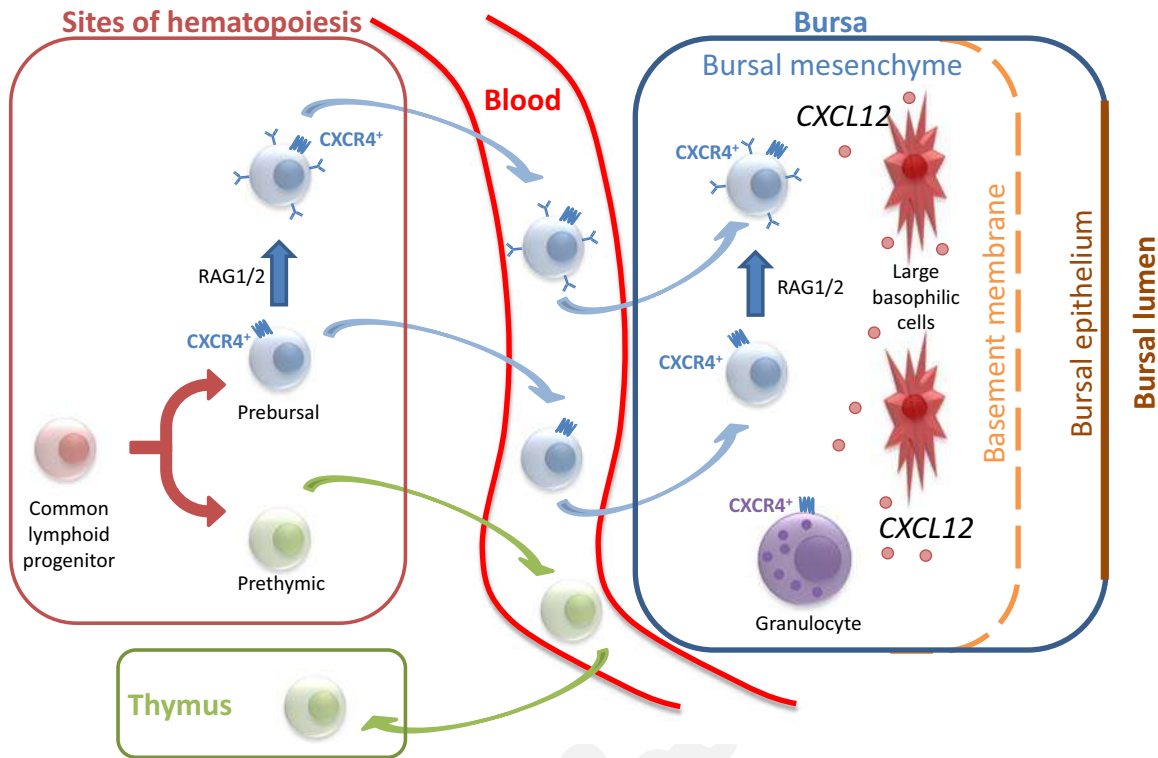


FIGURE 4.2 The early development of chicken B cells. Prebursal cells become committed to the B-cell lineage before migration into the bursa. Ig gene rearrangement is initiated prior to migration into the bursa and can continue in the bursal mesenchyme. The migration of prebursal cells into the bursa is regulated by CXCL12 expression in the bursal mesenchyme and by the expression of CXCR4 on prebursal cells.

This presents an interesting counterpoint to the fate determination of murine lymphoid precursors, where strong evidence indicates that activation of Notch 1 expressed on lymphoid precursors invokes a T-cell fate, whereas the lack of Notch 1 activation leads to the default B-cell fate (reviewed in Ref. [70]).

The high-level expression of Notch 1 ligands in the mammalian thymus provides a model in which uncommitted precursors that seed the thymus are induced into the T-cell lineage, whereas precursors remaining in the bone marrow develop along the default B-cell lineage [70]. In the chicken, the same situation might occur, that is, precursors that colonize the thymus are induced to develop into T cells, whereas precursors that do not colonize the thymus adopt alternative fate(s). That the default fate of lymphoid precursors in the absence of Notch 1 activation is the B lineage would suggest a restricted expression of appropriate Notch ligands in peripheral hematopoietic sites. Nonetheless, there is strong evidence that commitment to the B-cell lineage occurs early in embryonic life, prior to the colonization of the embryo bursa.

The rearrangement of immunoglobulin genes is an essential stage in the development of the B-cell lineage. Several lines of evidence have directly demonstrated that Ig gene rearrangement can occur before the B-cell progenitors colonize the bursa. Thus both Ig heavy- and light-chain

rearrangements have been isolated from a variety of hematopoietic tissues, and B lineage precursors with the capacity to colonize the bursa have been isolated from embryo bone marrow and shown to contain Ig rearrangements and to express surface immunoglobulin [23,71–73]. Therefore it is now clear that the bursal microenvironment is not required for the induction of RAG-1/2 expression and the rearrangement of chicken Ig genes.

4.3.2 Colonization of the bursa by B cell progenitors

The bursal epithelium develops as an outgrowth of the urodeal membrane early in embryonic life. It is colonized by B-cell progenitors in a single wave during the middle week of embryonic development. The colonization of the bursal microenvironment by B-cell progenitors represents a critical stage in B-cell lineage development. As discussed previously, there is strong evidence that the lymphoid progenitors that colonize the bursa are already committed to the B-cell lineage and can have already undergone Ig gene rearrangement. Nonetheless, many questions remain about the mechanisms underlying the bursa's functional colonization. It is important to consider *functional* colonization as occurring in two discrete stages. The first

represents the migration of B-cell progenitors into the bursal mesenchyme and the second represents the colonization of lymphoid follicles within the bursa.

Based on parabiosis experiments in which the blood circulation of two embryos is joined, it is clear that B-cell progenitors can migrate to the bursa through the blood [74]. This is further supported by the demonstration of prebursal cells in the blood containing Ig gene rearrangements [72]. Clearly, however, embryo blood contains many other cell lineages, so the question remains as to how the bursal mesenchyme selects B-cell progenitors as opposed to other cell types. While it is possible that all lineages traffic through the bursal mesenchyme, with only B-cell progenitors being retained, this seems unlikely based on the relatively high concentration of B-cell progenitors seen in embryo bursal sections. Rather more likely is that blood-borne B-cell progenitors selectively transit across vascular epithelia in the bursa into the mesenchyme. This may be analogous to the selective transit of naïve lymphoid cells across high endothelial venules (HEVs) in the lymph node or the transit of leukocytes across inflamed epithelia at sites of infection and will be discussed in more detail later.

Ig gene rearrangement can occur extrabursally, demonstrating that the bursal microenvironment is not required for gene rearrangement. Moreover, Ig gene rearrangement leading to surface Ig expression can be completed outside the bursal microenvironment. Nonetheless, analysis of the status of gene rearrangements in chicken B-cell progenitors isolated from the embryo bursa prior to about EID 15 shows a high frequency of nonproductive rearrangements [19]. Similarly, clones of B-cell progenitors isolated by retroviral transformation from EID 14 bursa frequently contain partial gene rearrangements: DJ_H alone, DJ_H and VJ_L, or VDJ_H alone [40]. It is therefore clear that, while V(D)J recombination can occur to completion extrabursally, the initial colonization of the bursal mesenchyme does not require surface (s)Ig expression. This makes it likely that those B-cell progenitors that colonize the bursa prior to completing V(D)J recombination can continue to rearrange in the bursal mesenchyme, consistent with the expression of both RAG-1 and RAG-2 in the embryo bursa [23]. Importantly, therefore, the process of V(D)J recombination can be completely dissociated from the regulation of bursal mesenchyme colonization.

Under normal circumstances, the bursa is colonized by prebursal cells from about day EID 8–14. Experimentally, the capacity of prebursal cells to home to the bursa can be demonstrated by the ability of embryo bone marrow cells to colonize the bursa of recipient embryos that have been treated with either irradiation or cyclophosphamide to ablate endogenous bursal lymphoid cells. It is remarkable that, while embryo bone marrow contains functional prebursal cells, the bone marrow after hatch does not [75]. In

contrast, prethymic cells can be demonstrated in both embryo and posthatch bone marrow. In effect, this means that the source of cells colonizing the bursa is only in the embryo and that the bursa is therefore colonized in a single wave during embryonic development.

4.3.3 Colonization of lymphoid follicles in the bursa

Among lymphoid cells, the initial colonization of the bursal mesenchyme is likely selective for B-cell progenitors, independent of whether they express cell surface immunoglobulin. However, there is a second and crucial level of selection that occurs during the colonization of bursal lymphoid follicles. Bursal follicles develop from the migration of B-cell progenitors across the basement membrane that separates the mesenchyme from the bursal epithelium. Once these precursors complete their migration, they begin proliferating, which drives follicle formation. We therefore consider our understanding of the requirements for translocation across the basement membrane before discussing the proliferation of bursal cells once they have translocated.

It has been clear for a number of years that, while B-cell progenitors containing nonproductive rearrangements can be isolated from the embryo bursa, bursal cells that are clonally expanding in bursal follicles contain only productive rearrangements and express cell surface Ig [19,20,76]. As a consequence, there was a widespread belief that sIg expression represents a critical checkpoint such that those B-cell progenitors expressing sIg translocated across the basement membrane, whereas those that did not express sIg did not translocate (Fig. 4.3). This would be consistent with the observation that productive rearrangements were selected during embryonic life in the bursa but not in the embryo spleen [76]. Several lines of evidence now support this contention, as will be discussed in more detail next.

The sIg receptor complex on B cells (B cell receptor/BCR) includes the Ig heavy and light chains noncovalently associated with the Igα/β heterodimer. As B cells develop, the first isotype of Ig expressed at their surface is IgM. SIgM includes two μ heavy chains, held together by disulfide bonds, each covalently associated with a light chain, again through disulfide bonds. The (μL)₂ tetramer represents the antigen-binding portion of the sIgM receptor and is held in the plasma membrane by a transmembrane region that is generally hydrophobic. The cytoplasmic domain of chicken sIgM is only three amino acids in length, as is the case in other species. This domain is too short to couple the sIgM receptor to the signaling molecules responsible for signal transduction downstream of sIgM, and so signaling through sIgM is a property of the Igα/β heterodimer. Both

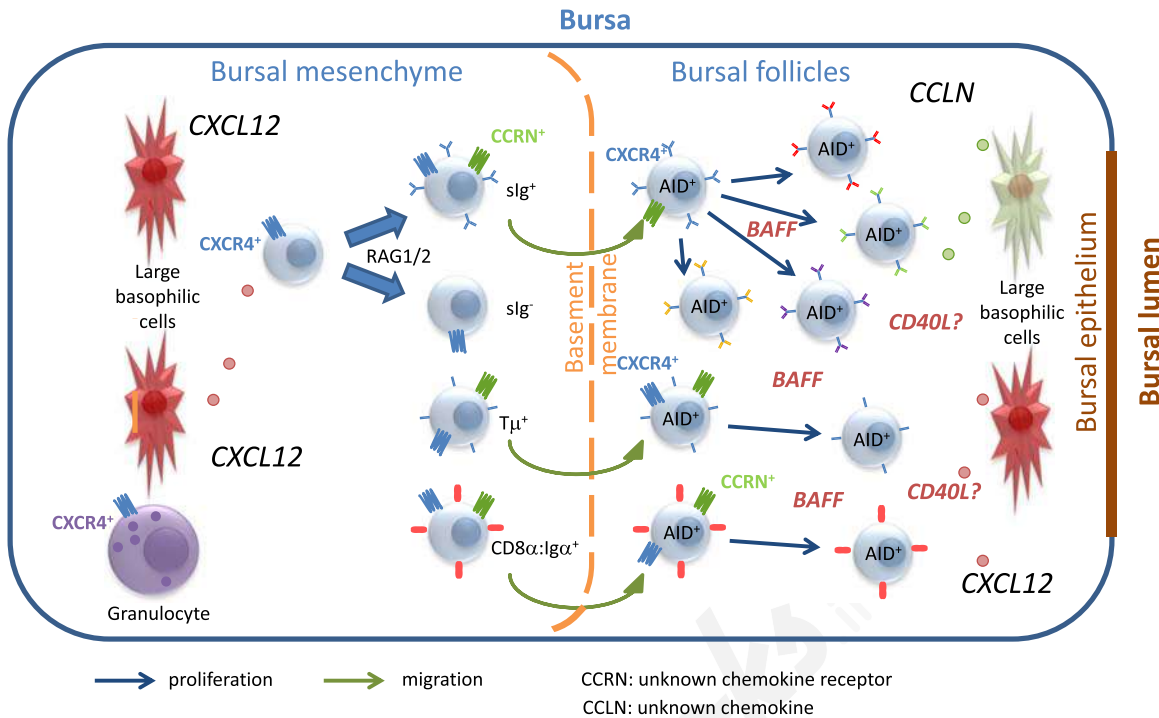


FIGURE 4.3 Colonization of bursal follicles by sIg-expressing B cells. Colonization of bursal follicles requires that B cells express sIg. This can be mimicked by expression of either truncated sIgM receptors ($T\mu^+$) or chimeric $CD8\alpha:Ig\alpha$ receptors. Migration is dependent on chemokine expression in bursal follicles and regulated chemokine receptor expression. CXCL12 is highly expressed in developing follicles but as only CXCR4⁺ B cells and not CXCR4⁺ granulocyte cross the basement membrane, involvement of another so far uncharacterized chemokine–chemokine receptor pair (CCLN–CCRN) is highly likely. Migration into bursal follicles is followed by induction of AID, resulting in the induction of gene conversion and proliferation that is supported by BAFF and may be driven CD40L. AID, activation-induced cytidine deaminase; BAFF, B cell-activating factor of the TNF family.

$Ig\alpha$ and $Ig\beta$ contain an extracellular Ig-like domain, a transmembrane region, and a cytoplasmic tail that contains conserved motifs implicated in signal transduction. $Ig\alpha$ and $Ig\beta$ are covalently bound to each other by an extracellular disulfide bond formed close to the plasma membrane. In chicken B cells, the stoichiometry of the association of $Ig\alpha/\beta$ with the $(\mu L)_2$ sIgM molecule is unclear. In murine B cells, however, evidence suggests that one $Ig\alpha/\beta$ heterodimer is associated with each $(\mu L)_2$ tetramer. At this point, there is no reason to expect the chicken BCR complex to be fundamentally different from the murine BCR complex. The signal transduction pathways downstream of the BCR complex have been explored in detail in mammalian B cells, and again there is no reason to expect fundamental differences between mammalian and avian BCR signaling. A detailed analysis of BCR signaling is beyond the scope of this chapter, but the reader is referred to other reviews and references contained therein such as Refs. [2,4].

In murine cells, expression of μ , light chain, $Ig\alpha$, and $Ig\beta$ is required to support the expression of the sIgM receptor complex at the cell surface. In the absence of any one of these polypeptides, the remaining chains are held up in the endoplasmic reticulum and degraded [77]. In chicken cells, the $(\mu L)_2$ receptor can be expressed at the cell surface

in the absence of $Ig\alpha/\beta$; however, without the signaling properties of $Ig\alpha/\beta$, the $(\mu L)_2$ receptor fails to transduce signals and can be considered biologically inert [3]. Molecular cloning of the components of the chicken BCR complex allows direct assessment of the requirements for sIgM expression in supporting the functional colonization of bursal follicles in the developing chick embryo.

Typically, surface IgM expression requires both full-length μ heavy chains paired with light chains. Surprisingly, however, light-chain knockout chickens contain B cells, the development of which is supported by a surface μ in the absence of light chain [78]. In these chickens, heavy-chain only antibodies can be generated in which the $C\mu 1$ heavy-chain domain is absent, likely as a consequence of alternative splicing. Since the $C\mu 1$ domain is critical in the association of the μ heavy chain with light chain, such $C\mu 1$ deleted heavy chains can be expressed at the cell surface. Since $C\mu 1$ deleted heavy chains are not seen in normal chickens, it is likely that heavy-chain only B cells are normally extremely rare and are amplified in light-chain KO chickens due to a lack of competition with normal B cells.

Since chicken Ig gene rearrangement generates minimal diversity, there has been considerable discussion as to

whether the chicken sIg receptor generated by rearrangement can recognize a ligand in the bursal microenvironment. This argument is based on an analogy between the mammalian pre-B-cell receptor, which contains the non-variable VpreB and $\lambda 5$ chains, and the chicken sIg receptor generated by rearrangement. In this model, recognition of a bursal ligand as a consequence of sIg expression would be required for sIgM-transduced signaling, leading to support of further B-cell development. Because this could only occur in the context of functional rearrangement of Ig heavy and light chains, this model would explain how the colonization of lymphoid follicles in the bursa could be limited to those bursal cells with functional BCR expression.

This issue was directly addressed using retrovirally mediated gene transfer *in vivo*. A truncated form of the Ig μ heavy chain was introduced into the RCAS (Replication-Competent Avian sarcoma-leukosis virus long terminal repeat with a Splice acceptor) retrovirus, a productive retroviral vector that can, *in vivo*, infect developing B-cell precursors. The truncated μ ($T\mu$) construct contained a deletion of the V_H and $C\mu 1$ domains and so did not associate with the light chain nor did it require the presence of the light chain for surface expression. Nonetheless, when expressed in B cells, this construct associated with the Ig α/β heterodimer, resulting in expression of a $T\mu$ complex that retained the capacity to transmit signals when appropriately stimulated by cross-linking. Infection of EID 3 chick embryos with the RCAS- $T\mu$ construct resulted in the presence of bursal cells in the neonatal chick that expressed the $T\mu$ receptor in the absence of endogenous sIgM. This formally demonstrated that recognition of a bursal ligand by the variable region of the surface IgM receptor complex is not required to support the progression of B-cell development. Still, since all bursal B cells in such chicks expressed either endogenous sIgM or the $T\mu$ construct, these experiments provided evidence that some form of BCR complex expression is indeed required for the normal progression of B-cell development [79].

The requirement for expression of the $T\mu$ receptor complex in these experiments nonetheless left open the possibility that a ligand in the bursal microenvironment can still recognize either the residual-constant-region domains of the $T\mu$ chain itself or the extracellular domains of Ig α and/or Ig β . These domains are expressed on either the surface of bursal B cells expressing the $T\mu$ receptor construct or on endogenous sIgM, consistent with the observed results. To address this possibility, chimeric constructs were generated in which the extracellular and transmembrane domains of murine CD8 α and CD8 β were fused to the cytoplasmic domains of chicken Ig α and Ig β , respectively. Coexpression of CD8 α and CD8 β preferentially leads to the formation of CD8 α/β heterodimers, and thus the cytoplasmic side of the

chimeric receptor complex mimics the sIgM receptor with the cytoplasmic domains of Ig α and Ig β , whereas the extracellular portion of the receptor does not contain any domains associated with either the $(\mu L)_2$ receptor or its associated Ig α/β heterodimer.

Coinfection of B-cell precursors in developing chick embryos with RCAS viruses encoding CD8 α :Ig α and CD8 β :Ig β fusion proteins resulted in the development of bursal B cells expressing the chimeric CD8 α/β heterodimer in the absence of endogenous sIg expression. Thus even under circumstances where no extracellular domains of the sIgM receptor complex were expressed, expression of the cytoplasmic domains of Ig α and Ig β was sufficient to support the normal progression of B-cell development. More detailed analysis demonstrated that membrane proximal expression of the cytoplasmic domain of Ig α is necessary and sufficient to support B-cell development [80] and that the signaling function of the cytoplasmic domain was required [81].

The importance of signaling motifs in supporting the colonization of bursal follicles with B-cell precursors expressing CD8 chimeric receptors firmly implicates biochemical activity as critically important for developing B cells to sense the expression of the functional BCR complex. However, since these signals can be provided by receptor constructs that have no Ig complex-related domains in the extracellular region, the notion of basal signaling as sufficient to support B-cell development has evolved. Surface receptors such as the BCR complex are constantly being phosphorylated, even under conditions where they are not actively stimulated. This is a consequence of the low-level constitutive kinase activity found in all cells. Moreover, phosphorylated receptors are constantly being dephosphorylated by phosphatases that also have significant constitutive activity. The levels of BCR phosphorylation in unstimulated B cells is therefore a consequence of the balance between receptor phosphorylation and dephosphorylation. The net result of these competing processes is that, at any given point in time, unstimulated B cells contain a small but significant proportion of phosphorylated BCR complexes that have the capacity to couple to downstream signaling pathways. Thus BCR expression, even in the absence of ligation, leads to a certain level of downstream signaling. At this point, the precise signaling pathways required for the progression of bursal B-cell development remain unclear [81].

An analysis of parabions in which blood-borne precursors could arise from either one of two joined embryos allowed assessment of the number of prebursal cells that colonize each bursal follicle. Under circumstances where the bursal mesenchyme contained precursors from both "strain A" and "strain B," a significant number of follicles contained exclusively strain A or strain B bursal cells while other follicles contained a mixture of both. This

demonstrates that each follicle is colonized by a small number of precursors, and the clearest estimates have put this number at between 2 and 5, with an average of about 3 [74]. Since follicles are normally colonized by cells expressing sIg, and sIg is allelically excluded, the distribution of cells expressing one or another IgM allotype in normal allotype heterozygous chicks also provides an estimate of the number of B-cell precursors per follicle. Again, a range of 2 to 5 with an average of about 3 precursors per follicle was consistently observed [71].

There are about 10,000 follicles in the average bursa, which means that the entire B-cell compartment in the chicken is derived from about 30,000 precursors that have undergone productive rearrangement at the heavy- and light-chain loci. Since only one in three light-chain rearrangements is in frame and only one in nine heavy-chain rearrangements is productive, only about 3% (i.e., 1 in 27) of B-cell precursors that have undergone rearrangement will contain both functional heavy and light chains. Therefore theoretical considerations place the minimum number of B-cell precursors in the developing embryo at approximately 10^6 , which is similar to the number that have been identified by quantitative polymerase chain reaction in the developing embryo [23].

The migration of B cells expressing surface immunoglobulin across the basement membrane to form B-cell follicles is a distinct process from the migration of B-cell precursors across the vascular epithelium into the bursal mesenchyme. Nonetheless, both processes require the directional migration of specific cells and it is likely that the selective expression of chemokines, chemokine receptors, and cell adhesion molecules plays a critical role in these processes as will be discussed in more detail later.

4.3.4 Growth of bursal B cells in bursal follicles

Following the migration of surface immunoglobulin-expressing cells into bursal follicles, there is a rapid proliferation among them. This proliferation coincides with the induction of gene conversion, resulting in the diversification of the VDJ_H and VJ_L sequences and leading to the generation of a diversified repertoire of B-cell specificities.

At this point, the molecule(s) responsible for inducing and maintaining this rapid proliferation are unclear. But, it was shown that all B cells in the bursa express CD40 and its ligand CD40L (CD154) strongly induces the proliferation of chicken bursal B cells in vitro [82], suggesting a contribution of the CD40-CD40L system to B-cell proliferation in bursal follicles.

The chicken homologue to the mammalian cytokine BAFF has been cloned and shown to be expressed at high levels in the chicken bursa [83,84]. Mammalian BAFF functions as essential survival factor for B cells [85]. Addition of recombinant BAFF to the cultures of bursal B

cells increases their survival [86] and expression of a BAFF decoy receptor that at least partially reduces the biological activity of BAFF in vivo leads to a reduction of bursal size [87]. It is likely therefore that the B-cell survival activity of BAFF is maintained in avian species.

During the final week of embryonic development, the number of surface immunoglobulin-expressing B cells in the bursa increases exponentially [88,89]. During this time, bursal cells typically undergo cell division approximately every 10 hours and while there is some cell death among them at this stage, the amount of cell death is substantially less than is seen in the bursa after hatch.

At this point, we can consider the role of bursal B-cell development in the embryo as an expansionary phase in which the bursal B-cell compartment goes from about 30,000 cells with a very limited repertoire to approximately 10^7 cells with a highly diversified repertoire. The bursal microenvironment is absolutely essential to the induction of gene conversion. This has been best demonstrated in chicks that have been "tailectomized" at EID 3. This procedure removes the tissue destined to become the bursa so that the bursal rudiment fails to develop. Remarkably, in such chickens relatively normal numbers of B cells develop in the periphery, suggesting that in the absence of the bursa, alternative sites exist in which B cells expressing surface immunoglobulin can develop [90,91]. Crucially, however, B cells developing in the absence of the bursa have a very restricted repertoire and their V genes do not show significant levels of gene conversion events [73].

A critical stage in the development of mammalian B cells is the functional elimination of those expressing self-reactive specificities [92–95]. In general, these mechanisms involve receptor editing, in which self-reactive specificities are removed by replacement of light-chain V gene sequences, clonal deletion, or the induction of an anergic state in which B cells are functionally inactivated. Several lines of evidence suggest that elimination of self-reactive B cells can occur in the bursa. As discussed previously, chicken B-cell development is supported by the expression of a CD8 α :Ig α homodimer in the absence of endogenous sIg expression [81]. The murine TL antigen is a cell surface molecule that is natural ligand for the murine CD8 α homodimer and using RCAS-mediated retroviral gene transfer in vivo, it was possible to express both TL and the CD8 α :Ig α homodimer in the same chicks. Critically as the levels of TL antigen increased, the frequency of cells expressing the CD8 α :Ig α decreased suggesting that ligation of the B-cell sIg receptor early in B-cell development results in B-cell deletion characteristic of negative selection [96].

As an alternative approach, antigen-specific chimeric B-cell surface Ig receptors were constructed by fusing the antigen-binding domain of the lamprey VLR with the

constant region of the truncated chicken IgM receptor ($T\mu$). In these experiments, chicken B-cell development was supported by a receptor in which a phycoerythrin-specific lamprey VLR was used as a model for a nonself-reactive surface receptor. In contrast, when the lamprey VLR fused to the chicken $T\mu$ receptor was specific for hen egg lysozyme (a chicken self-antigen), the resulting receptor led to B-cell deletion [96]. In conclusion, these results suggest that the developing chicken B-cell population is subject to self-tolerance.

4.3.5 Development of the bursa after hatch

By the time of hatch, the bursa contains about 10,000 follicles, each of which contains about 1000 bursal B cells. Each follicle is directly connected to the bursal epithelium, which separates the lymphoid compartment from the bursal lumen; the bursal lumen is itself connected to the gut lumen by the bursal duct. Shortly after hatch, the bursa goes through several changes that strongly suggest that its function after hatch may also be changing.

The first change in bursal structure is modification of the bursal epithelium overlying each follicle, resulting in the generation of “epithelial tufts” [97]. These structures, also called Follicle Associated Epithelium, are highly specialized and function to transport the contents of the bursal lumen into the bursa’s lymphoid compartment. Since the bursal lumen is connected to the gut lumen, this provides a means by which bursal B-cell development can continue in direct contact with molecules derived from the gut. In this regard, the cells of the bursal epithelial tuft appear to be structurally and functionally analogous to the M cells of mammalian appendix or Peyer’s patch [98,99]. The other major change that begins around the time of hatch is the segregation of bursal follicles into defined cortical and medullary regions (Fig. 4.4). Several lines of evidence suggest that these events are interconnected, as will be discussed next.

Prior to hatch, bursal follicles are rather homogeneous, with rapidly dividing B cells interspersed among a network of stromal cells. Surrounding the follicles at this time and interspersed in the interfollicular regions are cells expressing the intermediate filament protein desmin [100]. There

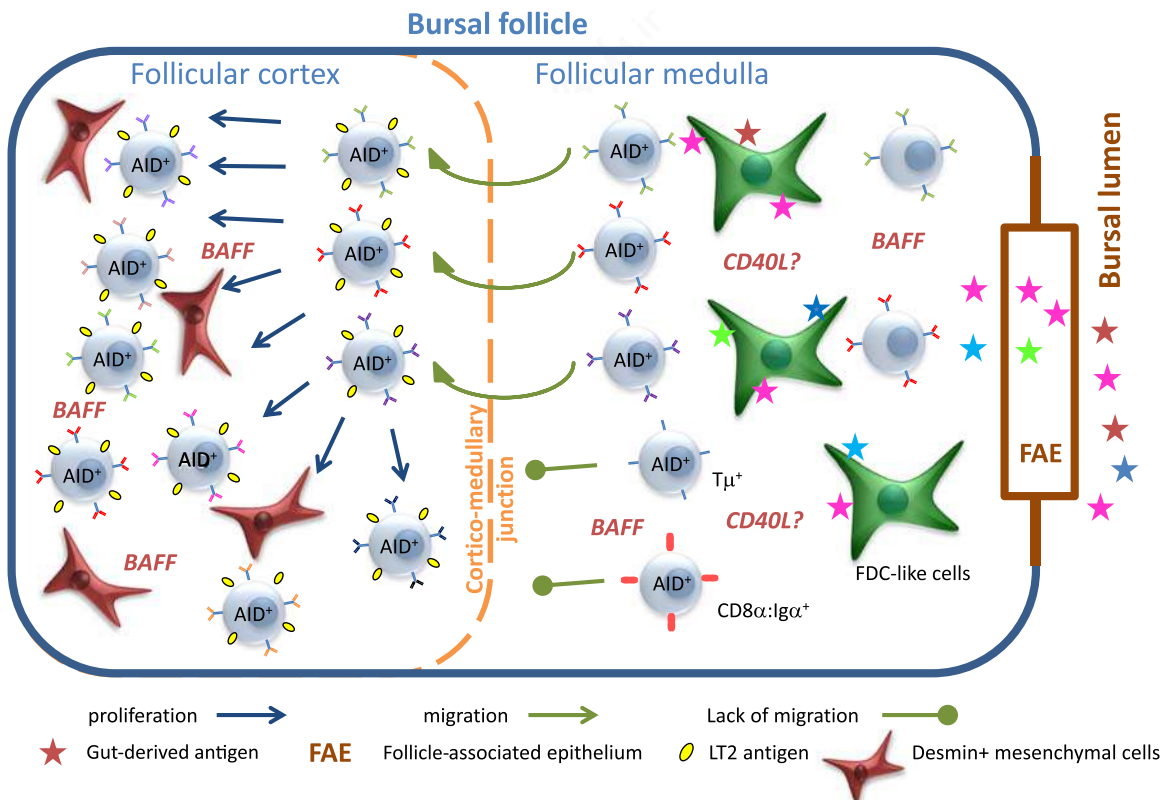


FIGURE 4.4 A model for antigen driven cortico-medullary redistribution of chicken B cells. Gut-derived antigen enters the bursal medulla through the FAE and is picked up and presented by follicular dendritic-like cells. Presentation of antigen to medullary antigen-specific bursal cells induces migration to the follicular cortex, which may be regulated by chemokine receptor expression. The CXCR4 ligand CXCL12 for instance is mainly found in the follicular cortex but at much lower levels in the medulla. In the cortex, bursal B cells proliferate and continue to diversify their immunoglobulin genes by gene conversion and express the LT2 antigen. FAE, follicle-associated epithelium.

is no clearly defined substructure to the follicle at this time. At around the time of hatch, however, some B lineage cells start to migrate back across the basement membrane and begin to proliferate among the desmin-positive cells between the basement membrane and the interconnective tissue that separates one follicle from another. Thus desmin-positive cells may form a scaffold for the developing cortex. Once the cortical and medullary regions have become segregated from each other, rapid bursal B-cell proliferation continues in the cortex but not in the medulla. Stathmokinetic analyses indicate that the rate of cell division in the cortex of bursal follicles is similar to what is seen in the embryo bursa, whereas the rate of cell division in the bursal medulla is reduced by at least an order of magnitude [89]. This is a strong indication that the medullary and cortical regions of the bursa have functional as well as structural differences.

Several lines of evidence suggest that the redistribution of bursal follicular B cells into cortical and medullary compartments is regulated in a fundamentally different way from the initial colonization of bursal follicles in the embryo. If bursal rudiments are grafted into the abdominal wall of normal embryo recipients, they become colonized by the recipient embryo's B-cell precursors. Follicles develop normally during embryonic life, but the engrafted bursa does not develop normally after hatch [101]. Similarly, if the bursal duct is ligated during embryonic development, thereby precluding the normal traffic of gut-derived molecules into the bursal lumen, bursal follicle development is normal in the embryo but their cortico-medullary structure fails to develop normally after hatch [102,103]. Taken together, these observations suggest that exposure to gut-derived molecules is critical for normal posthatch development of the cortico-medullary structure of the bursa.

In principle, these gut-derived molecules could be bacterially derived mitogens that directly induce maturation and proliferation of bursal B cells, or they could be bacterially derived products inducing stromal cells, perhaps via Toll-like receptor signaling, to produce cytokines supporting B-cell development. Alternatively, bacterial or gut-derived products could function as superantigens acting polyclonally on developing B cells or as specific antigens binding to bursal cell surface immunoglobulin. Any of these situations would lead to a requirement for gut-derived products to access the lymphoid compartment of the bursa, thereby explaining the importance of the connection to the gut lumen and the development of the epithelial tufts.

Interestingly, experiments with germ-free birds showed an undisturbed development of follicle architecture with cortex and medulla and unaffected numbers of B cells. Hence, it seems likely that gut-derived products, which induce cell proliferation in the cortex, are not of microbial origin (Kaspers and Härtle, unpublished data).

However, experiments in which the development of B cells was driven by retroviral transduction of either the $T\mu$ chain or $CD8\alpha:Ig\alpha$ chimeric receptors yielded important insights into the regulation of the later stages of B-cell development. Either the $T\mu$ receptor or the $CD8\alpha:Ig\alpha$ receptor supported all the early stages of early B-cell development, including colonization of bursal follicles, rapid oligoclonal expansion of B cells within the bursal follicles, and gene conversion [79,80]. This is also the case in chicks completely lacking a BCR mediated by knockout of the Ig heavy chain [104]. In addition, the cell surface phenotype of bursal cells supported by either $T\mu$ or $CD8\alpha:Ig\alpha$ receptors is indistinguishable from the phenotype of normal bursal cells from the same stage of development. As a consequence, it would seem unlikely that bursal cells supported by $T\mu$ or $CD8\alpha:Ig\alpha$ receptors would selectively lack the capacity to respond to a polyclonal signal that was independent of surface immunoglobulin expression. Nonetheless, neither the $T\mu$ receptor [105] nor the $CD8\alpha:Ig\alpha$ receptor [106] supported B-cell development after hatch. This means that the posthatch development of B cells is unlikely to be driven either by bacterially derived polyclonal mitogens acting directly on the bursal B cells themselves or by the induction of stromal cell-derived cytokines supporting B-cell development in the absence of surface immunoglobulin binding.

Conversely, these results support a model in which the development of B cells after hatch is dependent on the presence of Ig V region sequences on the bursal B-cell surface. This in turn would suggest that surface immunoglobulin receptor ligation, as distinct from receptor expression, is required for the later stages of B-cell development. We could envisage two potential explanations for this. One possibility is that the bacterial milieu of the gut contains superantigens which bind B-cell surface immunoglobulin independent of the specificity of the individual receptors. There is some precedent for B-cell superantigens recognizing particular V_H gene families in mammalian B cells [107,108], but at this point, there is no evidence for this occurring in the chicken and no candidate superantigens have been identified.

An alternative possibility is based on the probable antigenic heterogeneity of molecules present in the gut and therefore transported into the lymphoid follicles. By the time this transport is initiated, several days after hatch, there is substantial diversity of B-cell surface immunoglobulin receptors, so it is likely that in each follicle there are at least some bursal B cells that have immunoglobulin receptors specific for molecules transported from the gut. If surface immunoglobulin receptor ligation is required for the later stages of B-cell development, it follows that at any given point in time there are likely to be some B cells in each follicle with the appropriate specificity to recognize gut-derived antigens. This recognition

may be sufficient to support the cortico-medullary redistribution of bursal cells after hatch.

There is some evidence to support this possibility. In chicks transduced with the CD8 α :Ig α chimeric receptor, intrabursal introduction of anti-CD8 α antibodies maintained the presence of cells expressing CD8 α :Ig α after hatch, consistent with a posthatch requirement for surface immunoglobulin receptor ligation for B-cell development [106]. Similarly, in chickens expressing a T μ fused to the phycoerythrin-specific lamprey VLR, intrabursal application of phycoerythrin resulted in maintenance of VLR^{PE}T μ -specific B cells and supported their emigration to the periphery [109]. This observation provides evidence that ligation of bursal cell surface immunoglobulin could lead to positive selection as well as the negative selection discussed previously. Since the surface Ig receptor itself cannot know intrinsically whether it is interacting with a self-antigen or a foreign antigen, it begs the question as to how such distinct fates are regulated.

After hatch, the growth rate of the bursa slows, despite the fact that B cells in the cortex of bursal follicles are rapidly dividing [89]. Based on direct analyses of the rates of cell death among bursal lymphocytes and cell emigration from the bursa to the periphery, it has been estimated that about 95% of newly generated bursal cells die in situ by apoptosis [110,111]. Although it has been demonstrated that loss of cell surface immunoglobulin precedes apoptosis [112], it remains unclear why such a high proportion of bursal cells die in situ. In mammalian B-cell development, extensive cell death results from the accumulation of nonproductive rearrangements—cells that fail to express a surface immunoglobulin receptor die. This cannot be the situation in the bursa since all follicular B cells are derived from cells that express surface immunoglobulin. An alternative possibility is that the process of gene conversion might be highly error prone, leading to frequent out-of-frame V gene sequences. However, in B cells supported by the expression of the T μ receptor, an analysis of gene conversion events revealed an error rate of no more than 2%–3%, even under circumstances where nonfunctional gene conversion events were not selected against [44].

A further possibility is that, although gene conversion might generate predominantly in-frame products, combinations of heavy- and light-chain V regions could potentially be generated that fail to pair to form a functional immunoglobulin molecule. Evidence supporting this scenario comes from an analysis in which random combinations of full-length μ heavy chains and light chains were transfected into chicken embryo fibroblasts. Surprisingly, only about half of these combinations resulted in high-level surface immunoglobulin expression, with many others failing to be expressed at the cell surface (Neschadim and Ratcliffe, unpublished results). As a

result, even though gene conversion may not be intrinsically error prone, a substantial proportion of cells undergoing gene conversion may generate V_H/V_L combinations that are not expressed at the cell surface, leading to their apoptotic elimination. This may contribute to the high levels of cell death in the bursa.

While there is clearly trafficking of bursal cells from the follicular cortex to the medulla, it is not clear whether such trafficking is continuous or whether the cortex, once established, is simply maintained by in situ proliferation of cortical cells. If the establishment of the cortex requires surface Ig ligation, that is, positive selection in the medulla, the long-term maintenance of antigen in the medulla of bursal follicles suggests that bursal cell medulla-to-cortex trafficking may continue indefinitely. This in turn opens the possibility that once a bursal cell has migrated into the follicular cortex, it undergoes a finite number of cell divisions prior to either migrating to the periphery or undergoing cell death.

4.3.6 Role of cell adhesion molecules and chemokines in bursal cell development

B-cell development in the bursa is a sequence of events separated in time and location, in which highly regulated steps of B-cell migration can be observed. These are (1) the initial colonization of the bursal mesenchyme, (2) the migration into bursal follicle buds, (3) the redistribution of cells within the bursal follicle into cortical and medullary regions, and (4) the emigration of a small fraction of immature B cells into the periphery. Based on parallels with mammalian B-cell development, it is likely that these processes are regulated by the expression of chemokines, chemokine receptors, and cell adhesion molecules. This includes expression of specific adhesion molecules on the vascular epithelia of the bursa and complementary adhesion molecules selectively expressed on blood-borne B-cell progenitors.

Early investigations of potential adhesion molecules have shown that developing chicken B cells undergo changes in surface glycosylation during embryonic and posthatch development. Prebursal B cells express the carbohydrate epitope Sialyl Lewis^X (sLe^X), also known as CD15s, which is present in many glycoproteins and recognized by the selectin family of cell surface adhesion molecules [113]. In mammals, sLe^X-mediated binding to selectins allows circulating leukocytes to stick to and roll along endothelial cells lining blood vessels thereby enabling the leukocytes to accumulate at a site of vascular inflammation (reviewed in Ref. [114]). Along this line, sLe^X–selectin interaction could help prebursal B cells to leave the blood vessels in the bursa and colonize the bursal mesenchyme.

Interestingly, in addition to immigrating B cells Masteller et al. have described a chB6-negative population of larger cells with myeloid-like appearance that express high levels of sLe^X [115] and colonize the follicle buds in the bursa before the first chB6-positive B cells. Most likely these cells are the precursors of bursal secretory dendritic cells (BSDCs), which are the first leukocytes to seed the follicle buds [116].

Soon after migration into the bursa, sLe^X expression decreases and the cells begin to express the related carbohydrate epitope Lewis^X (Le^X/CD15), the expression of which identifies B cells that have started to undergo gene conversion [115,117]. Interestingly, loss of CD15s around embryonation day (ED)15 correlates with the time when developing B cells lose their ability to colonize a bursa [118]. Around hatch, surface glycosylation changes again. A fraction of B cells downregulates Le^X expression and becomes Le^X low, showing the same expression pattern as peripheral B cells [117]. In mammals, low-affinity homotypic and calcium-mediated Lewis^X–Lewis^X (Le^X–Le^X) interactions were proposed to be responsible for the initial steps of cell adhesion. In the bursa, this interaction could help to retain the cells in developing follicles, while down-regulation of Le^X expression would support migration into the periphery.

The young rabbit appendix is considered as homologue of the chicken bursa of Fabricius [119,120]. Strikingly, this does not only occur for the usage of somatic gene conversion to generate the antibody repertoire but also for the expression of sLe^X and Lewis^X [121]. sLe^X is expressed early on B cells in the blood and could mediate tethering and rolling by interacting with lectins on HEVs. Like in the bursa, expression switches to Le^X, at the time when cells start gene conversion. In addition, rabbit B cells with the capacity to colonize the appendix express CD62L (L-selectin), a homing receptor that binds ligands selectively expressed on specific vascular epithelia, and colonization of the rabbit appendix likely involves interactions between CD62L and PNAd (peripheral lymph node addressin) on the vascular epithelium [122]. In classical extravasation models, CD62L ligation typically induces a rolling behavior on the epithelial surface, promoting interactions with locally produced chemokines and stronger interactions involving integrin binding, which ultimately leads to extravasation [123]. Accordingly, it was shown for neonatal rabbits that blood B cells express the chemokine receptor CCR7 and CCL21, one of its ligands was found on HEV in the appendix. The same study suggests that LFA-1 and $\alpha_4\beta_1$ are the dominant integrins for B-cell recruitment into the rabbit appendix. To date, hardly anything is known about selectin expression in the bursa, but B cells in the bursa express β_1 integrin [124]. Together with the observed parallels in sLe^X/Le^X expression, it seems likely that

selectins and integrins involved in HEV extravasation in chickens are very similar to those found in rabbits.

In human and mouse, it was shown that CXCR4–CXCL12 interaction plays an essential role in early B-cell migration as it regulates stem cell migration into the bone marrow and migration of immature B cells from the bone marrow [125,126]. CXCL12 [127] and CXCR4 [128] were also described in the chicken. Recent work demonstrated the importance of this cytokine–cytokine receptor pair for both populating the bursa with B cells and migration of mature B cells into the periphery post hatch [129].

Around ED10, high and homogenous expression of CXCL12 is found in the mesenchymal part of the bursa anlage and prebursal B cells already express CXCR4 on the surface. The importance of CXCL12–CXCR4 interaction for B-cell migration into the bursa anlage and follicle formation was demonstrated by *in vivo* experiments with the CXCR4 blocking agent AMD3100. When the bursa anlage was transplanted on the chorioallantois membrane and injected with the CXCR4-inhibitor AMD3100, B-cell migration into the transplants was drastically reduced [129]. Furthermore, adoptive transfer of prebursal B cells into chicken embryos resulted in reduced follicle colonization when transferred B cells were pretreated with AMD3100 [130]. Consistent with a role for the CXCL12/CXCR4 axis in bursal colonization, granulocytes that also express CXCR4 are as well recruited into the bursa anlage.

After ED10, when CXCR4-negative hematopoietic precursors of BSDC have moved from the bursal mesenchyme into the surface epithelium of the follicle folds to initiate follicle bud formation by interacting with the epithelium [131], CXCL12 expression pattern changes and intraepithelial CXCL12 expression in the follicle buds became prominent. In parallel, between ED12 and ED14, the early CXCR4⁺ B cells migrate for the second time from the mesenchyme into the follicle bud, most likely guided by the high CXCL12 concentration.

At ED18 CXCR4 surface expression on bursal B cells is strongly increased as compared to earlier time points and high amounts of CXCL12 are found in the follicles [129]. Hence, it seems likely that high amounts of receptor and high CXCL12 abundance are responsible for retaining the developing B cells in the follicles, a CXCL12–CXCR4 function which is well-described for human B cells in the bone marrow [125].

Around hatch, in a third migration step, some B cells migrate back across the follicle surrounding basement membrane and start to proliferate between the encircling vimentin- and desmin-expressing mesenchymal reticular cells [100,132], leading to development of the follicle cortex and a clear separation of the follicle into cortex and medulla. At the same time, as B cells cross the basement membrane, CXCL12 expression becomes much stronger

in the developing cortex than in the central follicular region (medulla), until in the fully mature bursal follicle, CXCL12 is almost exclusively detected in the follicle cortex. This resembles CXCL12 expression in the mammalian GCs, where sequential modulation of the CXCL12-CXCR4 axis is essential to guide B-cell migration from the CXCL12-rich, highly proliferating dark zone into the CXCL12-low light zone for selection and back into the dark zone for additional rounds of proliferation and BCR mutation [133]. Another analogy can be found in the rabbit appendix. Here, B-cell follicles develop with a highly proliferating follicle base region and a nonproliferative apical region. In this apical region, follicular dendritic cells (FDCs) are located like in the bursal follicle medulla and the mammalian GC light zone [134] and the follicle base is the sole location of CXCL12 expression [135]. The observation that chicken bursal B-cell follicles show a CXCL12 expression pattern similar to mammalian GCs and B-cell follicles in the rabbit appendix argues for a contribution of CXCL12 and CXCR4 in the third migration step of bursal B to establish the follicle cortex/medulla architecture.

Around hatch, the final migratory step during bursal B-cell development is initiated with B cells emigrating from the bursa to seed the peripheral lymphatic structures. As mentioned earlier, at this time point, the follicle cortex is fully developed and is the site where the majority of B cells migrate [136]. Post hatch CXCR4 expression on bursal B cells decreases, a small subpopulation of bursal B cells closely resembling the very low level of CXCR4 on PBL B cells. A fraction of these CXCR4 cells has precisely the known phenotype of emigrating B cells (high surface expression of BCR and major histocompatibility complex (MHC) class II, positive staining for the OV antigen [137]) and are located next to those cortical capillaries enabling B-cell emigration [129]. This supports the model where bursal B cells have to downregulate CXCR4 surface expression in order to leave the CXCL12-high environment in the follicle cortex, a process also described for human and mouse B cells migrating from the bone marrow and the migration of GC B cells from the dark zone to the light zone [125,126,133,138].

As during initial follicle colonization only B cells leave the mesenchyme to migrate into the follicle buds but not the equally CXCR4⁺ granulocytes, it seems likely that further signals are required to initiate this second migratory step. Possible candidate signals are CXCL13 or CCL20 and their respective receptors CXCR5 and CCR6. Both chemokines and their receptors have been described in the chicken [139] and are important for B-cell migration in GC and gut-associated lymphoid tissue (GALT) structures in mice and humans [133,140]. In fact, CXCR5 is expressed on bursal B cells in varying amounts [141]. Interestingly, in contrast to a single CXCL13 protein in

mammals, three CXCL13 orthologues (CXCL13L1-L3) are identified in chickens, with CXCL13L3 showing the highest sequence identity with murine and human CXCL13 [139]. All three CXCL13 proteins efficiently induce chemotaxis of B cells [141] but detailed expression studies and functional analyses will be necessary to determine their role in bursal B cell development. Interestingly, recent studies demonstrated that CXCL13L1 is the real cellular orthologue for the Marek's disease virus (MDV)-coded CXC chemokine vIL8, which was initially be thought to derive from chicken IL8 but attracts B cells and T cells and no granulocytes [141].

In the rabbit appendix, CXCL13 is expressed already at birth and responsible for the attraction of B cells into the developing follicles [142]. Though CXCL13 expression in the bursa was not yet examined, proteomic analysis of ED10 bursa revealed the activation of the Vitamin A pathway [100]. This fits to the current model in mice, where retinoic acid is produced by neurons, induces CXCL13 in mesenchymal cells, which then attracts so-called LTis (lymphoid tissue inducer cells) and lead to the development of secondary lymphoid organs (reviewed in Ref. [143]). Hence, observations in rabbits and proteomic data make it tempting to speculate that a CXCL13 contributes to B-cell immigration into the developing follicles. Consequently, the CCLB and CCRB in Fig. 4.3 may represent CXCL13 and CXCR5, respectively.

4.3.7 Development of peripheral B cell populations

The function of the bursa is to generate naïve B cells for export into the periphery. Introducing the fluorescent dye fluorescein isothiocyanate (FITC) into the bursal lumen results in its uptake into bursal follicles and in situ labeling of bursal cells [110,137,144]. As labeled cells migrate from the bursa to the periphery, they can be detected by their fluorescence and defined as recent bursal migrants. Their direct quantization in the periphery indicates that the migration rate corresponds to approximately 1% of the peripheral-blood B-cell population per hour. This is consistent with 5% of newly generated bursal B cells migrating to the periphery.

In the juvenile bursa, most cell proliferation occurs in the follicular cortex. By analysis of bromodeoxyuridine incorporation following rapid uptake of label into the follicular cortex, bromodeoxyuridine-labeled cells were observed in the peripheral blood prior to their appearance in the follicular medulla [136]. This indicates that most recent peripheral-blood migrants are derived from migration directly from the cortex of bursal follicles (Fig. 4.5).

The life span of peripheral-blood B-cell populations has been assessed following surgical removal of the bursa. At

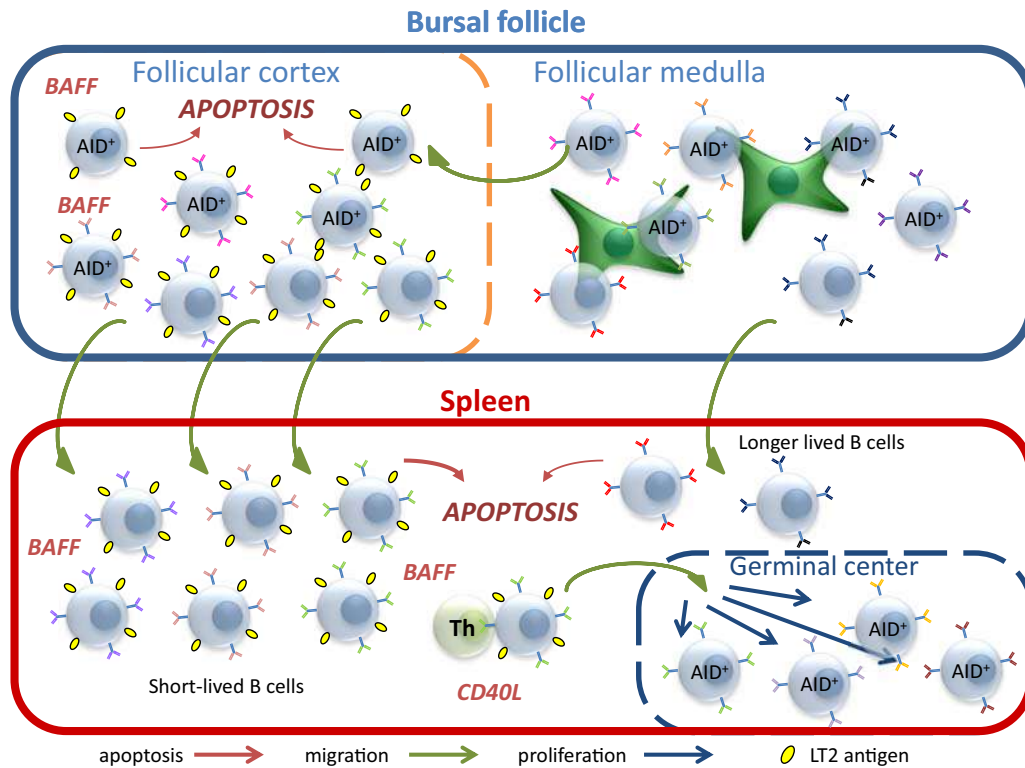


FIGURE 4.5 Heterogeneity of peripheral-blood B cells in the chicken. Most peripheral B cells emigrate from the cortex of bursal follicles as short-lived cells that express the LT2 antigen. A minority of bursal cells emigrate from the bursa as longer lived, LT2⁻ cells that may originate from the bursal medulla. In secondary lymphoid organs such as the spleen, antigen-specific B cells can be activated by exposure to antigen with the help of appropriate CD40L-dependent T-cell, leading to the formation of germinal centers in which B cells proliferate and induce the expression of AID, which results in further Ig diversification by gene conversion. *AID*, activation-induced cytidine deaminase.

3 weeks of age, approximately 60% of peripheral-blood B cells disappear, having an average life span of about 3 days. This accounts for the great majority of bursal cell migration from the bursa. The LT2 antigen is defined by the LT2 monoclonal antibody and stains about 60% of peripheral-blood B cells from 3-week-old chickens. Three days after surgical bursectomy, the frequency of occurrence of LT2-expressing bursal cells is reduced to less than 1%. This indicates that the short-lived population of recent bursal migrants expresses the LT2 antigen [145]. Although the LT2 antigen is a convenient marker of this population in the periphery, it has yet to be defined.

Following the initial loss of the short-lived B-cell population after surgical bursectomy, a further 30%–35% of peripheral-blood B cells disappear from the peripheral blood, having a life span of about 3 weeks. These cells do not express the LT2 antigen and therefore represent a functionally and phenotypically distinct peripheral-blood B-cell population [137,144,145]. Analysis of the distribution of LT2 expression on FITC-labelled recent bursal migrants indicates that about 90% expressed the LT2 antigen, whereas about 10% were LT2⁻. This is an important observation, as it suggests that functional differences between short- and longer lived B cells in the periphery

are established in the bursa and that B cells migrate already committed to being short- or longer lived. This suggests that at least two distinct pathways of B-cell maturation must exist in the bursa.

Most bursal cells migrate from the follicular cortex and express the LT2 antigen, consistent with the predominant expression of LT2 in the follicular cortex. Since the accumulation of gut-derived antigens in the bursa is restricted to the follicular medulla, this population of short-lived cells might represent a naïve repertoire of B-cell specificities. In contrast, approximately 10% of bursal emigrants do not express the LT2 antigen and likely represent the precursors of the longer lived peripheral-blood B-cell population. The source of these cells within the bursa is unclear, although, based on the distribution of bromodeoxyuridine labeling in pulse chase experiments, there is evidence that some bursal cells that have undergone division in the follicular cortex can ultimately migrate to the follicular medulla. Since the follicular medulla contains gut-derived antigens, it is tempting to speculate that the longer lived population of peripheral-blood B cells might represent a B-cell population that has been selected by exposure to antigen in the follicular medulla. The combination of short- and longer lived B-cell populations in the periphery might therefore

represent a combination of rapidly turning over and constantly replenished B cells with random specificities, and longer lived B cells selected for a repertoire defined by the range of antigens present in the bursa and therefore present in the environment.

Approximately 4 to 5 weeks after surgical bursectomy, the number of residual B cells in the peripheral blood stabilizes at about 5% of the original population. This level remains constant thereafter, and although they are not undergoing cell division in the peripheral blood itself, the B cells' rapid uptake of bromodeoxyuridine suggests that they are constantly being derived from rapidly dividing precursor cells [137]. Since this is observed in chickens in which the bursa has been ablated, the source of these cells must be extrabursal. Therefore, in addition to the short- and longer lived populations of bursal migrants that are nondividing in the periphery, the peripheral blood contains a third population of short-lived B cells that are derived from rapidly dividing extrabursal precursors. The proportion of this latter population is about 5% of peripheral-blood B cells at about 3 weeks of age. However, the frequency of this population appears to change with age. Thus, if the bursa is surgically removed earlier than 3 weeks, the frequency of this population decreases, whereas if it is removed later in life, the frequency increases. Therefore even though the immediate precursors to this population of B cells are extrabursal, this precursor population must be ultimately derived from the bursa.

This third population of peripheral-blood B cells may also be the source of peripheral-blood B cells in the older chicken, because the bursa involutes by about 6 months of age. The most likely source for these cells is the spleen. It has long been known that the spleen contains a population of cells that are functionally defined as post-bursal stem cells with the capacity to provide long-term reconstitution when transferred into cyclophosphamide-treated recipients. In the normal spleen, most B cells express the OV alloantigen; however, there is a small population of B cells that lacks this expression and have a characteristically larger size than the majority of splenic B cells. Following surgical bursectomy in 3-week-old chickens, there is a loss of splenic B cells, in parallel with the loss of peripheral-blood B cells; however, splenic B cells that do not express the OV antigen are not reduced and can become the major residual population. They are therefore a strong candidate for the postbursal stem cell population and are a likely source of the peripheral-blood B-cell population that is not sensitive to surgical bursectomy [144].

Mammalian B cells leave the bone marrow and first migrate to the spleen. Despite the differences in development between avian and mammalian B cells, it is probable that chicken B cells, after their emigration from the bursa, also migrate to the B-cell areas of the spleen [144].

These areas surround penicillary capillaries and are named periellipsoidal white pulp or periellipsoidal lymphoid sheath. The morphological discrimination between B-cell follicles and the surrounding marginal zone, a characteristic of mammalian B-cell areas, has not been seen in the chicken spleen, and at this point, there is no functional proof for the existence of follicular and marginal-zone B-cell homologues in the chicken. Further B-cell areas in the spleen are the GCs, which in the chicken are located at blood vessel branchings in the T-cell zone (periarterial lymphoid sheath, PALS), while additional single B cells are also interspersed in the PALS [146].

In addition to the spleen, which is directly connected to the blood circulation, chickens possess many mucosa-associated lymphoid tissues (MALT), including cecal tonsils, bronchus-associated lymphoid tissue, and the Harderian gland (see Chapter 11, for a detailed description). These secondary lymphoid tissues, the size of which is dependent on antigen exposure, do not contain distinct T- and B-cell areas but rather an admixture of both lymphocyte populations and large GC.

4.3.8 Activation of peripheral B cells

The molecular mechanisms underlying the activation of murine B cells have been well defined. Antigen picked up by BCR is processed, and antigen-derived peptides are presented in association with MHC class II on the B-cell surface. Helper T cells specific for these peptide/MHC class II complexes interact directly with the responding B cells and provide help through CD40L on the T cell interacting with CD40 on the B-cell surface. Implicit in this is the requirement for an MHC-restricted interaction between the helper T cell and the responding B cell. The naïve B cell is therefore activated by a combination of two signals, one through the BCR-binding antigen and the other through CD40-binding CD40L. This activates the responding B cell to the point where it can respond to cytokines, inducing proliferation and maturation to immunoglobulin secretion (reviewed in Ref. [147] and references therein).

At this point, the activation of chicken B cells to antibody secretion appears to be indistinguishable from the activation of mammalian B cells. Cyclophosphamide treatment destroys the lymphoid compartment of the bursa. Transfer of bursal cells into cyclophosphamide-treated recipients results in reconstitution of the bursal lymphoid population by donor-derived cells which can mature and reconstitute the periphery. The colonization of the bursa by donor cells is independent of the donor cell strain, and since recolonization typically takes place within a few days of hatch, allogeneic donor cells are not rejected. Following bursal and subsequent peripheral reconstitution, both syngeneic and allogeneic B cells can

be activated by polyclonal B-cell activators, an observation which demonstrates functional reconstitution by both sources of bursal cells. In contrast, only B cells sharing MHC class II with the host helper T-cell population are activated by T cell-dependent antigens. This is direct evidence that an MHC class II-restricted interaction between the helper T cell and the responding B cell is required for chicken B-cell activation [148].

When naive mammalian B cells have specifically bound antigen and receive appropriate T cell help, some of them form GC in the B-cell follicles of secondary lymphoid tissues. During the subsequent GC reaction, B cells receive signals from CD4⁺ helper T cells and FDCs, which induce proliferation of antigen-specific B cells, affinity maturation, and immunoglobulin class switching, resulting in the generation of both plasma cells and memory cells with a high-affinity BCR (reviewed in Refs [149,150]).

Birds also form GC, but in contrast to mammals, avian GCs are not located in splenic B-cell follicles but form as spherical structures, with a diameter of 50 to 175 μ m and a surrounding collagen capsule, in arterial branchings within the T-cell zone [151]. While mammalian GCs include a dark zone with densely packed proliferating centroblasts and a light zone with loosely packed centrocytes, FDCs, and follicular helper T cells [152], avian GCs look more homogeneous. Nonetheless, BrdU incorporation revealed a ring of strongly proliferating cells under the GC capsule and, by immunohistochemistry, CD4⁺ T cells and FDCs are detected in middle of the GC [146]. This suggests that chicken GCs have a zonal segregation like that seen in mammalian GCs with a subcapsular dark-zone homologue and a central light-zone equivalent. GCs in lymphoid tissues other than the spleen are typically larger, but with a similar composition.

The main functions of mammalian GCs are the induction of B-cell proliferation after antigen contact, affinity maturation, and immunoglobulin class switching. To date, there have been very few functional studies on avian GC, and definitive evidence for affinity maturation either in GCs or in overall antibody responses is lacking. Similarly, it is not clear whether GC B cells express the LT2 antigen (Fig. 4.5). Nonetheless, it has been shown that chicken GCs are oligoclonal, being derived from a small number of activated B cells, and that the BCR of GC B cells is modified by gene conversion and somatic hypermutation [153,154]. Furthermore, IgY- and IgA-positive B cells are detectable by immunohistochemistry in GC, indicating the occurrence of Ig class switching [146]. This suggests that, despite differences in localization and structure, the function of the chicken GC is probably equivalent to that of mammals.

There are two further points that should be raised. Since AID is required to initiate somatic hypermutation

and gene conversion, as well as class switching, it must be expressed in GC just as it is in the bursa. However, AID expression in the bursa results in hypermutation and gene conversion but not class switching. Therefore GC-specific factors other than AID are selectively required for class switching. In addition, while a major GC function is affinity maturation, which can be efficiently achieved by selection of B cells in which the BCR is modified by the point mutations that result from hypermutation, it is likely that the gene conversion events observed in chicken GCs subvert the process of affinity maturation by introducing too much variation in heavy- and light-chain V gene sequences. This may underlie the difficulty encountered in detecting significant affinity maturation of chicken antibody responses.

T cell-dependent B-cell activation leads to the generation of B-cell memory and enhanced secondary antibody responses. This is also the case in chickens, although the presumably long-lived memory B-cell population has not been unequivocally identified.

4.3.9 Plasma cell development

In the terminal differentiation step following antigen encounter, the B cell first becomes a plasmablast and subsequently a plasma cell, capable of secreting large amounts of IgM, IgA, or IgY. Because of the lack of distinct markers, chicken plasma cells are typically identified by their characteristic morphology (large lymphocytes, eccentric nucleus with cartwheel formation) and strong staining for Ig. As early as 48 hours after intravenous immunization, IgM⁺ antibody-secreting cells (ASC) are detectable in T-cell areas of the spleen. After 72 hours, these cells are mainly found in the red pulp of the spleen, and after 96 hours, the first IgY⁺ plasma cells appear [155]. Although the first IgM⁺ ASCs are presumably short-lived plasma cells, which develop similarly to mammalian extrafollicular plasma cells without the selective processes of a GC reaction, the later-occurring IgY⁺ cells probably originate from a GC [156,157].

Outside the spleen, a high frequency of plasma cells can be found in various MALT tissues. Cecal tonsils contain both IgY- and IgA-expressing cells, while in the Harderian gland, in addition to IgM⁺ cells, IgA⁺ plasma cells predominate, lying beneath the epithelium of the gland's excretory duct. In addition, many of these cells are located in the gut lamina propria, and plasma cells of each isotype can be found in the bone marrow. It remains unclear whether the chicken bone marrow stroma forms the specialized microniches that in the mammalian bone marrow produce specific survival factors like APRIL (TNFSF13, a proliferation-inducing ligand, see next section) to ensure long-term plasma cell survival [158].

4.3.10 Cytokines in chicken B cell development and activation

Activation, proliferation, and survival of B cells at all stages of differentiation are profoundly influenced by cytokines. In mammals, it is well known that several members of the tumor necrosis factor (TNF) and TNF receptor (TNFR) families are important in the homeostasis of lymphoid cells and tissues with the B cell-activating factor of the TNF family (BAFF/Blys/TNFSF13B) and CD40 ligand (CD40L/CD154/TNFSF5) playing an essential role in B-cell survival and activation. Recently, chicken homologues for BAFF [83] and CD40L [159] have been described and functional examination shows some similarities but also striking differences from BAFF and CD40L function in human and mouse.

BAFF was described in mammals as one of the most important cytokines for the survival and maturation of peripheral B cells. Its function in the homeostasis of recirculating and marginal-zone B cells is essential [160,161]; BAFF knockout mice have shown that immature B cells in the bone marrow develop in the absence of BAFF, while progression beyond transitional stage 1 (T1 B cells) is almost completely blocked [162,163]. Overexpression of BAFF in BAFF-transgenic animals leads to a massive enlargement of the peripheral B-cell pool and autoimmune diseases which strongly resemble systemic lupus erythematosus and Sjörger's syndrome in humans [164,165]. Thus physiologically expression and secretion of BAFF must be tightly regulated to ensure B-cell survival on the one hand and to avoid development of autoimmunity on the other.

The chicken homologue for BAFF has a 76% amino acid identity with human BAFF and is therefore surprisingly highly conserved [83]. Indeed, BAFF appears to be highly conserved in the whole class of Aves, with BAFF homologues identified in duck [166], goose [167], quail [168], and common ostrich (*Struthio camelus*) [169].

Mammalian BAFF can be constitutively expressed and/or induced. Stromal cells, including FDC from lymphatic tissues, constitutively produce BAFF, and this constant amount of cytokine is responsible for the maintenance of the peripheral B-cell pool [170]. During an immune response, BAFF production is also induced in monocytes, macrophages, dendritic cells, and granulocytes [171], which allows the accumulation of B cells at inflammatory sites [172,173]. Chicken BAFF mRNA was also detected in bursal B cells [84,86,174]. Expression studies with purified B cells and B cell-depleted bursal tissue clearly showed that most if not all BAFF in the chicken spleen and bursa is produced by B cells [86]. Autocrine BAFF production by chicken B cells contrasts with the lack of BAFF expression in murine splenic B cells, but, interestingly, under circumstances where the immune system is systematically activated in autoimmune diseases, human and mouse B cells can also produce

BAFF [175]. In addition, Yeramilli and Knight demonstrated that BAFF expression also predominated in rabbit appendix B cells [176]. Hence, autocrine BAFF expression by B cells seems not restricted to chickens but may apply in general to B-cell development in GALT.

Mammalian BAFF can bind to three different receptors: B-cell maturation antigen (BCMA, TNFRSF17), transmembrane activator, calcium modulator, cyclophilin ligand interactor (TACI, TNFRSF13B), and BAFF receptor (BAFF-R, BR3, TNFRSF13C) [162,177]. Expression of the different receptors is regulated during B-cell development [178,179]. BAFF-R is the receptor which mediates survival of peripheral B cells and is exclusively bound by BAFF [180,181]. BCMA and TACI can also bind the closely related TNF family member APRIL (TNFSF13), which is dispensable for normal B-cell development [182] but has been shown to play a role in CD40-independent class switching and the long-term survival of plasma cells [183,184]. In the chicken genome, functional homologues for BAFF-R and TACI have been identified, while the chicken BCMA homologue turns out to be a pseudogene [139,185]. Furthermore, there is no evidence either in the genome or in expressed sequence tag databases for a chicken APRIL homologue, which means that with only two receptors and one ligand, there is a considerably lower complexity of this interacting TNF–receptor–ligand group in the chicken than in mammals. Since in humans and mice, the long-term survival of plasma cells in the bone marrow is mainly mediated by APRIL binding to BCMA [183,186], and chickens probably lack both, the mechanism ensuring chicken plasma cell survival is unclear.

The expression of BAFF-R mRNA in the bursa during development was shown to increase in parallel with the number of B cells, while for TACI, a strong increase was detectable only post hatch [185], indicating a differential expression of BAFF receptors during chicken B-cell development. This may provide a mechanistic basis for the differential behavior of chicken bursal B cells before and after hatch. Thus embryonic bursal B cells maintained by sIg expression in the absence of ligation may require signaling through a different combination of BAFF receptors as compared to bursal cells after hatch, the maintenance of which requires sIg ligation.

Staining of BAFF receptors using recombinant cytokine revealed that receptor expression starts early in chicken B-cell ontogeny. As early as ED14, weak expression of BAFF receptors is detectable on both prebursal B cells in the spleen and on bursal B cells, with the later showing a continuous increase in BAFF receptor expression until hatch [86]. This contrasts with human and murine B cells, where BAFF-R expression is not initiated until the T1 B-cell stage [178], a more mature stage of development than that of chicken prebursal B cells.

BAFF neutralization, either by injecting the cross-reacting human decoy receptor BCMA as recombinant

protein [86] or by expressing huBCMA or chBAFF-R in RCAS-transduced chickens [87,185], strikingly reduced the size of bursal follicles and the number of B cells within bursal follicles. This demonstrates the necessity of BAFF for B-cell survival in bursal follicles and suggests that the cytokine is already important in the initial expansion of B cells after their migration into the bursal follicle. In contrast, BAFF overexpression using the RCAS retroviral gene transfer system did not noticeably affect B-cell numbers in the bursa [87]. In conclusion, these observations suggest that naturally occurring high levels of BAFF in the bursa may provide maximal support for B-cell proliferation. Hence, in the chicken, in contrast to its function in humans and mice, BAFF is essential for the immature stages of B-cell development. Interestingly, this parallels observations in rabbits, where B-cell development in the appendix is also BAFF dependent [176].

The introduction of recombinant BAFF- and the RCAS-mediated overexpression of BAFF induced strongly elevated B-cell levels in blood, spleen, and cecal tonsils, accompanied by increased levels of serum IgM and IgA [86,87]. Even more dramatic was the effect of BAFF neutralization, resulting in peripheral B-cell numbers that decreased to only a few percent of normal numbers. While these results could be explained by a role for BAFF in regulating bursal cell migration to the periphery, the parallel with mammalian species makes it more likely that chicken BAFF acts as a survival factor for peripheral B cells.

Similarities in BAFF functions on peripheral B cells in chickens and mammals suggest that mechanisms for maintaining the peripheral B-cell pool are conserved. Nonetheless, considerable differences in the role of BAFF in primary B-cell development exist between mammals and birds. Because B-cell survival in certain human and murine GALT structures such as Peyer's patches is also supported, at least in part, by BAFF [187], this could argue for a model in which the bursa as a primary lymphoid organ and possibly also the rabbit appendix evolutionarily originated from secondary lymphatic tissue.

Another receptor–ligand pair belonging to the TNF/TNF-R family with an important role in immunological processes is CD40 and CD40 ligand pair. CD40 is a potent costimulatory molecule on mammalian B cells. On B cells, the main function of its binding to CD40L is induction of cell proliferation, initiation and maintenance of a GC reaction, and, through this, induction of affinity maturation and immunoglobulin class switch. In the absence of CD40L, which in mice and humans is mainly expressed by CD4⁺ helper T cells, B cells are not capable of seeding GC, and the resulting antibody response is limited to low-affinity IgM [188,189]. In consequence, mutations in either CD40 or CD40L lead to hyper-IgM syndrome in humans, with dramatically reduced serum levels of IgG, IgA, and IgE, along with elevated amounts of IgM [190,191].

Interestingly, in the 1980s, there were reports of chicken lines with increased IgM and very low serum IgY levels—a phenomenon known as dysgammaglobulinemia [192,193]. Since, at that time, the function of CD40L had not been defined, the syndrome was never further characterized. However, it is thought that it might have been due to a defect in the chicken genes for CD40 or CD40L as subsequently identified by Young et al. [159]. CD40 is expressed on all chicken B cells, monocytes, and macrophages and to a lesser extent on thrombocytes [82,159]. In vitro stimulation of B cells from the spleen and bursa through CD40 induced massive cell proliferation and enabled long-term culture of splenic B cells for several weeks. CD40L stimulation also induced differentiation of peripheral chicken B cells toward a plasmablast-like phenotype with downregulated surface IgM and increased cytoplasmic immunoglobulin expression [82]. Thus the function of CD40 and CD40L in peripheral B cells seems to be largely conserved between birds and mammals.

However, the expression of CD40 on all bursal B cells and the proliferation of these cells after CD40L stimulation suggest an additional role for CD40/CD40L interactions during the earlier stages of chicken B-cell development. The effect of CD40L on early human and murine B-cell development is still controversial [194,195] but in rabbits there is evidence for an essential role of CD40L stimulation on early B cells. Rabbit appendix B cells proliferate upon CD40L stimulation [176] and inhibition of CD40 ligation by a soluble decoy receptor leads to a significant reduction of B cells in the appendix [196]. Usually, CD40L stimulation is mediated by CD4⁺ helper T cells. Though there are T-cell areas in the rabbit appendix, the source of CD40L for early B-cell proliferation is thought to be dendritic cells, macrophages, or epithelial cells [196]. This model could also be applicable for bursal B-cell development, where no T cells are present in the follicles. Similarly, to the survival function of BAFF, the proliferative effect of CD40L in the chicken bursa and rabbit appendix parallel their function in human and murine secondary lymphoid tissues and make it tempting to speculate that both bursa and appendix have evolved from secondary lymphatic tissue.

Next to TNF family members, many other cytokines are involved in the regulation of mammalian B-cell development and differentiation. Important B-cell cytokines such as IL7, IL10, and IL21 and their receptors [197–200] have been described and cloned in the chicken, and it will be interesting to see whether they exhibit rather conserved or more chicken-specific function.

4.3.11 Application of B cell cultures

In cell culture, chicken B cells are very fragile and lipopolysaccharide, the classical stimulus for murine B cells,

does not work on chicken B cells. However, the availability of chicken CD40L and its strong proliferation-inducing capacity have led to new possibilities for ex vivo cultures with long-term cultivation of splenic B cells up to 3 weeks [82]. As chicken B cells are the target for several economically highly relevant viruses, these cultures greatly expand the possibilities to study interactions between viruses and target cells which so far relied on cultures of embryo fibroblasts or animal experiments. Usage of CD40L-driven B-cell cultures were established for infection with MDV, where it is possible to precisely reflect the life cycle of this oncogenic herpesvirus in vivo and evaluate transcription of viral genes in detail in an easily accessible system [201,202]. Field strains of infectious bursal disease virus so far had to be propagated in embryonated eggs, but now this can also be done in CD40L-stimulated cultures of bursal B cells and was used for a better description of viral gene expression [203–205].

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Structure and evolution of avian immunoglobulins

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5.1 The basic structure of immunoglobulins

Immunoglobulins (Ig) are glycoproteins that have antibody (Ab) activity and are found in the blood, lymph, and vascularized tissues of all the jawed vertebrates [1,2]. Their basic unit structure (Fig. 5.1) consists of four polypeptide chains, two heavy (H) and two light (L), that form the monomeric unit (H_2L_2). Each class of immunoglobulin can form a membrane-bound antigen receptor or a soluble secreted immunoglobulin (Fig. 5.1). Immunoglobulin G (IgG) consists of the basic unit but more complex molecules, such as IgM and IgA, are made up of multiples of the unit—for example, $(H_2L_2)_n$. The H and L chains are formed from domains, each of about 115 amino acids, which have highly conserved cysteine and tryptophan residues and an intradomain disulfide bridge that confers the functionally important tertiary structure. The H and L chains are joined by interchain disulfide bonds.

Domains at the amino terminal are highly variable (V), and the V_H and V_L domain pairings create the antigen (Ag)-binding site that confers the Ab specificity. The Ag-binding cleft accommodates an epitope (antigenic site) of 6–9 amino acids or carbohydrates. Since pairs of H and L chains joined by disulfide bonds form the basic “monomeric” Ig unit (H_2L_2), it possesses two Ag-binding sites (Fig. 5.1). Very little genetic variability is found in the other domains, and these are referred to as the constant region domains (CH or CL). H chains typically have 2–4 C region domains, and the L chains have a single C region domain. The biological properties are dependent on the C domains for membrane transportation; these include half-life and initiating secondary effector functions, such as complement fixation and opsonization. For a detailed description of Ig structure, the reader is referred to Burton et al. [3] and Turner et al. [4].

Advanced vertebrates possess several Ig classes, distinguished by H chains that have separately encoded C regions. These are found to be antigenically distinct when compared using antisera specific to the C_H regions of the various Ig classes. In mammals, most studies have been carried out on human and mouse, and five Ig isotypes, or classes, have been identified: IgM, IgA, IgG, IgD, and IgE, which are defined by their distinct H chains (μ , α , γ , δ , and ϵ , respectively). These different isotypes have characteristic patterns of multimerization; for instance, IgM is generally a pentamer and IgA is a dimer. Each isotype-specific C region is encoded by a distinct gene, and multiple heavy-chain isoforms can be produced by alternative pathways of RNA processing, such as the secreted Ig and membrane Ig forms of all H chains, or full-length and truncated H-chain isoforms, as has been reported for some avian species (see Section 5.2.2). Multimeric forms of Ig contain a polypeptide joining (J) chain, which has a molecular weight (MW) of 15 kDa [5]. The J chain does not seem to be essential for Ig polymerization [6] and is probably incorporated as a result of, rather than as a prelude to, Ig assembly. Evolution of the various Ig classes has probably made a significant contribution to functional diversity, as there are considerable differences in function between the human Ig classes in terms of Ag processing and recruitment of effector mechanisms. In some mammals, different subclasses of the Ig isotypes such as IgG1 to IgG4 in humans have been reported. These are encoded by separate genes but have H chains with regions of similarity; as such they may represent recent gene duplication events. In addition to isotype (class) and subclass variability, allelic variation in different individuals can give rise to different Ig allotypes.

L chains have been highly conserved in evolution [7–9], but different types of L chains can be used. In

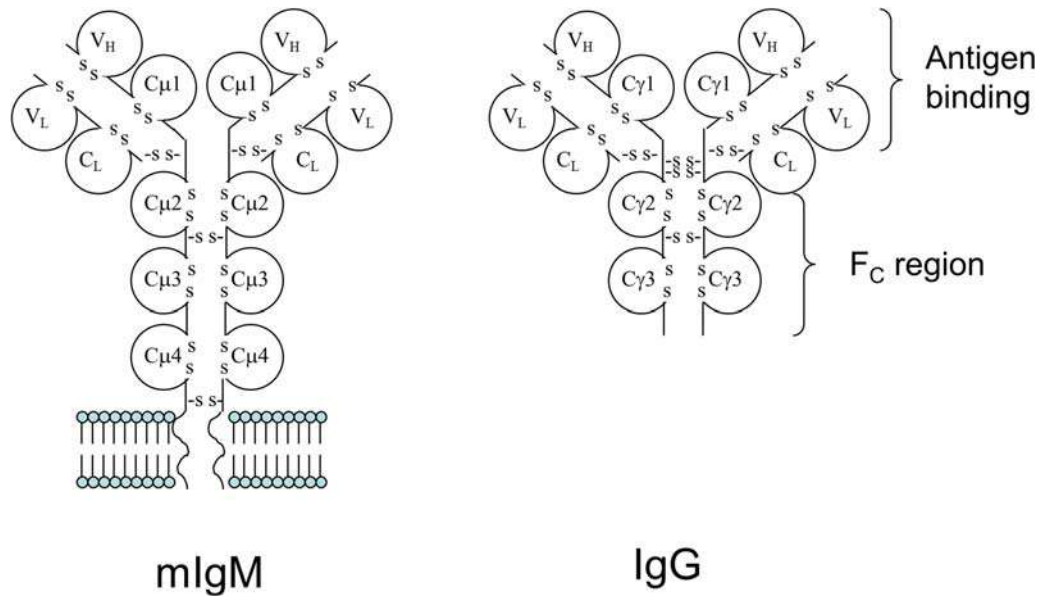


FIGURE 5.1 Schematic of the structure of the membrane-bound IgM (mIgM) molecule and IgG. The various domains of the heavy (H) and light (L) chains are shown as variable (V) and constant (C). The C domains of the heavy chain of the IgM molecule (C_μ) and IgG molecule (C_γ) are numbered.

mammals, κ and λ have been identified. Many species possess both of these isotypes but use them in markedly different ratios [7]. The varying species utilization of the κ and λ isotypes is not fully understood but, since they are encoded on different chromosomes (also different from the chromosome carrying the H-chain locus) and utilize different pools of V genes, there may be advantages, in terms of the Ab repertoire, in using both. However, a single Ig molecule uses only one L-chain isotype, just as it uses a single H-chain isotype, (e.g., $\gamma_2\lambda_2$), and a single plasma cell normally produces Ig utilizing single H- and L-chain isotypes, providing a single Ag specificity. The suppression of the unutilized isotypes is referred to as “allelic exclusion.”

5.2 Avian immunoglobulins

Using immunocytochemical and genetic techniques for a long time, only three classes of avian Ig had been described as the homologues of mammalian IgM, IgA, and IgG. Detailed reviews of their structural, physical, and chemical properties, as well as their biological functions, can be found elsewhere [10–16]. Very recently genomic analysis has revealed an additional IgD homologue in the ostrich and some Neoaves [17]. Most extensive studies have been performed on the chicken (reviewed in Higgins [12]) and duck [18–27], but there are also descriptions of Ig from other species such as turkey [28–30], pigeon [31,32], pheasant [33–36], quail [33–36], numigall (a chicken/guineafowl hybrid) [37], ostrich [17,38], and penguins [39,40].

5.2.1 Avian IgM

Chicken IgM is structurally and functionally homologous to its mammalian counterpart. It is the predominant B cell antigen receptor, and during embryonic development, it is the first isotype to be expressed. Physical determinations of chicken IgM under various conditions have suggested a MW in the range 823–954 kDa, with an average value of 890 kDa. However, the H chain has a MW of ~ 70 kDa, while the L chain has 22 kDa [12,41], which indicates that chicken IgM is more likely to have a tetrameric $(\mu_2L_2)_4$ rather than a pentameric $(\mu_2L_2)_5$ configuration; perhaps it is a mixture of the two. Small amounts of monomeric (7S) IgM in normal chicken serum have been reported [42,43]. Free μ chains have been found in sera from bursectomized chickens [43,44] and in the survivors of infectious bursal disease [45]. Recently, it was shown that sera from L-chain knockout birds also contain secreted H-chain-only antibodies. These μ chains lack the CH1 domain and their antigen-specific production is induced by immunization [46]. However, at present it is still unclear whether Hc-only antibodies are only found under pathological and artificial conditions or might also play a role under physiological conditions.

Surprisingly, analysis of the ostrich and emu genome sequence revealed two functional μ genes (μ_1 and μ_2), which share an overall amino acid identity of almost 60% and exactly the same CH4 sequence. The μ_2 gene is located downstream of $C\alpha$ and transcriptomic analysis demonstrated expression of both genes, though μ_1 was expressed to a much higher level [17].

The mean serum concentrations of IgM range between 1 and 2 mg/mL (see Table 5.1) [42,56]. The MW of duck IgM is 800 kDa and, with its components H (86 kDa) and L (22 kDa) chains [22], this also suggests a tetrameric structure.

Invariably, IgM is the predominant isotype produced after initial exposure to a novel antigen. As in mammals, this response is usually transient [34,60–63], although after chronic bacterial infection, such as *Bordetella avium* in turkeys, IgM is reported to be active for several weeks [64]. Research into the biology of avian IgM has been somewhat limited because of the apparent evolutionary conservation of the IgM molecule and its transient role in the protective immune response.

5.2.2 Avian IgY (IgG)

Phylogenetic studies have shown that the avian, low-MW, homologue of mammalian IgG has similarities with both mammalian IgG and IgE and is probably equidistant between them. This avian isotype is the predominant form in sera, produced after IgM in the primary antibody response, and it is the main isotype produced in the secondary systemic antibody response. It is often referred to in the literature as IgY, as originally proposed by Leslie and Clem [41]. Salt precipitation studies indicate that the low-MW avian form of Ig has different biochemical properties from those of mammalian IgG—hence, the name IgY. Since the chicken isotype shares homology in function, others have suggested that the term *avian IgG* should be retained [65]. As a result, both IgY and IgG are used interchangeably in the literature. We prefer to use the name *IgY* because this Ig appears to be the evolutionary predecessor of both IgG and IgE [16,66], and it shares homology with each of these mammalian isotypes; also, this nomenclature is now well established for all nonmammalian vertebrates and well used for amphibians and reptiles.

The major difference between chicken IgY and its mammalian homologue is the longer H chain in the chicken molecule. Avian IgY consists of five domains (V, C1–C4), as opposed to the four that are found in mammalian IgG, and the avian molecule does not possess a genetically encoded hinge. Instead, there are “switch” regions with limited flexibility at the C ν 1–C ν 2 and the C ν 3–C ν 4 domain interfaces. The limited flexibility of the avian IgY may account for some of its unique biochemical properties, such as the inability to precipitate antigens at physiological salt concentrations, which is seen in chickens and ducks. For example, the two arms may be so closely aligned that they preclude cross-linking of epitopes on large antigens (reviewed in Ref. [67]). The C ν 2 domain may have been condensed in subsequent evolution to become the genetic hinge found in mammalian IgG.

Next to two μ genes, recent sequence analysis of the ostrich genome revealed a second ν gene, which shares only about 50% sequence identity with the ostrich ν 1 gene. Transcription studies demonstrated a very low ν 2 expression in young ostriches but a high RNA abundance in the spleen of adult birds [17]. A second ν gene has also been identified in the pigeon genome and both ν genes seem to be expressed at almost equal levels [32].

Although IgY is the major avian systemic Ab active in infections, detailed protein characterization has only been carried out in the chicken and the duck. Chicken IgY in serum is monomeric, H $_2$ L $_2$, with MW 165–206 kDa [41,47,59,68,69], although a 19S polymer has been detected in sera of day-old chicks [43]. The MW of the ν chain is 67–68 kDa [37,41]. The mean serum concentrations of chicken IgY averages 5–7 mg/mL [42,52]. For detailed information on the physical, chemical, and antigenic properties of chicken IgY, the reader is referred to other reviews [10,12]. The amino acid sequence of the ν chain and its

TABLE 5.1 Characteristics of chicken Ig.

Property	IgY	IgM	IgA	
Molecular weight (Kd)	165–206 [41,47,48] ^a	820–950 [12,41]	Serum	170–200 [41,49]
			Intestine	300–500 [50]
			Bile	350–900 [49–51]
Serum half-life (days)	3–4 [52,53]	1,7 [52]	2 [53,54]	
Concentration (mg/mL) in				
Serum	5–7 [42,52,55]	1–2 [42,56]	0,35–0,65 [42,56]	
Yolk	7, 9–25 [57,58]	minimal	minimal	
Albumen	–	0,15 [58]	0,7 [58]	
Configuration	Monomer [41,59]	Tetramer/pentamer [12,41]	Monomer/trimer/tetramer [50]	

^aSee references.

disulfide-bonding patterns have been deduced from the cDNA nucleotide sequence [66]. Partial characterizations of turkey [29,30] and pigeon IgY [31] show similarities with that of the chicken. Radioimmunoassay analyses have indicated antigenic similarities between avian IgY molecules [70] and have confirmed a lack of antigenic relationship with mammalian IgG or IgD [71,72]. A relationship with mammalian IgA [73] has been reported, but this is now discounted. A problem encountered with structural studies on IgY from many avian species is the unusual pattern of degradation in response to reducing agents and proteolytic enzymes. The molecules dissociate following mild reduction in the absence of a dispersing agent [34], and papain hydrolysis yields predominantly $F(ab')$ fragments and dialyzable peptides, rather than the $F(ab')_2$ and Fc fragments obtained with IgG [35]. Recently, an IgY fragment containing the C ν 3 and C ν 4 domains was expressed, and its structure was determined to 1.75-Å resolution by crystallography (Protein Data Bank entry 2W59). The structural features share similarities to both mammalian IgG and IgE [74].

As mentioned previously, ducks produce two “isoforms” of IgY [15,67]. The larger is structurally analogous to chicken IgY and has been referred to as “7.8S IgG” [18–21]. The smaller isoform (5.7S IgG) is mostly referred to as IgY(Δ Fc) since it has only three H-chain domains (V, C $_1$, and C $_2$) and resembles, structurally and antigenically, an $F(ab')_2$ fragment of IgY [20,21,26,75]. Further details on IgY(Δ Fc) and duck antibodies can be found in Chapter 19 and in Magor [76].

The full-length and truncated ν chains [plus a third species that occurs as a transmembrane (TM), Ag receptor form on the surface of some lymphocytes] are the products of a single gene (Fig. 5.2) [23]. The three ν chains

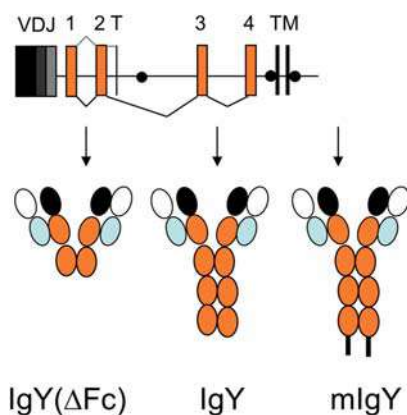


FIGURE 5.2 Schematics of the full-length ν and truncated $\nu(\Delta$ Fc) chains of duck IgY as the products of a single gene. Alternate splicing to generate the truncated $\nu(\Delta$ Fc) chain includes the first two C exons and alternate terminal exon T, as shown above the line. Splicing to generate the full-length ν chain skips the alternate internal exon, as shown below the line. Splicing to generate the transmembrane form includes the TM exons, which are spliced into a cryptic splice site near the end of exon 4. Each transcript uses a different polyadenylation signal sequence. TM, transmembrane.

arise from the use of different polyadenylation cleavage sites. In the case of the $\nu(\Delta$ Fc) chain, the cleavage site follows a small exon (Term) lying in the intron between the C ν 2 and C ν 3 exons. The Term exon encodes the two terminal amino acids (glutamate–phenylalanine) of the $\nu(\Delta$ Fc) chain, which are the only amino acids occurring in this polypeptide that do not also occur in a comparable position within the full-length ν chain.

The evolutionary origins of the truncated IgY are unknown. It is not yet known whether it occurs in all waterfowl or only ducks and geese; however, it is thought to be present in all Anseriformes. A truncated IgY was also found in several reptile species such as lizards, turtles, snakes, and salamanders, but at least for the turtle it was shown that full-length and truncated IgY are encoded by separate genes (reviewed in Ref. [77]). Some other species (lungfish, nurse shark, and skate) also have truncated Ig; however, these are not IgY, suggesting that truncated Ig has arisen more than once during evolution [78].

5.2.3 Avian IgA

The predominant form of Ab activity in bodily secretions is IgA. In mammals, IgA is a dimer, $(\alpha_2L_2)_2$ linked by a J chain that binds to a receptor on the tissue surface of epithelial cells [79,80]. This receptor becomes integrated into the IgA molecule as secretory component (SC), and the IgA complex is then transported through the epithelial cell and secreted into the lumen of the organ in question [81]. SC promotes adhesion of IgA to the epithelial surface and protection from proteolytic degradation within cells.

IgA has been found in all mammals and, based on molecular genetic evidence from chicken [82], duck [25], and pigeon [32], it is probably present in all avian species. The phylogenetic origins of IgA are not known. IgA is absent in lower jawed vertebrates, but amphibians and bony fish express specialized mucosal antibody isotypes. Hsu and colleagues [83] have reported that the African clawed frog (*Xenopus laevis*) has a secretory molecule (IgX) that has retained more of the physical and antigenic properties of IgM and is secreted into the intestinal tract. However, it does not associate with the J chain nor has a SC been identified in frogs. Bony fish express more primitive IgT molecules [84] which also carry out specialized functions at mucosal surfaces [77]. Due to large sequence differences, both IgX and IgT are not considered as homologues but as analogues of IgA which evolved independently by convergent evolution [84,85].

Several workers have demonstrated the presence of a structurally and functionally homologous form of mammalian IgA in chicken secretions, especially bile [42,86–88]. cDNA encoding the chicken α chain has been sequenced, and the inferred amino acid sequence confirms overall homology with mammalian α chains [82]. There are some

differences, however; the chicken α chain possesses four C domains with no genetic hinge, suggesting that (as with the relationship between avian and mammalian IgG) the primitive C_H2 domain could have been condensed to generate the hinge region of the mammalian molecule. The molecule contains the J chain [89] and SC [90–93]. Chicken IgA, extracted from secretions such as bile, is usually larger than the IgA found in mammalian secretions, suggesting that the avian form is a trimer $(\alpha_2L_2)_3$ or a tetramer $(\alpha_2L_2)_4$ [50]. The mean concentration of IgA in chicken serum is 0.35–0.65 mg/mL [42,56]. Cloning of cDNA encoding the chicken J chain [94] and polymeric Ig receptor [95] confirms that the origins of secretory Ig predate the evolutionary divergence of birds and mammals.

Molecules cross-reacting antigenically with chicken IgA have been identified in guinea fowl, pheasant, Japanese quail, turkey, and pigeon, but not duck [96]. IgA has since been confirmed biochemically and antigenically (but not yet characterized at the molecular level) in the turkey [28,29,97] and the pigeon [31,98].

A secretory form of Ig found in the bile of ducks and geese resembles IgM, both physically and antigenically [22,99,100]. Yet, in terms of ontogeny, this form appeared in the bile several weeks after IgM had appeared in the serum [22,101]. Using cDNA cloning and sequencing, Magor et al. [25] demonstrated that the H chains resemble chicken α chains, and studies on the expression of α -chain mRNA have confirmed the delayed ontogeny of this molecule. A cDNA corresponding to the polymeric Ig receptor and J chain was also identified in duck [102].

5.2.4 Avian homologues of IgD and IgE

Although there were some early reports of a chicken homologue of IgD on the surface of chicken lymphocytes [103], it was generally accepted that there is no avian homologue for IgD, with the majority of chicken B cells expressing only IgM. Further, the duck IgH locus showed the absence of a δ gene (see later). However, recent genomic studies revealed a functional δ gene in the ostrich and screening of further avian genome sequences found δ sequences in 18 bird species from 12 orders but not in Galloanseres [17].

IgD function in mammals is still only partially understood. Whether the presence of IgD has a functional impact on B cell development in the respective bird species and B cell development between IgD-positive and IgD-negative birds differs has to be elucidated.

So far, no IgE isotype has been described for birds and even the extensive genomic analysis of 48 bird species has provided no hint of its existence, making IgE an isotype exclusively present in mammals. Regarding the function, it is known that avian IgY can sensitize tissues [104–107], although the parameters of sensitization and activation differ from those of mammalian reagenic Ab

[108]. Nevertheless, it seems likely that functions ascribed to mammalian IgE are performed by avian IgY.

5.2.5 L chains

The L chains from a variety of avian species have been studied [7,24,109–118]. Most interest has concerned the mechanism of Ab diversity generation (see Chapter 4) rather than the structural biochemistry. Early studies suggested that chicken L chains were like the mammalian κ chain [7]; however, it is generally agreed that the avian L chains align with the λ L chains of mammals. There is some physicochemical and antigenic evidence that avian species have additional L chain isotypes [112], which could reflect heterogeneity within the λ class (e.g., due to glycosylation).

Lately, analyses of bird genomes have demonstrated a differing number of functional V λ in different bird species. While ostrich, zebra finch [119], and turkey [117] like the chicken possess only a single functional V λ gene, four functional V λ genes were identified in pigeons [118] and nine in Pekin ducks [27]. All functional V λ genes are combined with different numbers of pseudogenes (V ψ λ), upstream of the functional V λ s in pigeons and interspersed in ducks, supporting a conserved mechanism of generating λ diversity by gene conversion. As birds with a single V λ are found in all three avian phylogenetic groups (Palaeognathae/ostrich, Galloanseres/chicken and Neoaves/zebra finch) [120], it seems likely that the common bird ancestor possessed a single functional V λ gene, which in some species was the base for gene duplication.

5.2.6 Genomic organization of the IgH and IgL locus

With three isotype genes available, Southern blotting was used to map the duck IgH. The picture that emerged from these Southern analyses suggested an unusual organization, with the ν gene 3' in the locus. Overlapping λ phage genomic clones were isolated and mapped to determine the gene order. The chain gene and the ν gene were determined to be in inverse orientation [121]. Subsequent complete sequencing of the overlapping genomic clones showed that the duck locus was organized with α between μ and ν , and α in inverse transcriptional orientation (Fig. 5.3A) [122]. The contig generated extended from a D segment, included a single J segment, followed by μ , α , and ν H chain genes. The single J segment suggested that ducks, like chickens, undergo limited rearrangement events by VDJ recombination. It was also apparent that there is no δ gene in the locus to encode an avian homologue of IgD. Class switching to α involves an unusual intermediate requiring the inversion of a segment carrying μ and α , rather than the deletional switch circle seen in mammals (Fig. 5.3B).

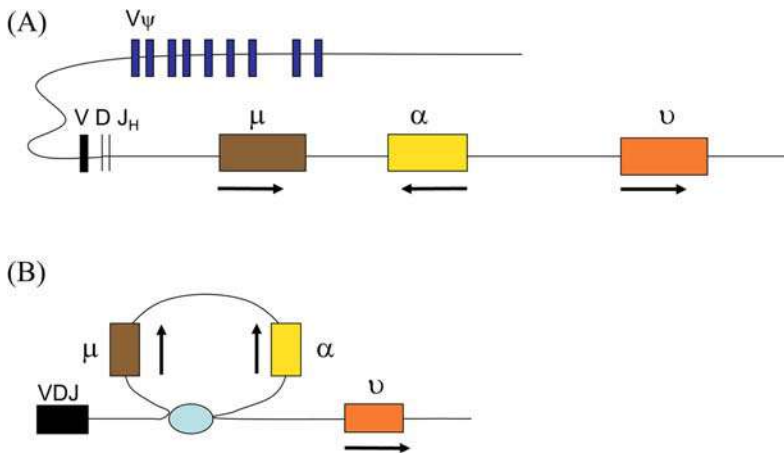


FIGURE 5.3 (A) Schematic derived from overlapping genomic clones of the duck immunoglobulin (Ig) locus, showing the gene organization with α between μ and ν , and α in inverse transcriptional orientation. Upstream pseudogenes are inferred from data showing that ducks undergo limited VDJ rearrangement events and are diversified by gene conversion. (B) Class switching to Ig α involves an unusual intermediate requiring the inversion of a segment carrying μ and α , rather than the deletional switch circle in mammals. Class switching to ν would proceed through the excision of a switch circle.

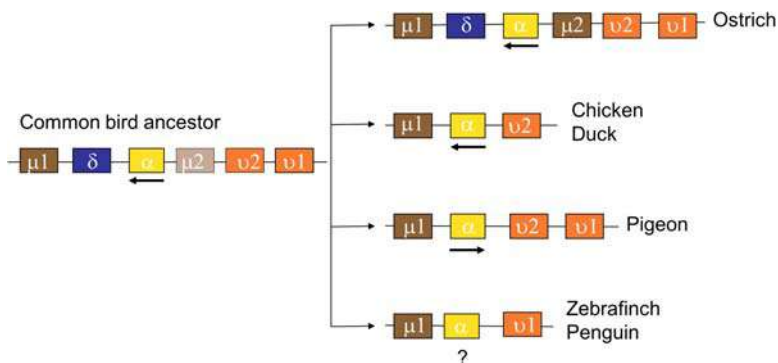


FIGURE 5.4 Schematic diagram of the IgH gene evolution in birds. Constant region genes are indicated as colored boxes. Arrows below are used to indicate the transcriptional orientation of the α gene. Phylogenetic evidence suggests that two ν subclass genes originated before divergence of ostrich and pigeon. The evidence for potentially two μ genes in the locus of the common ancestor of birds is based on the organization inferred for ostrich and crocodiles. Modified from Huang T, Wang X, Si R, Chi H, Han B, Han H, et al. Identification of a transcriptionally forward α gene and two ν genes within the pigeon (*Columba livia*) IgH gene locus. *J Immunol.* 2018;200(11):3720–3728.

Despite its importance, the chicken IgH locus has received less scrutiny than that of the duck. Long-range PCR on genomic DNA confirmed a similar organization of the ν and α genes of the chicken [123]. Although bacterial artificial chromosome clones were identified that carried the ν gene, these extend almost 100 kb downstream and do not carry the α or μ genes [123]. Previous work examined the germline configuration of the chicken μ gene and demonstrated the presence of a single JH segment in the chicken H chain locus [124]. At the time of writing, the chicken IgH locus has not yet been assembled from the current draft of the chicken genome.

As mentioned earlier, both ostrich and pigeon have two ν genes; $\nu 1$ and $\nu 2$ genes are located downstream of all other H chain isotype genes. Interestingly, the pigeon $\nu 2$ gene is more related to the ostrich $\nu 1$ gene and the single zebra finch and penguin ν gene, while pigeon $\nu 1$ and ostrich $\nu 2$ show a closer relationship to the chicken and duck ν gene. Phylogenetically, this is indicative for a common bird ancestor with two ν genes, from which $\nu 1$ was deleted in chicken and ducks and $\nu 2$ in the zebra finch branch (Fig. 5.4) [32].

The chicken L chain locus has recently been mapped to chromosome 15, in a region syntenic to the human λ light-chain locus (http://www.ensembl.org/Gallus_gallus/). The locus contains a single functional VL and JL region upstream of one C region. Upstream of the locus are approximately 25 V region pseudogenes, which lack the recombination signal sequences required for VDJ recombination, and have 5' and/or 3' truncations. The pseudogenes are used in a “hyperconversion” mechanism to generate the diversity at the Light chain, involving gene conversion of fragments of the pseudogenes into the rearranged functional V region gene [114].

The generation of the chicken Ig repertoire is reviewed in Chapter 4. Similarly, a 2006 review has examined duck VH and VL region sequences in expressed sequences. The data suggested that the same gene conversion paradigm for the development of the immune repertoire holds true in ducks [102].

Avian Ig allotypes

A number of allotypic variants associated with chicken μ , α , and ν chains have been reported (reviewed in Refs.

[14,125,126]). These may have some importance in immune regulation [125,126]. Allotypic forms of IgY have been reported to exist in the sera of turkey and pheasant [127], based on reactivity with Ab against eight allotypes associated with chicken IgY but not other avian species examined. However, nucleotide and amino acid sequence analysis of ν cDNA has indicated the probable occurrence of allotypic variants in duck IgY [26]. In comparisons of cDNA and genomic sequences for the duck, allotypes were observed for all genes; however, there were 37 polymorphic positions in the α gene, compared with 10 and 7 in μ and ν , respectively [122]. Many of these polymorphic positions were within the C α 2 domain, suggesting that this region is evolving rapidly or under less constraint.

5.3 Ig half-life

Turnover rates of chicken Ig have been determined by several investigators using radiolabeled proteins. Ivanyi et al. [128] prepared chicken gamma globulin (chGG) using ammonium sulfate precipitation of serum proteins and labeled this protein fraction with ^{131}I . The biological half-life was determined to be 2 days in 14-week-old males and 1.55 days in adult females. Using a similar approach, Patterson et al. [129] determined chGG half-life in newly hatched chickens and laying hens and reported values of 3 days and 1.45 days, respectively. The increased turnover of chGG in laying hens can be explained by the transfer of IgY from the circulation to the developing ovarian follicles (see Section 5.5). A more detailed study was carried out by Leslie and Clem [52] using purified radiolabeled chicken IgY and IgM to measure serum elimination curves in roosters. They observed a rapid clearance phase during the first 48 hours followed by a slower elimination with first-order kinetics. The initial rapid clearance was attributed to intra- and extravascular equilibration and the removal of aggregated or denatured proteins. Serum half-lives were extrapolated during the second phase and found to be 4.1 days for IgY and 1.7 days for IgM. In agreement with this study, Heryln et al. [53] calculated the half-life of purified non-aggregated IgY in 2- to 9-month-old chickens to be 3.3 ± 0.7 days. Values for turkey Ig have been provided in only one study [28]. Turkey IgY had an average half-life of 5.91 days in laying hens. The values for IgM, measured in only two hens, were 2.69 and 2.74 days. This study also reported the half-life of IgA for two birds as 1.92 and 1.68 days. Comparable data for IgA in two chickens were 3.3 and 1.9 days [54].

As an alternative approach, the decline of antigen-specific maternal antibody titers in newly hatched chicks has been used to study IgY elimination kinetics. Maternally derived infectious bronchitis virus-specific

antibodies exhibited a linear decline, with a mean half-life of 5–6 days [130]. This observation was confirmed by Fahey and colleagues [131], who reported a half-life of 6.7 days for IBDV-specific maternal antibodies. Maternal IBDV-specific antibodies were reported to decline to background levels within 28 days of hatching [132]. Similar elimination kinetics were reported for Newcastle disease-specific maternal antibodies. Using hemagglutination inhibition assays, Kaleta et al. [133] estimated the half-life of maternal antibodies in chicks as 5.2 days. Injection of antibodies into maternal antibody-negative chicks led to nearly identical results: a half-life of 4.9 days. This study also demonstrated that 4-week-old chicks and adults have more rapid elimination kinetics (3.5 and 3.4 days, respectively) than do very young chicks [133]. The faster elimination kinetics observed in antibody injection studies compared with studies based on naturally transferred maternal antibodies may partly be due to the removal of denatured proteins in the injected preparations. Collectively, these studies give some guidance on calculating titers of antigen-specific maternal antibody at a given time point, on the basis of single quantitative analysis.

5.4 Natural antibodies

Natural Abs have been defined as antigen-binding Abs present in nonimmunized individuals. In mammals, these antibodies are preferentially derived from the B1 B cell population located in the peritoneal cavity, along the intestinal tract and in the bone marrow and to a smaller extent from marginal zone B cells in the spleen [134–136]. Natural antibodies have broad specificity but only low binding affinity. In mammals, these Abs are mostly IgM, although IgG and IgA isotypes have been reported (for review, see Refs. [137,138]). Natural Abs are considered a bridge between innate and adaptive immune system, and it has been suggested that in mammals they cooperate with the complement system as a first line of defense [139]. They can perform various other functions, such as clearing foreign, dead, or catabolic materials (e.g., polysaccharides); enhancing antigen uptake; and processing and presentation by B cells or dendritic cells. Natural IgG was shown to collaborate with serum lectins to elicit effective antimicrobial activity [140]. It has also been suggested that natural antibodies contribute to self-tolerance, as a considerable part of the binding repertoire of natural Ab in humans is directed against autoantigens, such as thyroglobulin, myoglobulin, ferritin, transferrin, albumin, and cytochrome C [141].

Parmentier and colleagues [142] investigated the phenomenon of natural Ab in the plasma of chickens that had been divergently selected for high or low specificity responses to sheep red blood cells (SRBC). Using a range

of different self and foreign antigens (chicken albumin, ovalbumin, myoglobin, thyroglobulin, transferrin, keyhole limpet hemacyanin (KLH), and bovine serum albumin), these workers concluded that natural Abs of predominantly IgM, but also IgY and IgA isotypes are present in chickens that have not been immunized. They reported an increase in the levels of natural Ab with age, perhaps due to environmental sensitization, and higher levels in those birds that had been selected for better antibody responses to SRBC. In a further study [143], they isolated natural Ab that bound to a specific antigen (KLH) from the plasma of nonimmunized adult hens and adoptively transferred it to young cockerels that had not been immunized. Adoptive transfer of the natural Ab enhanced a subsequent IgM and IgY response to KLH, whereas, when specific antibodies isolated from birds that had been immunized with KLH were adoptively transferred, the “naïve” recipients showed no enhanced humoral response after KLH immunization. Recent studies with chicken lines selected and bred for high or low natural antibodies demonstrated that high amounts of natural KLH-binding antibodies are heritable, associated with higher survival and increased avian pathogenic *Escherichia coli* resistance in early life [144,145]. As there are no known structural differences between natural and adaptive IgY, it is not too surprising that natural maternal IgY is transferred to the chick via egg yolk [146]. Lammers et al. [143] concluded that as in mammals natural Ab could play an important role in both initiation and regulation of specific humoral immune responses of poultry. Recent genome-wide association studies have identified a region on chromosome 4 which has a major impact on both total IgM and natural IgM antibodies to KLH and an SNP in the Toll like receptor (TLR)1A gene seems to be the most likely candidate for this variation [147]. This is especially interesting, as TLR1/2 activation of murine B cells does enhance plasma cell differentiation [148] and marginal and B1 B cells, the source of natural antibodies, exhibit a very robust response to TLR stimulation (reviewed in Refs. [149,150]). The functional relationship between natural Ab and specific antibodies remains unclear, and it would be useful to investigate the genetic and functional relationships between these two types of Ab. The role of natural Ab in the regulation of immune responses and its importance for disease resistance, especially in the neonate chick, deserves further investigation.

5.5 Maternal antibodies

The immune system of the newly hatched chick is only partially mature and therefore not capable of providing complete protection against pathogens on its first encounter with the external environment after hatching. Innate

immune mechanisms seem to be fully functional in the neonate, but optimal adaptive immune responses only develop during the first few weeks after hatching. B lymphocytes first emigrate from the bursa to seed secondary lymphoid organs around hatching, whereas the first population of T lymphocytes leaves the thymus around embryonic incubation day (EID) 15, with the second and third waves of migration taking place after hatching (discussed in Chapter 3). Transfer of maternal antibodies helps to protect the offspring until adaptive immune responses become fully effective. This was first described in 1893 by Felix Klemperer [151], who observed that, in birds, immunity against tetanus bacteria is transmitted via the egg yolk. It was later addressed by Brierley and Hemmings [152], who demonstrated the selective transport of antibodies from the yolk sac to the circulation of the embryo and hatched chick. These and other studies laid the foundation for the concept that, in birds, maternal antibodies are deposited into the egg and subsequently absorbed by the developing embryo [153]. The phenomenon has been exploited in vaccination strategies by the poultry industry; point-of-lay pullets are usually boosted with vaccines to raise their circulating antibody levels during lay so that protective maternal antibodies are transferred to the offspring. For a fuller discussion of practical vaccination strategies (see Chapter 18).

IgY is selectively secreted from the circulation of the hen into the egg yolk [129,154]. The amount of IgY transferred across the follicular epithelium into the yolk is proportional to the IgY concentrations in serum [155–158] and is mediated by an active transport mechanism [57]. Antigen-specific IgY antibodies are found in the newly laid egg with a delay of 5–6 days in comparison with the serum antibody concentration. This is explained by the time required for follicular development and oviposition [129]. Yolk IgY concentrations were reported to be in the range of 20–25 [58], 7.9 [57], and 10 mg/mL [159] for the chicken, depending on the analytical method used. For turkey and pigeon, values of 5.1 and 5.4 mg/mL, respectively, have been reported [97,160]. The rate of accumulation of IgY in the egg is proportional to the increase in mass of the developing oocyst and estimated to be 45 mg/day at 2 days before laying [57]. The mechanism of this transfer is still not known but is likely to be receptor-mediated [156]. Studies in the duck further indicate that a functional Fc portion is involved in IgY secretion into the yolk since IgY Δ Fc, the naturally occurring truncated form of IgY in duck serum, is only poorly transferred to the yolk [161].

The second step in maternal antibody transfer requires absorption of IgY across the yolk sac membrane into the embryonic circulation. This transport process begins at a slow rate around EID 7 [155] and increases steeply during the 3 days before hatching, to reach a capacity of

600 µg/day [57]. It has long been proposed that this process is Fc receptor-mediated since chicken IgY—but not mammalian IgG—is capable of being transported and binding of IgY to the yolk sac membrane is pH-dependent [162]. In 2004 the receptor was cloned and named FcRY [163] (see Section 5.4.2).

The total amount of IgY absorbed by the embryo represents only 10% of that deposited into the egg yolk. The fate of the remaining 90% is not known, though most likely it is digested proteolytically along with the residual yolk contents [57]. This may explain the approximate eightfold lower antigen-specific antibody titers in neonatal serum compared with those in yolk. Total serum IgY levels increase to their maximum value about day 2 post-hatch, reaching 1–5 mg/mL [57], and subsequently they decline until de novo synthesis of IgY becomes evident around day 10 posthatch (see Fig. 5.5). Comparable kinetics have been described for the duck, with peak levels at day 5 and minimum levels at day 14 posthatch [161].

While IgY is found in the yolk but not the albumin, the converse is true for IgM and IgA antibodies. Only minute amounts of these two isotypes can be detected in the yolk [164], with most being secreted into the egg white [165], reaching concentrations of 0.15 and 0.7 mg/mL for IgM and IgA, respectively [58]. During embryonic development, these isotypes are distributed to the yolk sac and the amniotic fluid [166] but are not transferred into the embryonic circulation [12]. Since the amniotic fluid is imbibed by the embryo at about EID 10–12, both IgM and IgA are found in the gut at hatching. Additional Ig is delivered to the intestine when the remaining contents of the yolk sac are transferred through the yolk stalk 1–2 days post hatch; they probably play an important role in the early protection of the gut. Endogenous production of IgM and IgA in the chick starts, such as IgY production, around day 10.

Interestingly, offspring from Agammaglobulinämia hens that were deprived of maternal antibodies showed significantly reduced total serum levels of IgY, IgM, and

IgA [54]. Chicks in which the uptake of maternal IgY from the intestine was inhibited by embryonic bursal duct ligation showed strongly reduced levels of naturally occurring antibodies against SRBC, *E. coli*, and *Brucella abortus* [167], and they reacted with a limited antibody response to immunization with SRBC [168]. Though the exact underlying mechanism is unclear, the magnitude of the produced Ig seems to be influenced by the presence of immune complexes on bursal secretory dendritic cells (BSDC) in the bursa of Fabricius, which are formed after uptake of maternal Ig and stimulate proliferation of bursal B cells and BSDC maturation [169,170].

Transfer of maternal antibodies to the offspring via egg yolk is evident in all avian species studied so far [28,58,160,161]. However, some species have developed additional pathways to protect their hatchlings; the best characterized example is that of the pigeon [98]. Birds such as pigeons, penguins, and greater flamingos produce a specific secretion in the crop sac that is regurgitated to feed to the squabs. Crop milk is rich in fats and proteins, although, unlike mammalian milk, it does not contain lactose. Moreover, the production of crop milk is under the control of prolactin [171]. Crop milk is also rich in IgA, with concentrations in the range of 1.5 mg/mL, but it contains little IgY. Uptake of IgA from crop milk leads to IgA accumulation in the intestinal tract, where it presumably provides protection against pathogenic gut microorganisms. Depending on the study, very little [160] or no [98] IgA is transferred across the intestinal wall into the circulation; this represents an important local immunity in the gut.

5.6 Fc receptors

In the chicken, so far five FcR or FcR-like proteins have been described. These include the chicken polymeric Ig receptor (gg-pIgR), an FcRn homologue designated FcRY, an FcR-like protein that seems to lack IgY reactivity (FcRL), and two receptors binding to chicken IgY: ggFcR and CHIR-AB1.

5.6.1 Chicken polymeric Ig receptor

The gg-pIgR, cloned in 2004 [95], is located on chicken chromosome 26 in close vicinity to the chicken IL-10 and IL-19 genes (IL-10 cluster). It shares remarkable homology with its mammalian counterparts, including the Ig-binding motif in the first Ig domain. In contrast to the five Ig domains present in mammalian pIgR, gg-pIgR contains only four, with no domain resembling the second mammalian Ig domain. Its expression has been demonstrated at the mRNA level in the liver, intestine, bursa of Fabricius, and Harderian gland [95,172]. Functionally, it is believed that gg-pIgR interacts with the J chain of

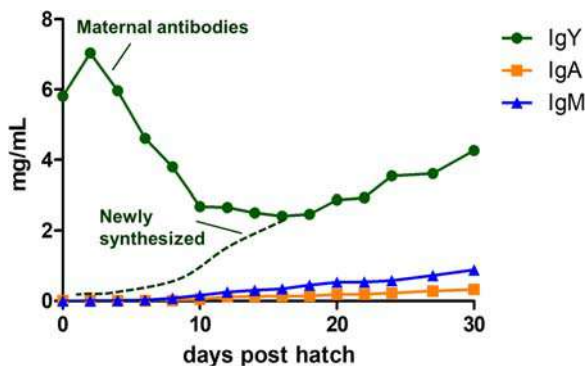


FIGURE 5.5 Kinetics of maternally transferred antibodies and endogenous Ig production post hatch.

polymeric IgA at the basolateral epithelial surface, mediates transcytosis, and is released onto the mucosal surface by cleavage of the receptor.

5.6.2 Chicken FcRn homologue

Maternal Ig transfer from egg yolk to chicken embryo is mediated by the FcRY [163]. In contrast to mammals, which use the FcRn with homology to MHC class I, the FcRY belongs to the mannose receptor family. It consists of N-terminal cysteine-rich and fibronectin II domains followed by eight C-type lectin-like domains. Structural analyses have revealed symmetric binding, where each CH4 domain of IgY is contacted by the FcRY [173]. Binding occurs only at slightly acidic pH of about 6.5, present in intracellular vesicles, and IgY is released at basic pH [174]. The mammalian FcRn is also responsible for binding to albumin and for albumin's long half-life. It is not clear if chicken FcRY mediates similar responses, and it has also not been reported if chicken FcRY, like its mammalian counterpart, is expressed on endothelial cells.

5.6.3 Chicken Fc receptor cluster

The human Fc receptors are all present on chromosome 1, with the exception of CD89, the Fc α RI receptor that is located in the human leukocyte receptor complex on chromosome 19. A syntenic region could be identified in the chicken. However, it harbors only a single gene with homology to mammalian FcR [175]. This receptor contains four extracellular Ig domains and a short cytoplasmic domain. For cell surface expression, it is dependent on the FcR γ chain. It has homology to mammalian FcR and FcRL, but, since it lacks detectable binding to IgY, it is classified as chicken FcRL.

5.6.4 ggFcR

A novel chicken FcR has been described as ggFcR on chicken chromosome 20, a region in mammalian genomes where no FcRs are present [176]. It consists of four Ig domains, a TM region with a basic residue that allows association with the FcR γ chain, and a short cytoplasmic domain. It binds to IgY with low affinity. ggFcR mRNA expression is detectable in thrombocytes and macrophages. The binding site of ggFcR, as identified by point mutations, is located in the CH2 and CH3 domains and closely resembles binding of mammalian IgG to FcR [177].

5.6.5 CHIR-AB1

The chicken leukocyte receptor complex harbors a vastly expanded multigene family designated chicken Ig-like receptors. One subgroup of these receptors is characterized

by a single Ig domain, a positively charged TM residue that is essential for association with the FcR γ chain, and a long cytoplasmic domain containing an ITIM. These features suggest an inhibitory or activating function, and the receptors have been designated CHIR-AB [178,179]. One particular member of this family, CHIR-AB1, binds IgY with high affinity [180]. In further studies, it was demonstrated that CHIR-AB1 belongs to a group of highly homologous receptors that vary in their binding to IgY, from undetectable to high-affinity binding [181]. The crystal structure of the CHIR-AB1 Ig domain and mutational analyses revealed that the interaction of CHIR-AB1 with IgY takes place at an interphase formed by the CH3 and CH4 domains of the IgY molecule, a binding mode resembling the human Fc α RI-to-IgA interaction rather than the interaction of IgG with its FcR [182,183].

5.7 Avian antibody responses

As in mammals, IgM is the predominant isoform produced in the avian primary humoral response, while IgY is the predominant form in the secondary response [184]. Most studies on avian Ab responses have used the chicken. A wide variety of antigens and assays have been employed [10], but most of the published works relate to protective immunity generated in response to pathogens or vaccines. It has long been recognized that chickens have a prodigious ability to produce antibodies in response to specific antigens [185]. However, there can be marked differences in the abilities of different inbred lines to produce Ab responses to the same antigen or vaccine (for further discussion, see Chapter 7). Studies of Ab production and function in other avian species have been much less comprehensive. Bearing in mind that the class *Aves* is both large and diverse, it seems highly likely that there will be considerable species variation, both in the ability to produce Ab and in the properties of the primary and secondary Ab responses.

Measurement of Ab responses to complex antigens such as those found on pathogens or vaccines provides little information on the intricacies of Ab responses, including the contribution of the different Ig isotypes during the course of a primary or secondary response. The response to defined Ag, such as purified proteins or heterologous red blood cells, can be more useful for studies on Ig function, on the ontogeny of the humoral immune system, or as indices of the immunosuppressive effects of toxins. Single antigenic determinants or "haptens" [e.g., dinitrophenol (DNP), trinitrophenol (TNP), or fluorescein] are required for more precise analyses of Ag–Ab interactions that yield information on affinity, valency, and response kinetics. Such approaches have been limited to the chicken, with few studies carried out on other avian species.

Immunization studies of chickens with SRBC have shown that in prime-boost experiments, the primary antibody response peaked at 5–7 days p.i. and consisted mainly of IgM antibodies. The peak of the secondary response was not reached more quickly, but the antibodies were mostly of IgY isotype [184,185]. Interestingly, several studies, using SRBC, TNP, *B. abortus*, or an inactivated NDV vaccine, showed that the secondary antibody response did not reach significantly higher antibody titers than the primary response when the boost was given in the declining phase of the primary response [184,186–188]. A significantly increased antibody titer to nonreplicating antigens was obtained if the second immunization was performed at the peak of the primary response [188].

Information on the duration of antibody responses is limited by the fact that most studies were performed with replicating or persistent pathogens, which may induce continuing stimulation of the B cell system. For instance, in infection models with *Salmonella typhimurium*, constant LPS-specific IgY serum concentrations were demonstrated for at least 20 weeks p.i. [189]; for an IBV live vaccine, HI titers remained high for 10 weeks p.i. [130]. For model antigens such as SRBC and TNP-SRBC, a rapid drop of antibody titers within 2 weeks p.i. was shown for the primary response and a more prolonged course after the secondary immunization [184,188].

It seems that, in contrast to antibody responses in mammals, repeated immunization of chickens does not lead to very high and long-lasting antibody titers. This could be indicative of differences in the generation and maintenance of long-lasting plasma and memory cells, a hypothesis that has to be verified by more detailed studies.

Early biochemical studies on the precipitation of chicken IgY indicated an unusual feature. Optimum precipitation, assessed by the titer and intensity of precipitin lines, occurs in a high-salt ($10\times$ physiological) or low-pH (<5.0) environment [69,190,191]. Precipitating Abs from pheasant, quail, and owl have similar requirements, although those from duck, pigeon, and turkey do not [185,192]. It is generally considered that salt and low pH (by undefined mechanisms) support the extensive cross-linking needed to generate insoluble complexes of Ag and avian Ig. Higgins [193] reviewed the subject, eliminating most of the explanations. He concluded that high-salt concentration and low pH affect the steric arrangement of the Fab arms of the avian IgY and alter the shape of the molecule producing functional bivalency.

Analysis of the literature reveals that avian species show a broad spectrum of humoral immune responsiveness. However, it is pertinent to point out here that, in direct comparisons of the Ab responses between chickens and other bird species, Ab responsiveness is in the order: chicken $\gg$ pheasant/partridge $\gg\gg$ turkey $>$ pigeon $>$ quail $>$ duck [193]. This is discussed more fully in Chapter 19.

Secretory IgA provides a first line of defense against many pathogens. Preferential stimulation of secretory IgA production probably requires delivery of the Ag to the mucous membranes and the use of live organisms. Secretory IgA against mycoplasmas [194,195], coccidia [196,197], *E. coli* [198], and NDV [199] has been detected in chicken secretions. By comparison, there have been few studies on IgA in other avian species. Turkeys develop IgA antibodies to *B. ordetella avium* [64], and ducks produce bile IgA to influenza A viruses after experimental infection [101]. In the chicken, Abs against NDV are found in respiratory secretions after intranasal, but not intramuscular, administration of vaccine [200]. In duck bile, the secretory IgA response to orally administered live influenza viruses is independent of systemic Ab [101]. Unfortunately, secretions rich in IgA are not readily obtained from live birds.

The contribution made by affinity maturation to the development of specificity and effectiveness of avian Ab is not clear. Affinity maturation is one outcome of the somatic mutational events that occur in maturing B cells during the immune response. With the generation of an increasingly diverse repertoire of Abs, some will have higher affinities for the Ag and will dominate subsequent responses. In mammals, this occurs as the Ab response switches from IgM to IgG.

A major question is whether the avian variable region genes, constructed by recombination and gene conversion, have the ability to undergo further diversification during subsequent immune responses. In chickens, Ig gene diversification in germinal centers has been examined only for the light chain, as the upstream VL pseudogene sequences are entirely known. Mutation due to gene conversion continues in chicken splenic germinal centers, with evidence suggesting that more than one event accumulates in a single V region sequence [201]. The accumulation of gene conversion events in the VL chain sequences appeared more targeted than seen in a similar analysis of bursal gene conversion events [202]. In addition, there was a much greater diversity of sequences following immunization with fluorescein isothiocyanate-BSA, a larger antigen, than with dinitrophenyl-BSA. While this is suggestive of affinity maturation, there is no evidence to confirm that these mutational events result in antibodies of higher affinity. Reanalysis of these data suggests that the diversification in chickens (and rabbits) tends toward more mutation and less selection than in mammals, reflected in bushier tree structures [203]. Somatic mutation, seen as unlinked point mutations, was apparent in chicken splenic VL region sequences, suggesting that this mechanism of diversification is operating in chicken Ig genes [201,202]. It therefore seems possible that continuing gene conversion can contribute some additional diversity that is provided by somatic mutation in mammals.

Nevertheless, the efficiency of this system, in terms of both repertoire development and increasing affinities, might be very low.

5.8 The chicken egg as a source of antibodies

As previously pointed out, immunization of hens leads to the generation of antigen-specific IgY antibodies that are efficiently transferred into the yolk [165]. Extraction of these antibodies from freshly laid eggs can be easily accomplished without the need for regular blood sampling or specialized equipment. Here we give a brief overview of the generation, purification, and application of chicken egg antibodies.

Since the chicken egg yolk contains 8–10 mg IgY per mL [57], about 100–200 mg of total IgY can be extracted from a single egg [204], with 2%–10% being antigen-specific [205]. Specific chicken antibodies have been successfully raised against a wide variety of antigens, including proteins, peptides, lipid hormones, and carbohydrate components from a large variety of species, including viruses, bacteria, fungi, plants, and animals [206]. A major advantage of the chicken is that antibodies can be raised against antigens that are highly conserved in different species of mammals. This has been attributed to the phylogenetic distance between birds and mammals [207,208]. In addition, chicken IgY antibodies show little cross-reaction with mammalian Ig [73] and do not bind to mammalian Fc receptors [209] or activate the mammalian complement pathway [209]. Avian antisera can therefore be used to avoid the interference normally associated with the use of mammalian antibodies in various immunological assays [210,211], flow cytometry [212], and immunohistology [213]. Earlier studies have reported that IgY antibodies require unique buffer systems for agar gel immunoprecipitation assays, but subsequent work has shown that immune-complex formation can be achieved under the same conditions as used for mammalian antisera [214,215].

Chickens are most frequently immunized into the pectoral muscle with antigens in combination with adjuvants. Use of complete Freund's adjuvant (CFA) was reported by some authors to be well tolerated, producing no local inflammatory reactions [207]. However, detailed histological studies have clearly documented that CFA elicits all signs of inflammation and leads to the development of a persistent granulomatous myositis [216]. Despite attempts to develop alternative adjuvants with comparable immunogenicity, but less inflammatory potential, CFA still remains the standard adjuvant in many laboratories. New technologies such as DNA vaccination may well overcome some of the problems associated with immunization

with conventional antigens and facilitate the generation of yolk antibodies against specific antigens [217]. As in rabbits, initial immunization with soluble antigens is usually followed by a booster immunization 2–3 weeks later. In most cases, a specific and high-titer IgY response develops 1–2 weeks later and collection of eggs for IgY extraction from yolks should be started 2–3 weeks after the booster injection. Subsequent booster immunizations may help to maintain the antibody titer over long periods of time [58,218], but rarely leads to a significant increase in titer.

Several methods have been described for large-scale antibody purification from the egg yolk. In the first stage, it is essential to remove the lipids, either by precipitation without [219] or with chemical additives [220,221] or using lipid solvents such as ether [129,154], chloroform [222], or isopropanol [215]. Usually this is followed by one or more protein precipitation steps [220,221] or the use of chromatographic methods for further purification.

5.8.1 Avian antibodies as tools for research

Antibodies extracted from egg yolk have found widespread application in biomedical research [223]. Certain proteins are so highly conserved between different mammalian species that it is impossible to evoke xenogenic responses. In contrast, birds have sufficient evolutionary distance from mammals for the mammalian proteins to be recognized as foreign and for avian humoral immune responses to be evoked. For instance, it has proved extremely difficult to produce mammalian antibodies against the abnormal form of the prion protein PrP, whereas antibodies can be raised in chickens and quantities harvested from eggs for use as diagnostic tools in studies on bovine spongiform encephalopathy [224].

There has been little emphasis on the development of avian monoclonal antibodies. A chicken B cell line (HU3R27), deficient in thymidine kinase activity, was developed as a fusion partner for the production of chicken monoclonal antibodies, but the resulting hybridomas soon lost the ability to produce antibody in culture [225]. An improved fusion cell line (R27H4) was subsequently developed by fusing HU3R27 with spleen cells from a chicken immunized with KLH; the resulting hybridoma was reported to secrete highly reactive IgY and weakly reactive IgM for over 6 months [226]. Since then a small number of other chicken monoclonal antibodies have been produced using either R27H4 or the related R27H1 cell line as fusion partners [227–230], although this technology seems to have found only limited use. Perhaps this is because avian antibodies are required as tools only for relatively few specialized assay systems and sufficient amounts of these purified Ab can normally be provided from egg yolk.

Newly available chicken transgene technology has allowed for a completely new application of the chicken as platform for the generation of human antibodies. Therefore H-chain and L-chain loci of wildtype chicken were disrupted by homologous recombination followed by site-specific insertion of single functional human VH and VK or VL genes. These were designed to splice to the endogenous chicken constant regions, to ensure proper formation of the B cell receptor complex. From these birds, paired human light- and heavy-chain variable regions can then be recovered and cloned as fully human recombinant antibodies, which show an epitope coverage similar to wild type chickens [231,232].

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Avian T cells: Antigen Recognition and Lineages

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6.1 Introduction

Antigen recognition by T cells is a sophisticated process mediated by the T cell receptor (TCR). Two key features distinguish T cell antigen recognition from most surface receptors that are precommitted to recognition of a specific ligand. First, the antigen-binding region of the TCR is generated by a process that involves modification of DNA at the TCR locus with rearrangement of gene segments as well as junctional addition and deletion of nucleotides. This results in an immense diversity in the antigen-binding regions of the TCR chains. Second, the receptor represents a very sensitive antigen recognition receptor. For example, the classical, TCR $\alpha\beta$ form of the receptor recognizes peptides complexed to major histocompatibility molecules at a density of only 0.1–1 molecules/ μm^2 [1].

Most of the paradigms for T cell function have been established in man and mouse. Although birds and mammals evolved from a common ancestor more than 300 million years ago, many aspects of mammalian T cell structure and function also apply to avian T lymphocytes. Nevertheless, studies on the chicken indicate some unique T cell features. Here we will summarize various aspects of TCR structure and function and try to integrate some of the recent key findings concerning mammalian TCR.

6.2 T cell receptor structure and lineages

Chicken T cells use a heterodimeric surface receptor for antigen recognition. Each TCR chain is composed of two immunoglobulin (Ig) superfamily domains. The membrane distal variable (V) region of TCR is composed of a V-type Ig domain linked to diversity (D) and junctional (J) segments (for the larger TCR chains; β and δ) or a V

domain linked to a J domain (for TCR α and γ). The sequence at this VDJ or VJ junction (known as the complementary determining region-3 or CDR-3) exhibits extensive sequence diversity generated by the process of somatic DNA recombination [2]. All TCR chains contain a single membrane proximal constant (C) domain—as opposed to up to four C domains in antibodies—which defines the “class” of the TCR chain. The C regions are anchored in the plasma membrane by a connecting peptide and a hydrophobic transmembrane region that has one or two basic residues. The TCR chains have very short cytoplasmic regions, which do not confer a direct signaling capability. Signaling for all TCR is mediated by a complex of molecules known as the CD3 complex.

All jawed vertebrates have four distinct TCR chains, the α , β , γ , and δ TCR which form either TCR $\alpha\beta$ or TCR $\gamma\delta$ heterodimers. The α and γ chains of the TCR are slightly smaller molecules than the β and δ chains of the TCR. A cysteine in the region known as the connecting peptide forms an intermolecular disulfide bridge between two of the chains to generate the $\alpha\beta$ TCR or the $\gamma\delta$ TCR, which define the two major T cell lineages. Additional surface molecules are used to further subdivide $\alpha\beta$ T cells into the CD4⁺ T helper population that recognizes peptide complexed to MHC class II molecules and CD8⁺ cytotoxic T cell population that survey peptide-MHC class I molecules. Additional T cell subsets may be defined by the patterns of cytokine secretion, expression of cell surface molecules, or transcription factors.

6.2.1 Somatic DNA recombination

The Ig diversity of chicken B cells is generated by gene conversion, a process taking place during bursal

development [3]. T cells develop from precursors in the thymus (details of chicken T cell ontogeny and thymic development are described in Chapter 3). Somatic DNA recombination and junctional modification create variability in the TCR thus ensuring a diverse repertoire of CDR-3 sequences. The principles of somatic DNA recombination in chicken and mammalian T cells are identical. For a comprehensive description of the molecular events leading to somatic DNA recombination, the reader is referred to excellent reviews covering the subject [4–7]. Briefly, the process of somatic recombination involves the processing of various segments encoded in the TCR loci associated with modification of the DNA sequence at the junctions between these segments. The chromosomal regions encoding TCR clusters each possess a similar organization with a set of 5' V gene segments, followed by a number of diversity (D) (in the case of TCR β and TCR δ), and joining (J) gene segments and one or few 3' constant (C) genes. This reservoir of V(D)J gene fragments is randomly assembled during thymic development to form a complete gene encoding a TCR. During the process of recombination the sequence at the V(D)J junction is modified by addition and deletion of nucleotides to form an incredibly diverse set of TCR. Moreover, the functional TCR on the surface of cells comprises two chains ($\alpha\beta$ or $\gamma\delta$) and these are also combined randomly. Hence the phenomenal diversity of TCR recognition is generated by three processes: recombination, junction modification, and pairing of the TCR chains.

In the case of the α and γ TCR genes, a randomly selected V gene segment is combined with a J gene segment and for TCR heavy chains β and δ , an initial DJ rearrangement is succeeded by a VDJ combination. The regions between the V (D) and J genes are excised. The process of somatic DNA recombination is mediated

by the conserved enzymes encoded by the recombinase-activating genes 1 and 2 (*RAG1* and *RAG2*) that are directed by special recombination signal sequences located in the vicinity of each V, D, and J gene. During transcription, the combined V(D)J genes are spliced to the 3'C gene to form a complete TCR. The peptide MHC binding site is specially generated by protruding loops of the V(D)J junction, which are homologous to the Ig complementary-determining regions (CDR) of antibodies and display extensive diversity. Although the CDR1 and CDR2 regions are encoded by the V gene segments, the CDR3 region is generated by the juxtaposition of V(D)J genes. During the process of somatic DNA recombination, the joining of gene fragments is imprecise and subjected to base deletions by exonuclease activity, nonencoded base insertions (N nucleotide additions), and insertions of short palindromic sequences (P additions). The major enzymes mediating these processes, such as RAG and terminal deoxynucleotide transferase (TdT), have been characterized in the chicken [8,9]. Thus the CDR1 and CDR2 of the TCR are germline-encoded, whereas the CDR3 is generated randomly due to base insertions and deletions and shows the highest degree of variability. Analysis of crystal structures of TCR has revealed that the CDR1 and CDR2 mainly contact the MHC α helices, and in the case of CDR1 also the end of the antigen peptide. The CDR3, however, is localized directly over the center of the MHC peptide binding groove and makes contact with the peptide bound in the groove, as well as the MHC α helices [1,10]. The diversity of T cells is fixed after thymic development and is not changed after antigen recognition. This is different to B cells, which undergo somatic hypermutation in germinal centers after antigen recognition [6]. The organization of the individual chicken TCR clusters will be described below.

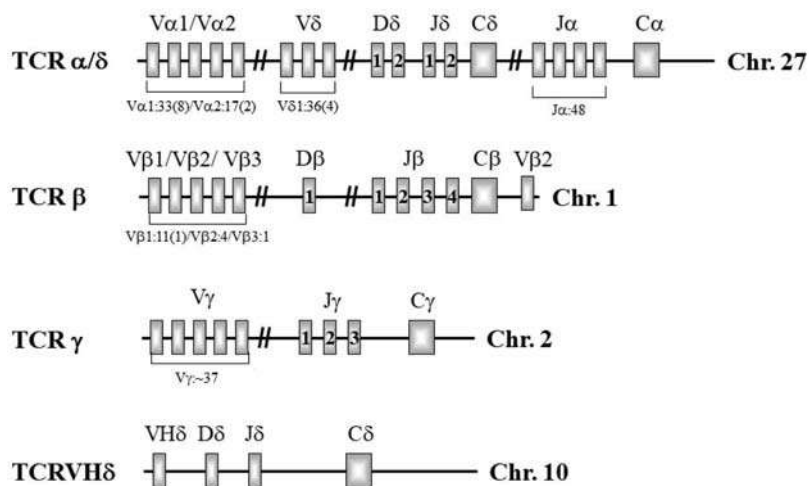


FIGURE 6.1 TCR gene loci. Schematic representation of the chicken TCR loci. Note that the boxes indicate the relative position of the elements but do not represent the exact number where more than four segments are present. For those, the number of functional genes is indicated below the sequence (number of pseudogenes in parenthesis). More details are found in Table 6.1. TCR, T cell receptor.

6.2.2 Organization of the T cell receptor clusters

TCR α / δ cluster

In mammals the TCR α and TCR δ genes are encoded by a single cluster designated TCR α / δ . All nonmammalian TCR α / δ gene clusters characterized so far seem to share this configuration [11–13]. In this TCR α / δ cluster the classical TCR δ encoding VDJC genes are located between the V α and J α sequences. Therefore, somatic recombination of the TCR α locus leads to the excision of the entire classical TCR δ cluster. This TCR δ deletion has been found on both alleles of all chicken $\alpha\beta$ T cell lines tested [13].

Southern blot and sequence analyses indicated that there are two V α families with a total of at least 25 members (Fig. 6.1) [13,14]. All T cell lines examined to date share a V α member from one of these two families, suggesting that there are only two V α families and these two V α families share less than 30% amino acid homology. In 1997 a cosmid clone representative of the 3' end of the TCR α / δ locus was sequenced and contained six J α genes and a single C α gene composed of four exons [15]. Southern blot analysis suggests that there are more J α sequences upstream of this region [14].

The sequence of the entire TCR α / δ locus located on chromosome 27 and spanning about 800 kb has been annotated and published by two groups with slightly different numbers for the gene segments [16,17] (Table 6.1). Parra et al. identified a total of 60 V α elements grouped into two subfamilies, whereas Liu et al. described 54 V α elements and 10 subfamilies. The V α 1 genes identified by Parra all share an unusual feature: they are encoded by a single exon [14,17]. Likewise, the number of J α gene segments is reported to be 48 and 67, respectively, by Parra et al. and Liu et al. All of the J α elements are

flanked by a canonical 12-pb Spacer recombination signal sequence. There is a single C α .

The TCR δ locus is located between the V α and J α gene segments and contains 36 or 31 V δ gene segments according to Parra et al. and Liu et al., respectively. It has been argued that a large number of V δ gene segments is typical for $\gamma\delta$ high species, such as birds, pigs, and cattle [19]. The V δ gene segments are followed by two D δ , two J δ , and a single C δ gene. The D δ gene segment displays an upstream 12-bp and downstream 23-bp spacer recombination signal sequence. In mammals, there is a single conserved V δ gene in reverse orientation downstream of the C δ gene, which is missing in the chicken. The V α elements display a mean nucleotide length of 340 bp and thus are slightly shorter compared to the V δ gene fragments with a mean length of 370 bp.

The complex organization of the TCR α / δ locus increases the combinatorial diversity, such that some V α and V δ gene fragments can contribute to either TCR α or TCR δ and are referred to as V α / δ . This has been proven for at least eight V α and two V δ elements [16]. All V α and V δ elements are followed by a canonical 23-bp spacer recombination signal sequence. The D δ elements may be utilized during the rearrangement with extensive variability in all possible combinations. So, V α and V δ gene segments have been found to be rearranged directly to J δ lacking a D δ segment or they were rearranged to either one of the two D δ elements or alternatively to both D δ segments in tandem. The J δ genes were found to be equally represented in embryonic $\gamma\delta$ T cells, whereas in adult $\gamma\delta$ T cells there was a bias toward J δ 1 usage [13].

Interestingly, in galliform birds, a second TCR δ -like locus was identified on chicken chromosome 10 that contained a single VH δ , D δ , J δ , and C δ cassette with the V segment more closely related to IgH V segments (and

TABLE 6.1 Summary of chicken T cell receptor loci.

	Length (kb)	Chromosome	V α	D	J	C	Reference
TCR α	800	27	60 [10]/V α 1:33 [8], V α 2: 17 [2]		48	1	[17]
			54/V α 1:1, V α 2:21, V α 3:11, V α 4:14, V α 5:1, V α 6:1, V α 7:1, V α 8:2, V α 9:1, V α 10:1		67		[16]
TCR β	210	1	16 [1]/V β 1:11 [1], V β 2:4, V β 3:1	1	4	1	[18]
	120	1	8/V β 1:4, V β 2:4	1	4	1	[16]
TCR γ	82	2	37/V γ 1:6, V γ 2:7, V γ 3:2, V γ 4:5, V γ 5:2, V γ 6:4, V γ 7:4, V γ 8:2, V γ 9:3, V γ 10:1, V γ 11: 1		3	1	[16]
TCR δ	800	27	36 [4]/V δ 1:36 [4]	2	2	1	[17]
	800	27	31/V δ 1:14, V δ 2:6, V δ 3:10, V δ 4:1	2	2	1	[16]

^aTotal number (number of pseudogenes)/subgroup: total number of genes in subgroup (number of pseudogenes in subgroup). TCR, T cell receptor.

thus termed VH δ) than to other TCR V segments [20]. In the zebra finch, this VH δ segment is present in the conventional TCR α/δ cluster. The impact of having a TCRVH δ locus is not known but could be related to the specificity of ligand recognition and/or the function of TCR $\gamma\delta$ T cell subsets.

TCR β cluster

The chicken TCR β genes were the first nonmammalian TCR genes to be isolated [21]; initially identified as containing 10 V β gene segments. Based on their nucleotide homology these 10 V β sequences were further divided into six V β 1 and four V β 2 family members (Fig. 6.1). Analysis of one of the earlier genome assemblies led to the identification of nine V β 1 and four V β 2 segments a single D β , four J β , and single C β [22]. Members within a family share over 90% homology, whereas the homology between the families is less than 40% [23].

Two recent publications have annotated the TCR β genes located on chromosome 1 and spanning up to 210 kb [16,18] leading to slightly different results regarding the number of V β elements. Although Liu et al. describe eight V β fragments grouped into two subfamilies, Zhang et al. identified 16 V β elements, with one of them forming a novel V β 3 subfamily. Interestingly, the V β 2.4 elements are located downstream of the single C β in reverse transcriptional orientation. The V β 1 elements are preferentially used during recombination followed by V β 2 elements and the single V β 3 member is used infrequently [18,23]. There may also be a bias toward V β 1 segments located at the 5' end of the TCR β locus.

Two $\alpha\beta$ TCR-specific monoclonal antibodies (mAb) have been generated [24,25]. The TCR2 mAb specifically recognizes all V β 1 family members, and the TCR3 mAb the V β 2 family members [23,26]. It is not known, if the novel V β 3.1 element is recognized by any of the existing anti-TCR β mAb. A single 14 nucleotide D β element is located downstream of the V β sequences [21,27]. The recombination signal sequences have been conserved as described for the TCR α/δ locus.

The potential combinatorial diversity that results from only few V β families with high intrafamilial homology and a single D β element seems to be reduced in comparison to the mammalian TCR β cluster. The TCR V genes encode the CDR1 and CDR2 that are mainly responsible to contact the MHC α helices. The chicken MHC locus differs markedly from its mammalian counterpart because it is very compact with only few genes and it is less polymorphic [28]. It is therefore interesting that the TCR chain contacting the MHC has also reduced germline diversity; a minimal essential TCR locus to match the minimal essential MHC (Chapter 7). The CDR3 region contacting the MHC bound peptide, however, is generated

by the sequences around the D β element. All three reading frames of the D β are utilized and they encode a glycine that may be involved in the formation of the CDR3 loop [29]. The D β gene is followed by four J β sequences [18,27]. Extensive N- and P-nucleotide additions, as well as nucleotide deletions, are observed at the junctions, thus creating highly diverse CDR3 sequences. It has been shown that the CDR3 β length ranges between 7 and 21 amino acids with an average of 13 amino acids. This is a much wider CDR3 β range as compared to mammals, but similar to the situation in duck [18,19]. Due to the upregulation of the TdT, the extent of N region addition increases during embryonic development. During rearrangement, the intervening chromosomal DNA is excised and forms so-called deletion circles that have also been detected for the TCR β and TCR γ locus. These deletion circles are used as a marker for recent thymic emigrants [30,31]. Moreover, as in mammals, germline transcripts of TCR β were found before the detection of rearrangements, indicating that transcription of unrearranged TCR genes precedes the recombinase activity. Both V D rearrangements and D J rearrangements were detected at identical time points indicating that the chronology of rearrangement steps may not be strictly regulated in the chicken [32]. In mammalian thymocyte development the TCR β chain first pairs with a nonpolymorphic partner designated pre-T α which provides an important developmental signal. A chicken pre-T α homolog has been described with mRNA expression demonstrated in double negative and double positive, but not single positive thymocytes [33].

TCR γ cluster

The TCR γ cluster is located on Chromosome 2 and spans approximately 82 kb, which is significantly smaller compared to mammals [16,34] (Fig. 6.1). The original work on the V γ locus grouped the V γ sequences into 3 families [34] (V γ 1, 2 and 3) although the availability of deep sequencing technologies and genome sequence covering this region has enabled this to be revisited. More recent work has identified a large number of V γ segments in the locus and current estimates range between 38 and 40 V γ segments (with 13 pseudogenes) [16,35]. These V γ sequences can be subgrouped; with Liu et al. [16] proposing 11 subgroups whereas Dixon et al. [35] propose four families (V γ 1 to V γ 4) with the V γ 2 family subdivided into 4 subgroups (A, B, C, D) according to the conventions laid out by IMTG. Dixon et al. [35] used a deep sequencing approach to assess TCRV γ repertoire and most segments were found to be expressed although their contribution to the overall repertoire in thymus and peripheral tissues varied widely. There are three J γ genes in the locus and a single C γ . The genomic organization is

unlike many other vertebrates which have a cassette-based organization with groups of V segments arranged with specific J and C segments.

In mouse and man, different waves of $\gamma\delta$ T cells with canonical, public $V\gamma$ $J\gamma$ rearrangements occurring early during thymic development populate various tissues, such as $V\gamma3J\gamma1C\gamma1$ T cells homing to the skin and $V\gamma5J\gamma1C\gamma1$ present in the intestine. This situation is different in the chicken where as early as embryonic incubation day 10 of development members of all of the three $V\gamma$ families described by Six et al. start to rearrange [34]. The thymic (adult) and peripheral tissue repertoires of $V\gamma$ were much more similar in chickens compared with mice or humans although in all cases a substantial portion of the repertoire was public, and found in most, or all, individuals [35]. The biology of the TCR γ repertoire will be important in defining the function of these cells and in chickens there are substantial public and private compartments that differ in size depending on the $V\gamma$ usage. The diverse intestinal $\gamma\delta$ T cells appear to be derived from early thymic emigrants forming a long-lived, self-renewing population [36,37].

The genomic T cell receptor regions in other birds

Apart from the chicken TCR genomic sequences, other TCR loci in birds remain elusive with the exception of the white Peking duck and zebra finch [19,20]. The zebra-finch TCR α/δ locus on chromosome 27 contains two C δ and a single C α . Approximately 10 $V\alpha$ and about four $V\delta$ gene segments are located upstream of single D δ , two J δ ,

and a first C $\delta1$ segment. Further 3' an addition D δ , J δ , and C δ is found. Furthermore, as outlined above, the atypical VH δ is located within the TCR α/δ locus and not separately on a different chromosome as in gallinaceous birds [20].

An extensive analysis of the genomic TCR loci has been performed in the white peking duck. Briefly, 49 $V\alpha$, 30 $V\delta$, 13 $V\beta$, and 15 $V\gamma$ gene segments were classified. While most TCR loci followed the architecture as observed in the chicken, the TCR β locus displayed an unusual organization with several $V\beta$ followed by a single D β , two J β , and a single C β and further 3' another four J β and a second C β [19].

6.3 CD3 signaling complex

6.3.1 Mammalian CD3

The CD3 molecules serve at least three functions. They are important during the assembly of the TCR complex, they control the surface expression of the TCR complex by targeting incomplete complexes to degradation and they trigger signal transduction following TCR ligation. In mammals, the CD3 complex consists of CD3 γ , CD3 δ , CD3 ϵ , and a $\zeta\zeta$ homodimer. The CD3 chains form two dimers, a CD3 γ -CD3 ϵ and a CD3 δ -CD3 ϵ heterodimer before assembly with the TCR. The extracellular domains of the CD3 molecules are composed of a single Ig-like domain, the transmembrane regions harbor one acidic amino acid that is important for the assembly and the intracellular domains contain an immunoreceptor tyrosine-based activation motif (ITAM) that is essential

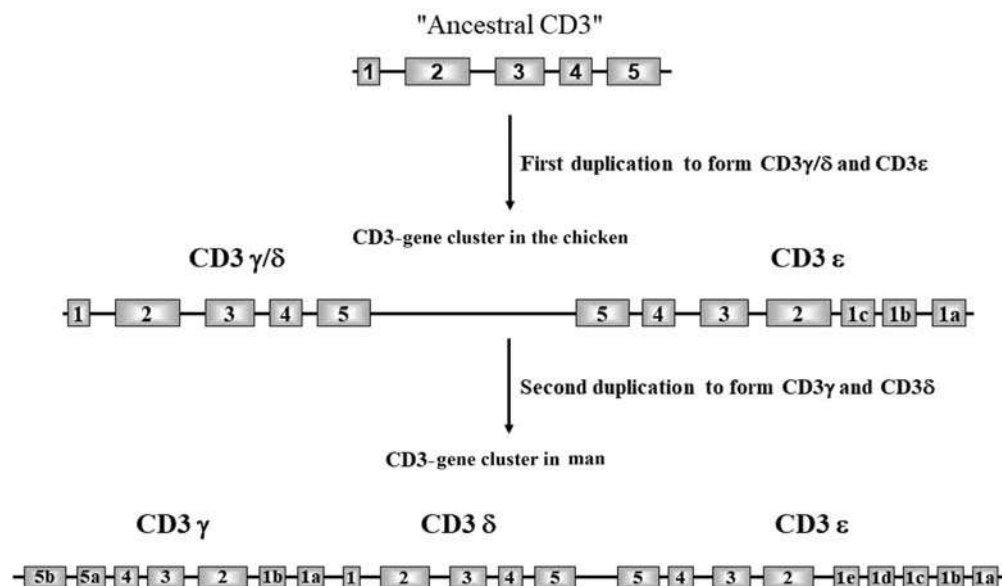


FIGURE 6.2 Model of CD3 gene evolution. Hypothetical model depicting the stepwise evolution of CD3 genes in vertebrates. The number of exons of each CD3 gene is shown. Sizes and distances are not drawn to scale.

for signal transduction. The ζ chain has only nine extracellular amino acids, its transmembrane domain also contains an acidic residue and a cysteine for dimerization and the long cytoplasmic tail has three ITAM. Various aspects concerning assembly, structure, and function of the mammalian CD3 complex have been summarized in excellent reviews [38–42].

6.3.2 Chicken CD3 γ/δ and CD3 ϵ

In contrast to mammals, only two chicken CD3 genes exist, a CD3 ϵ homolog and a the CD3 γ/δ gene with equal homology to mammalian CD3 γ and CD3 δ [43–46]. The genes encoding chicken CD3 γ/δ and CD3 ϵ are located on a 9.5 kb region on chromosome 24 and are flanked by two unrelated genes (ZW10 and EVA) [47]. The precise genomic organization and the exon/intron structures are depicted in Fig. 6.2. Since the two CD3 genes in the chicken are tightly clustered and transcribed in opposite orientations it is likely that both genes arose from an ancestral form by gene duplication. The exon/intron structures of all CD3 genes also support this hypothesis. Although there are variations at both ends of the CD3 ϵ and mammalian CD3 γ locus, their core structure has been preserved. We have therefore proposed that the three CD3 genes present in mammals arose by a stepwise evolution from an ancestral CD3 gene [45,47]. This CD3 ancestor was duplicated to form CD3 γ/δ and CD3 ϵ that are present in fish, amphibians, and chickens [48–52]. Only in mammals, has a second duplication taken place to form CD3 γ and CD3 δ . Interestingly, many features that have been described for either mammalian CD3 γ or CD3 δ are combined in the chicken CD3 γ/δ . For example, certain sequence features depend on glycosylation and a cytoplasmic internalization motif [47]. The unique properties of the chicken CD3 γ/δ and CD3 ϵ proteins were also revealed by a structural analysis demonstrating a unique dimer interface which allows the assembly to both the

TCR α and TCR β chain. It was also shown that two CD3 γ/δ and CD3 ϵ copies are a prerequisite for binding of the $\zeta\zeta$ homodimer [53].

6.3.3 $\zeta\zeta$ homodimer

In addition to the two CD3 genes, the $\zeta\zeta$ homodimer is an essential part of the chicken TCR. The $\zeta\zeta$ homodimer functions as an adaptor molecule that is used by various surface receptors including the TCR. It is not encoded in the same area of the CD3 genes but on chicken chromosome 1. Structural characterization of the $\zeta\zeta$ homodimer revealed that the two transmembranous aspartic acid residues are critical for the dimerization [54]. The cytoplasmic domain contains three repeats of the ITAM. An ITAM is defined as two YXXL motifs spaced by eight amino acids [55]. The tyrosine residues of the ITAM become phosphorylated during cellular activation and mediate downstream signaling events (see below). The chicken $\zeta\zeta$ homodimer has been so well conserved that it is able to associate with the mammalian TCR and to restore its function [56]. In mammalian TCR an alternatively spliced version of the ζ chain designated η chain is sometimes assembled to form a $\zeta\eta$ heterodimer; however, this has not been characterized in the chicken.

6.3.4 T cell receptor complex—structural models

The TCR complex consists of the TCR heterodimer and the signaling components. As stated above, all molecules (TCR heterodimer, CD3 γ/δ , CD3 ϵ , and $\zeta\zeta$ homodimer) are essential for surface expression. The valency (number of TCR heterodimers in a TCR complex) and the stoichiometry (molar ratios between subunits) are still unresolved and two models are proposed: a monovalent model with a single TCR heterodimer and a bivalent model consisting of two TCR heterodimers. Moreover, no crystal structures of the entire complex exist. Biochemical studies, however, indicate that different multiunit receptor complexes on hematopoietic cells such as the killer inhibitory receptors, certain Fc, and collagen receptors, are assembled by similar mechanisms [57]. All of these receptors are characterized by basic transmembrane residues in the ligand-binding domain and acidic residues in the signaling dimer [42,58]. These residues favor the formation of a three-helix interface composed of two acidic and one basic residue (Fig. 6.3). This formation of trimers would imply that for the chicken a CD3 γ/δ -CD3 ϵ signaling dimer is associated with TCR α or TCR β to form a TCR α -CD3 γ/δ -CD3 ϵ trimer and a TCR β -CD3 γ/δ -CD3 ϵ trimer. In the following step these two trimers ($\alpha - \gamma/\delta - \epsilon$ and $\beta - \gamma/\delta - \epsilon$) are assembled and the free basic charge on TCR α aids formation of a third trimer together with the $\zeta\zeta$ dimer. Although the assembly of the

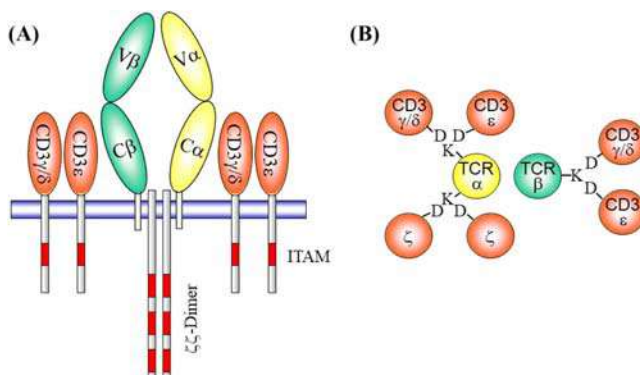


FIGURE 6.3 Monovalent chicken TCR structure. The model is based on current concepts and structural analyses of the mammalian TCR. TCR, T cell receptor.

chicken TCR complex has not been studied in great detail the conserved sequence features across species suggest similar assembly mechanisms. Apart from the transmembrane residues that are essential for the building of a complete TCR complex, the interactions of the extracellular Ig domains are important for proper assembly [59]. Again the similarity between the chicken and mammalian TCR complex is remarkable: the TCR heterodimer has a 20–26 amino acid long connecting peptide, whereas the CD3 chains are only 5–10 amino acids. The CD3 chains all contain a highly conserved R(K)XCXXCXE motif close to the transmembrane region that has been implicated in the signaling of the TCR complex [59] and forms a metal coordinated cluster [60]. Despite these highly conserved sequence motifs it must be recognized that the overall protein homology between the chicken and mammalian TCR chains is very low. It is not surprising, therefore, that the chicken CD3 and TCR chains are not able to substitute for the respective mouse chains [61]. Notably, while all components of the TCR complex are essential for the surface expression of the $\alpha\beta$ TCR, the murine $\gamma\delta$ TCR contains only CD3 γ -CD3 ϵ dimers and lacks CD3 δ [62]. Information about the chicken $\gamma\delta$ TCR is not available, though probably it also contains two CD3 γ / δ -CD3 ϵ heterodimers.

6.3.5 T cell receptor signal transduction

Signal transduction has also been intensively studied in mouse T cells, but neglected in the T cells of the chicken. Because of strong conservation of intracellular signaling motifs, it can be predicted that signaling through the chicken TCR complex is similar to that of the mammalian complex. Central to the signaling capability of the TCR complex, following crosslinking by peptide MHC ligand, is signal amplification by 10 cytoplasmic ITAM located in the cytoplasmic domains of the various CD3 chains and the $\zeta\zeta$ homodimer. ITAM are not unique to the CD3 chains; they are utilized by many different cell surface receptors. As for the TCR complex, the ITAM in these molecules are not located in the cytoplasmic domain of the ligand-binding receptor chain, but they are assembled into the complex by association with adapter molecules such as CD3, $\zeta\zeta$ homodimer, Fc γ chain, or DAP-12. In all cases the ITAM is encoded by two exons that are flanked by a type 0 intron, thereby forming a flexible two exon set. It has been speculated that this symmetric two exon ITAM set was used in many receptors as a signaling building block that could be inserted or deleted without disturbing the reading frame [41].

The initial event taking place after ligand binding is mediated by an Src family kinase such as Lck. This Src kinase activity leads to the phosphorylation of the ITAM. Fully phosphorylated ITAM bind with high affinity to the

tandemly arranged SH2 domains of ZAP-70, a member of the Syk family protein tyrosine kinases. ZAP-70 is also transphosphorylated by Lck, leading to an increase of its catalytic activity. Due to the presence of 10 ITAM in the signaling units of one TCR complex, TCR triggering leads to signal amplification by recruiting up to 10 ZAP-70 molecules. Moreover, several unique residues apart from the ITAM in the cytoplasmic domains of each CD3 chain have been implied in differential signaling activities of individual CD3 subunits. Especially the highly conserved proline-rich sequence in CD3 ϵ may be involved in early signaling events. ZAP-70 finally phosphorylates adapter proteins such as LAT and SLP-76. Under steady-state conditions, basal levels of protein tyrosine kinase activity are balanced by the activity of protein tyrosine phosphatases such as CD45, however, this ratio is shifted following activation. It has been postulated that the concentration of TCR complexes and coreceptors in lipid rafts leads to the exclusion of CD45.

6.4 CD4 and CD8

The protein tyrosine kinase Lck, critically required to phosphorylate the ITAM, is associated with the cytoplasmic domains of the CD4 or CD8 coreceptors. Binding of CD4 and CD8 to MHC class II and MHC class I molecules, respectively, brings Lck into proximity with the ITAM. CD4 and CD8 thus constitute essential components of a functional TCR complex and have the major function of bringing the Lck kinase close to the ITAM. Both chicken CD4 and CD8 have been initially characterized by specific mAb [63]. These mAb analyses have demonstrated that CD4 and CD8 are expressed on mutually exclusive subsets of T lymphocytes in the periphery, while they are coexpressed on a major thymocyte population, see also [Chapter 3](#). The ratio of CD4⁺ to CD8⁺ peripheral T cells has been detected for many different chicken lines and seems to be dependent on the MHC haplotype [64,65].

Both CD4 and CD8 were cloned by a COS cell expression system [66,67]. Chicken CD4 is a single chain molecule with four extracellular Ig domains and a cytoplasmic domain with a conserved Lck binding site. Although the binding site to MHC has not been conserved a positively charged amino acids in the amino-terminal domain maybe involved in the interaction with MHC class II β chain.

Chicken CD8 is composed of two chains, CD8 α and CD8 β , which can form the two isoforms designated as CD8 $\alpha\alpha$ homodimer and CD8 $\alpha\beta$ heterodimer [66]. Both CD8 chains are composed of a single extracellular V-like Ig domain. The CD8 α chain contains the conserved Lck binding site, whereas CD8 β has only a short cytoplasmic domain. The comparative analyses of the CD8 α crystal

structure in chicken, swine, and cattle revealed four highly conserved aromatic residues important for homodimerization and three conserved amino acids that form hydrogen bonds to the MHC molecule [68]. The CD8 α gene seems to be polymorphic, especially in the putative MHC class I binding site [69]. Furthermore, the chicken CD8 locus contains multiple duplicated CD8 α like genes with unknown function [70]. Two mAb have been generated that recognize either the CD8 α chain (CT8) or the CD8 β chain (EP42), respectively [63,66]. As in mammals, most of the T lymphocytes bear the CD8 $\alpha\beta$ heterodimer. In young chickens, there is a distinct population of $\gamma\delta$ T cells with CD8 $\alpha\alpha$ homodimers, mainly in the spleen and intestine (see also Chapter 2); however, in the intestine there is an additional population of CD8 $\alpha\beta$ $\gamma\delta$ T cells that is not found in mammals (see also Chapter 11).

Most lines of chicken express single positive CD4 or CD8T cells in the periphery; however, there is evidence for the coexpression of both molecules on a large proportion of peripheral T cells in some chicken lines [71]. All of these cells carry the CD8 $\alpha\alpha$ homodimer. The function of this cellular subset is not known at present.

6.5 Costimulatory molecules

Optimal signaling through the TCR requires secondary signals through many different costimulatory and -inhibitory receptors that regulate the extent, quality, and duration of the T cell activation. Structurally these receptors can be divided into IgSF members and members of the tumor necrosis family (TNF). The IgSF members include the activating receptors CD28 and ICOS, whereas CTLA4, PD1, and BTLA represent inhibitory receptors that contain cytoplasmic ITIM [72]. In the chicken CD28, ICOS, and CTLA-4 [73] are clustered together on chromosome 7, PD1 is located on chromosome 9, and BTLA on chromosome 1 (unpublished observation).

CD28 and CTLA-4 (CD152) have a single V-like Ig domain typical for the CD28 family and they contain the typical B7 binding site MYPPPI in the FG loop [73,74]. In addition, CTLA-4 has a GNGT motif in the g strand that is required for high-affinity binding to the B7 family. The membrane proximal connecting peptide of CD28, but not of CTLA-4, lacks a typical C that is required for dimerization. In the cytoplasmic domain both CD28 and CTLA-4 harbor a PI3 kinase binding motif (CD28: YMXM; CTLA-4: YVKM) [73,74]. To date, no mAb or functional studies have been described for chicken CTLA-4. Chicken CD28 is expressed on $\alpha\beta$ T cells and a small $\gamma\delta$ T cell subset [74,75]. Antibodies crosslinking CD28 have a costimulatory effect on phorbol myristic acid-activated T cells [76] and certain mAb against CD28 induce T cells to proliferate and secrete interferon- γ

(IFN- γ) without any costimulation. This situation is similar to rat and human.

CD28 and CTLA-4 share the ligands designated B7-1 (CD80) and B7-2 (CD86). Binding to these ligands, however, delivers opposing signals. Triggering CD28 leads to T cell activation and survival, whereas CTLA-4 ligation inhibits T cell responses and regulates peripheral T-cell tolerance [72]. This complex situation is regulated by the affinity between receptors and ligands and the different expressions of the receptors. CD28 is constitutively expressed on T cells and has a low affinity to B7 ligands. In contrast, CTLA-4 has a high affinity to B7 molecules, and it is rapidly upregulated by activated T cells. In the past decade, a number of additional molecules with similarity to either CD28 or B7 have been described and most of them are also conserved in the avian genomes of turkey, zebrafish, and chicken. Additional CD28 family members include ICOS, PD-1 [73], and BTLA, whereas the B7 family of genes consists of at least nine distinct members [77,78]. A mAb against B7.1 has been described that reacts with cells of bursa and spleen [79].

In contrast to the IgSF members of T-cell costimulatory molecules, there is only limited information regarding the TNF receptor (R) family members. Homologs of mammalian CD30, CD30 ligand, OX40, OX40 ligand, and GITR, GITR ligand have been characterized for the chicken [80–83]. Chicken CD30 has only low homology with its mammalian counterparts and harbors four instead of three extracellular TNFR repeats [81]. The cytoplasmic binding motifs for signal transducing TNFR-associated factor (TRAF) 1, 2, and 3 molecules have been conserved but not the site for TRAF6/TNFR-associated protein. A 22 amino acid long cytoplasmic region is highly conserved in human, mouse, and chicken; however, its function is not known. Interestingly, chicken CD30 is strongly upregulated on cells that have been transformed with the Marek's disease virus [81]. This situation is similar to the diverse range of Hodgkin's and non-Hodgkin's lymphomas which overexpress CD30—in fact CD30 was previously known as “Hodgkin's disease antigen.”

The ligands of the TNFR family members are type II transmembrane molecules that share high similarity at the C-terminus and most of them are expressed on activated T cells. Chicken CD30 ligand (CD30L or CD153) has been cloned by a subtractive hybridization approach and shared about 33% identity with its mammalian counterparts [80]. It is mainly expressed in spleen, thymus, bursa, lung, and a monocyte-like cell line. Unfortunately, the interaction of CD30 with its potential CD30L and the functional consequences of this interaction have not been studied.

The chicken OX40/OX40 ligand pair has been identified in the genome and successively cloned. Their mutual interaction was demonstrated and by virtue of an

OX40-Fc receptor hybrid molecule the tissue distribution of OX40 ligand was investigated. Although nonstimulated cells did not express OX40 ligand, activation with IL-2 and IL-12 induced the upregulation of OX40 ligand on T cells [82].

The chicken GITR/GITR ligand molecules were also characterized recently and their mutual interaction was proven. Using an antichickens GITR specific mAb, a strong upregulation on T cells was detected following activation, and, as in mammals, a subpopulation of $CD4^+CD25^+$ T cells constitutively expressed GITR [83]. These results point to an important potential conservation of GITR expression on regulatory T cells. In contrast to mammals, chicken GITR seems to be expressed on all erythrocytes.

6.6 T cell lineages

Three major chicken T cell lineages can be distinguished, based on their TCR usage and mAb reactivity. The TCR1 mAb recognizes all $\gamma\delta$ T cells, whereas the mAb TCR2 and TCR3 react with $\alpha V\beta 1$ and $\alpha V\beta 2$ TCR, respectively [23–25,84,85]. $\alpha V\beta 1^+$ T cells are most abundant, in the peripheral blood of young chickens, whereas $\gamma\delta$ T cell frequency increases with age. As outlined above, CD4 and CD8 serve as additional markers to further divide these lineages into subsets. Additional mAb against various surface antigens such as CD5, CD6, costimulatory molecules may either define more subsets or activation states of T cells. The ontogeny, tissue localization, and functional aspects have been the subject of reviews [26,86].

The expression of CD4 and CD8 on $TCR\alpha\beta^+$ T cells broadly differentiates helper/regulatory T cells and cytotoxic T cells typically restricted by MHC class II and class I molecules, respectively [87,88]. For instance, after infection of chickens with infectious bronchitis virus, MHC-restricted $CD8^+ \alpha\beta$ T cells have been identified as effector cells [89]. Similarly, in vitro studies using reticuloendotheliosis virus (REV)-infected target cells have provided evidence that the cytotoxic effector cells are MHC-restricted and reside in the $CD8^+ \alpha\beta$ T cell population, whereas no cytotoxicity was found in the $\gamma\delta$ T cell population [90]. In another study using a novel cytometry-based method, antigen-specific cytolytic activity was mainly confined to $V\beta 1$ $CD4^+$ and $CD8^+$ cells [91]. Finally, analyses of REV-transformed cell lines carrying Marek's disease virus genes have also provided evidence for MHC restriction [92].

The function of $V\beta 1$ and $V\beta 2$ expressing T cells has been analyzed by depletion studies using the specific mAb. Both populations were found to be involved in cytotoxic T cell responses against REV [90]. In contrast, IgA production in the gut was more dependent on $V\beta 1^+$ T cells [93] although this may reflect the relative paucity

of $V\beta 2^+$ T cells at this site [94]. Depletion of $CD4^+$ or $CD8^+$ cell populations indicated that $CD4^+$ cells were required for the development of autoimmune-mediated thyroiditis, while $CD8^+$ cells were not so important [95]. The role of TCR $V\beta 3$ -expressing T cells remains unknown.

Although the exact role of the different $\alpha\beta$ T cell subsets in many infections of chickens remains unresolved it is obvious from these studies that the subsets may be secreting discrete sets of cytokines. The basic concept of clonally defined T_H1 versus T_H2 T cell subsets, which are distinguished by the secretion of either $IFN-\gamma$ or interleukin-4 and IL-13, has not been confirmed for the chicken, mainly because of the inability to clone antigen-specific T cells. These cytokines have all been characterized in the recent years and tools to measure them by quantitative RTPCR, bioassays, or antibody-based techniques are being developed [96,97]. There is good evidence for a functional T_H1 system, since the basic mechanisms leading to a functional T_H1 response are well conserved in the chicken [98]. Vice versa T_H2 -like responses have been defined in some models in the chicken [99]. To our knowledge, there have been no efforts to correlate T_H1 - or T_H2 -type cytokine secretion with TCR $V\beta 1$ or $V\beta 2$ subsets.

There is also evidence for a T_H17 like subset. Two members of the IL-17 family have been cloned and expressed in the chicken [100]. Following generation of IL-17A and IL-17F specific mAb, intracellular cytokine staining revealed that IL-17A is expressed in both $\alpha\beta$ - and $\gamma\delta$ T cell subsets [101].

Regulatory T cells have also been described phenotypically and functionally in the chicken [102]. They share the $CD4^+CD25^+$ phenotype, the suppressive activity, and the IL-10 production with their mammalian counterparts. The recent discovery of chicken Fox-P3 now allows further investigation of this important T cell subset. It thus seems plausible to state that the major T cell subsets discovered in the mammalian system are also present in the chicken.

Chickens belong to a group of animals that have high frequencies of $\gamma\delta$ T cells, compared with mice and humans. Frequencies of $\gamma\delta$ T cells in the chicken can reach up to 50% of peripheral lymphoid cells [103,104]. Chicken $\gamma\delta$ T cell development is strictly thymic dependent but differs dramatically in many aspects from $\alpha\beta$ T cell development, such as the embryonic age of migration of precursors in the thymus, the phenotype of $\gamma\delta$ thymocytes, and the inability of cyclosporine A to block $\gamma\delta$ T cell development [86]. A significant androgen-induced expansion of $\gamma\delta$ T cells is detectable in male chickens at 4–6 months of age [105]. As in mammals the functional role of $\gamma\delta$ T cells has not been elucidated. The broad tissue distribution of $\gamma\delta$ T cells suggests that they have

important immune surveillance functions. There are only few reports that have analyzed chicken $\gamma\delta$ T cells in infection models. Following oral administration of various *Salmonella enterica* Typhimurium strains, a strong increase of CD8⁺ $\gamma\delta$ T cells with activated phenotype was observed [106–109]. $\gamma\delta$ T cells were also found to participate in graft versus host reactions [110]. In the chicken, it is not known if $\gamma\delta$ T cells recognize classical peptide MHC antigens or, as in mammals, bind to alternative antigens. The recent technical developments to produce knock-out chickens will soon allow studies on the functional roles of the different chicken T-cell lineages [111].

6.7 Methods to study T cell function

Novel methods to gain insight into T cell function are urgently needed in chicken immunology. Here, we will briefly summarize some of the methods that have either been developed recently or should be feasible in the near future by adapting mammalian methods to study chicken T cell function.

For phenotypic analysis of T cell subsets, there is an increasing portfolio of mAb, reacting with defined surface markers. This panel of mAb allows to detect and differentiate various T cell subsets and activation states. In addition to the above mentioned novel mAb, other examples include specific mAb against CD25, CD28 [75], CD45 isoforms [112], SLAM genes [113], CRTAM [114] among others. There are limitations in respect to TCR reagents. Besides the TCR-specific mAb that are only reactive with chicken, there are no V-specific mAb available. A highly conserved intracytoplasmic CD3 ϵ epitope allows the detection of CD3 ϵ in many bird species either using intracytoplasmic staining or immunohistology [52].

There is a particular need to address recently activated pathogen-specific T lymphocytes. As in mammals it should be feasible to utilize MHC tetramers given that the chicken MHC is less complex compared to mammals and the binding motifs have been clarified (Chapter 7). The complete annotation of the chicken TCR loci as described above sets an important basis for TCR profiling methods and the detection of CDR3 profiles. Some studies regarding CDR3 length using spectratyping and/or sequencing have been published [22,115,116] and parallel sequencing approaches have been developed and are being applied to various circumstances.

Concerning T_H subsets and their signature cytokines, there are mAb available, which are suitable for ELISpot and cytoplasmic staining. IFN- γ and IL-17A can be detected intracellularly [101,117].

There is a constantly growing list of recombinant chicken cytokines, some of which are available commercially, although they are not all tested for biological

activity. Nevertheless, the availability of recombinant IL-2, IL-12, IL-17, and IL-18 have been instrumental to study T cells [100,118–121]. These cytokines should be very helpful to clone antigen-specific chicken T cells in the future.

There are several methods to monitor T cell proliferation, such as the classical ³H thymidine and MTT assays [122], but also methods including BrdU incorporation [123], CFSE labeling [124], and EdU-based flow cytometry assay [125]. In addition, CD107a a highly conserved lysosomal membrane protein can be used to detect degranulation of cytotoxic T—and NK cells [126], and Annexin V staining can be used to detect apoptosis.

In conclusion, there are quite a lot of novel techniques available to study T cell phenotype and function, but there is still a need to develop additional methods, in particular in vitro culture systems to propagate T cell clones.

6.8 Perspectives

The chicken TCR serves as a paradigm for the nonmammalian TCR complex. Its components, structure, and function have been elucidated in some detail during the last decade. The ongoing efforts to finalize the chicken genome, the characterization of genes encoding chicken CD, and cytokine homologs have set an important basis to study the chicken T cell system in more detail. These tools have been used to identify the influence of gut microflora on TCR β repertoires [22], the clonal structure of Marek's disease tumors, and the CD8⁺ T cell response to Marek's Disease Virus [127]. Major tools that are being, or need to be, developed include mAb defining surface markers to study, as yet undefined, T cell subsets such as NK T cells or regulatory T cells; MHC tetramers to follow antigen-specific T cells after infections; in vitro culture methods to clone chicken T cells; mAb for intracellular cytokine staining; infection models for the analysis of T cell function in vivo and immune evasion mechanisms; methods to inactivate specific genes in T cells or T cell subsets in vitro and in vivo. Although the list of goals to be achieved is long, the chicken still provides an excellent model for T cell research, since it provides an one of the best-characterized animal models with many advantages over other animal models. These include easy access of the embryo during egg development; advanced EST databases and availability of the chicken genome database; a relatively small body size so experimental subjects can be accommodated in biosecure high-containment facilities; finally, a wealth of well-defined naturally occurring infectious diseases and, resistant and susceptible genotypes for studying host–pathogen interactions. The development of transgenic and knockout chickens (Chapter 10) has the potential to revolutionize our understanding of the roles for different

T cell subsets (and their restriction elements). All these factors provide excellent prospects for many exciting future discoveries in the field of chicken T cell research.

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The avian major histocompatibility complex

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7.1 Introduction

The major histocompatibility complex (MHC) was discovered in mice as the genetic locus responsible for rapid tissue allograft rejection, but it has been clear for decades that the typical mammalian MHC is an enormous genomic region with hundreds of genes, of which only a few highly polymorphic classical MHC genes are responsible for graft rejection [1]. These classical MHC class I and class II genes have central roles in immune responses to pathogens, vaccines, and cancers, and are found to be important for other biological functions as well [2,3]. It has also become clear that these highly polymorphic MHC molecules are found in all jawed vertebrates but thus far have not been found in jawless fish or invertebrates [4,5].

Most detailed understanding of the MHC outside of mammals has come from the domestic chicken, where decades of research have been driven ultimately by the economic importance of the poultry industry [6–9]. Unexpected discoveries arising from the study of the chicken MHC have included fundamental principles for structure/function relationships of MHC molecules and for genomic evolution, and it is likely that continued examination will lead to many discoveries of interest to the poultry industry, the biomedical community, and the field of comparative immunology.

This chapter in previous editions [6,7] focused on reviewing the detailed evidence and the history of discovery, but the updated version of this chapter will consider new findings and current controversies about the chicken MHC, as well as the beginning of detailed molecular research into other birds to give a more complete picture of the various forms of the avian MHC.

7.2 The biology of the major histocompatibility complex

The MHC was discovered and named for transplantation phenomena that are largely due to the highly polymorphic classical class I and class II molecules. However, the basic function of these classical MHC molecules is to bind short peptides within a cell and present them on the cell surface for inspection by immune cells, particularly T cells with T-cell receptors (TCRs) composed of α and β chains (i.e., $\alpha\beta$ T cells), which allows appropriate immune responses to occur [10].

Classical MHC molecules can differ at many amino acid positions in the peptide-binding groove, so that different alleles bind different repertoires of peptides [10]. The primary driving force for the high polymorphism is a molecular arms race with infectious pathogens. A common scenario is that a pathogen peptide presented by an MHC allele confers protection to the host, pathogen variants are selected which have changed the sequence of the peptide so that that MHC allele is no longer protective, a new MHC allele is selected which can present a peptide that confers protection, and so on. This situation leads to what evolutionary biologists call the Red Queen scenario, in which both the host and pathogen run as fast as they can to keep in one place. The selection pressures include heterozygote advantage and rare allele advantage, manifesting as overdominance and frequency-dependent selection [11–13].

The MHC molecules are not just ligands for the TCRs on canonical $\alpha\beta$ T cells. Some so-called nonconventional $\alpha\beta$ T cells have TCRs with particular sequences that allow them to recognize nonclassical class I molecules which can present a variety of ligands, including lipids,

bacterial metabolites, and bacterial peptides [14,15]. T cells with TCRs formed of γ and δ chains ($\gamma\delta$ T cells) can recognize classical and some nonclassical class I molecules, and they are even reported to recognize some classical class II molecules [16]. Natural killer (NK) cells express a variety of NK receptors [17], some inhibitory receptors that bind classical class I molecules and some nonclassical molecules (thus sensing the absence of class I molecules) and some activating receptors that sense the presence of certain nonclassical class I molecules (for instance, those that are expressed after infection or stress, see Chapter 8.4). Pathogens evolve to escape the immune response, not just by varying their sequences, but also by whole-scale immune evasion. A common scenario is a virus downregulating MHC molecules to escape T-cell responses, which is countered by NK cells recognizing cells which lack normal levels of the class I molecules, which is countered by viruses evolving MHC decoy molecules to fool the NK cells, which is countered by NK recognizing the viral MHC decoy molecules and so on.

Moreover, the biology of the MHC does not just include transplantation and responses to infectious pathogens. The most numerous and strongest genetic associations for the human MHC by far are with autoimmunity, allergy, and immunopathology, with nearly every autoimmune disease having the strongest association in genome-wide association studies (GWAS) with particular classical MHC alleles [2,18]. These damaging diseases against self-antigens and harmless nonself-antigens are thought to be due to the precarious balance between responding strongly enough to virulent pathogens and overreacting with damage to the host, with explanations for some autoimmune diseases being rooted in selection of T cells for self-tolerance in the thymus [19]. There is also a long history of MHC associations with aspects of reproduction, including mate choice and placental physiology [17,20,21], which all seem likely to be part of the ongoing struggle between males and females to control the outcome of reproduction. Finally, there are many other biological phenomena that may be determined by variation in the MHC other than in classical MHC genes, such as in complement components (including both immune responses and central nervous system development by synaptic pruning), cytokines such as TNF, and enzymes such as steroid hydroxylase [2,22]. There are many such examples from the human MHC, so genetic associations with a genetic region such as the MHC must be treated with caution until there is strong mechanistic evidence for the particular causative variation.

The B locus of chickens was discovered as a serological blood group [23] but was found to be associated with a variety of functional properties emblematic of the MHC in mammals, including graft rejection, graft-versus-host response, mixed lymphocyte response, autoimmunity, and

control over antibody responses to immunogens with limited epitopes [24–28]. The B locus has also been found to influence autoimmunity in chickens [29,30], but thus far there are no reports of involvement with reproduction. Perhaps the most striking phenomena associated with the chicken B locus are the strong associations with resistance and susceptibility to infectious diseases. There are decades of evidence for a variety of infectious pathogens, starting with the recognition that the B locus determined response to Marek's disease (MD) [31–34], and by now including different kinds of viruses, bacteria, and parasites [8,9,34].

Although much work has been done on the chicken immune system, including MHC molecules, $\alpha\beta$ T cells, $\gamma\delta$ T cells, and NK cells, understanding the complex immune responses involving the MHC at the level of mammals is still some ways off. The use of B locus recombinants showed that the BF-BL region rather than the BG region is responsible for resistance to MD [34,35], but the BF-BL region contains several closely linked genes so that determining which gene is responsible has not been easy. A particular pair of recombinants at the edge of the BF-BL region has implicated a polymorphic BG gene in MD resistance [36], but the classical class I gene BF2, the class II genes BLB1 and BLB2, and the NK receptor BNK (sometimes called Blec2) have all been suggested to be involved [8,37–39]. The striking properties of BF2 molecules from MD-resistant versus susceptible haplotypes also suggest involvement in resistance [8,40,41], and BF2 alleles are implicated in resistance to Rous sarcoma virus, based on vaccination with peptides selected using the BF2 motif from a protective MHC haplotype [42,43]. GWAS using single-nucleotide polymorphisms (SNPs) has identified the BF-BL region as strongly associated with the level of bacterial carriage by *Salmonella* and *Campylobacter*, but again the exact genes involved remain unclear due to strong linkage disequilibrium [44–46]. The difficulties in determining the genes involved in disease resistance are not just technical but are part of a wider conceptual issue, as discussed in the next section.

7.3 The major histocompatibility complex: a genomic region or a biological unit?

Most of what is known about the structure and function of the MHC and MHC molecules comes from research into mammals, particularly humans, and biomedical model species such as mice. The typical mammalian MHC is an enormous genetic region containing hundreds of genes and pseudogenes, of which only a few encode the highly polymorphic transplantation antigens for which the region

is named. Already decades ago, the question of whether these genes are all “MHC genes” was raised [47], and this issue is at the heart of a schism of nomenclature and interpretation about the chicken MHC.

As an example of a typical placental mammal, the human MHC [2,3] is roughly 4 million base pairs (Mbp) of physical length and 2–4 centimorgans (cM) of recombination, consisting of a class III region flanked by the class I region and the class II region. The class I region contains only three classical class I genes (HLA-A, B, and C) along with three nonpolymorphic nonclassical class I genes (HLA-E, F, and G), interspersed among many genes of highly disparate structure and function. The class II region encodes three to four classical class II molecules (one or two HLA-DR, one DQ, and one DP), three nonpolymorphic nonclassical class II molecules (HLA-DM, DO, and DQA2), and the antigen processing and peptide-loading molecules for the class I system [PSMB7, PSMB8, transporter for antigen presentation (TAP)1, TAP2, and tapasin or TAP-binding protein (TAPBP)] and the serine-threonine kinase BRD2 (also known as RING3). The class III region has many genes encoding molecules of disparate structure and function, including two nonclassical class I genes (MIC-A and MIC-B); cytokines (TNF α , TNF β 1 or LT α , TNF β 2 or LT β); and components of the complement cascade (C4A, C4B, C2, and factor B); among many others.

The human MHC is often described as flanked by an “extended class I region” and an “extended class II region,” adding many hundreds of other genes over 10 Mbp [48]. Among the genes found in the extended class I region are butyrophilins, tripartite motif (TRIM) genes, scavenger receptors, and olfactory receptors, ending with a nonclassical class I gene involved in iron metabolism (HFE). The equivalent region of the mouse MHC contains many nonclassical class I genes involved in functions ranging from immunity to nervous system function and behavior.

Some of these genes behave as a biological unit, involved in a particular set of functions and generally staying together during evolution. The class I gene system includes the classical class I genes, the inducible proteasome components (PSMBs or LMPs), the TAP peptide transporters, and the bespoke chaperone tapasin (or TAPBP). The class II gene system includes the classical class II genes and the chaperone DM along with the negative regulator DO. Some other genes are found in the MHC of most nonmammalian vertebrates, including the complement components and the BRD2 kinase. However, many of the genes come and go in evolution around these core components. For instance, the number and functions of nonclassical class I genes in and around the MHC differ markedly between humans and mice [3], and some TRIM genes found near the chicken classical MHC genes

are found on different chromosomes in humans and mice [49].

Some nonclassical class I genes in mammals are found amid other paralogous genes on different chromosomes, for instance, the CD1 and MR1 genes on human chromosome 1 and the FcRn gene on chromosome 19 (although these genes are dispersed over a huge distance [50]). Other nonclassical class I genes are found elsewhere in the human genome (ZAG, EPCR, and ULBPs). The closest relatives to MIC-A and MIC-B found in the human class III region are found as Mill genes in the NK complex (NKC) in mouse [51]. On the other hand, regions of nonclassical class I genes are found at some distance from the classical MHC genes in mouse, chicken, and the frog *Xenopus*, a parallel already mentioned long ago [47]. Thus it seems clear that even genes related to the classical MHC genes can move through the genome over evolutionary time.

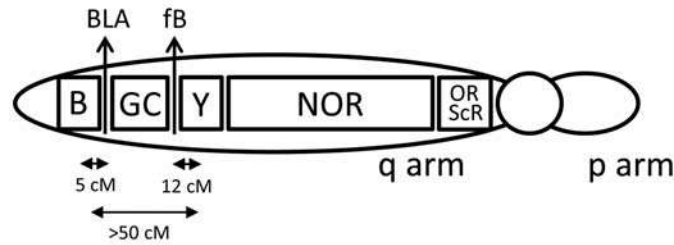
The concepts of a biological unit (genes that can travel together and form part of a pathway with biological significance) and a syntenic region (genes that can travel together in evolution without necessarily being functionally related) provide simple ways to consider these issues. As mentioned earlier and discussed with examples in a recent review [5], the classical class I system and classical class II systems form biological units of the MHC, with other genes coming and going as part of the MHC syntenic region.

7.4 The chicken major histocompatibility complex and the major histocompatibility complex syntenic region

As described earlier, the B locus was discovered as a serological blood group but was found eventually to determine a variety of properties previously described for the MHC in mammals. Comparison of different chickens as well as different cell types revealed that the blood group antibodies recognized cell surface molecules encoded by two genetic loci, now known as the BG region and the BF-BL region. The BF-BL region determined molecules that looked like class I and class II molecules, while the BG region determined what were considered to be erythrocyte antigens (until it was later found that they are present on lymphocytes and epithelial cells as well) with different structural properties [52–56]. The B locus, and the BF-BL region in particular, was found to determine resistance and susceptibility to MD and other economically important infectious diseases [9,34,35].

A combination of genetics, cytogenetics, and molecular biology has led to the current picture (Fig. 7.1A) of

A. Chicken chromosome 16



B. The B locus

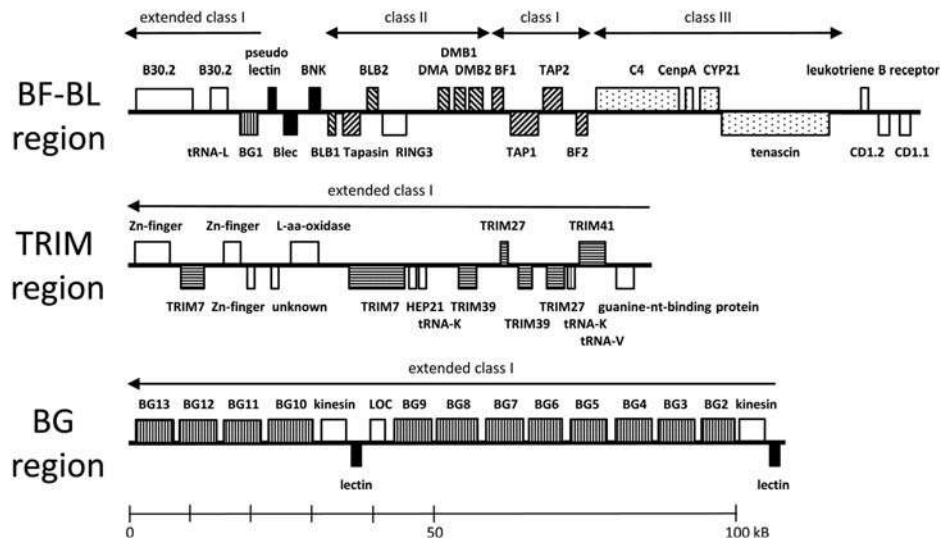


FIGURE 7.1 Organization of regions on chicken chromosome 16, as currently understood. (A) Depiction of chromosome 16, based on analysis by FISH, radiation hybrids, genetics, southern blotting, and sequencing. B, B locus; GC, G + C-rich region of PO1 repeats; Y, Rfp-Y region; NOR, nucleolar organizer region; BLA, class II A gene; fB, factor B gene; ORs, olfactory receptor genes; SRCRs, scavenger receptor with cysteine repeat genes. Double-headed arrows indicate recombination frequencies between B and BLA, fB and Rfp-Y, and B and Rfp-Y. (B) Region of the B locus currently sequenced and annotated, including the BF-BL region, the TRIM region and the BG region. Genes represented by boxes. Rising and falling stripes indicate genes of the classical class I and class II presentation system, respectively; stippled indicates class III region genes; black indicates lectin-like genes and pseudogenes; horizontal stripes indicate TRIM family genes; vertical stripes indicate BG genes. Names of genes above indicate transcription from left to right and below indicate transcription from right to left. *Figure modified from Kaufman J. The avian MHC. In: Schat KA., Kaspers B, Kaiser P., editors. Avian immunology. 2nd edition. Elsevier Ltd; 2013. p. 149–167.*

the B locus on the long arm of a microchromosome, named chromosome 16. The B locus is telomeric to a region of PO41 repeats, which are followed by a region discovered to have nonclassical class I and class II B genes along with genes encoding lectin-like cell surface molecules, which was originally named the Rfp-Y region and later simply the Y region. On the centromeric side of the Y region is the NOR that encodes ribosomal RNA, followed by regions containing olfactory and scavenger receptor genes.

Cloning and sequencing has separated the B locus into three well-characterized regions (Fig. 7.1B): BF-BL, TRIM, and BG [57–61], although extended sequencing beyond the BG region is now awaiting careful annotation

[62]. Similar analyses have started for the Y region [63]. The BF-BL region seems to be relatively stable with a very low rate of recombination [64–67], while the TRIM, BG, and Y regions seem very dynamic [61,63,67]. As a well-characterized example, the tandem arrangement of BG genes in the same transcriptional orientation allows unequal crossing-over and deletion, giving rise to expansions and contractions of the BG region [61], while many gene pairs in the BF-BL region (BF1 and BF2, TAP1 and TAP2, BLB1 and BLB2, BNK and Blec) are in opposite transcriptional orientation so that recombination leads to inversion rather than loss of essential genes [58,68]. Crucially for the arguments developed below, the region of PO41 repeats leads to so much recombination that the

B locus and the Y locus are genetically independent [69,70] and could be located in far different places in the genome. However, the reference genome of the chicken annotated by ENSEMBL shows Y region genes very close to the BG region, along with various genes that are found in the human MHC. If this assembly of chromosome 16 is accurate, then the region between the B locus and the Y locus is in fact very small.

The BF-BL region is relatively small and compact, with 25 genes (mostly with small introns and small intergenic distances) in about 110 kB, but contains the polymorphic genes of the classical MHC class I and class II system [58–60]. Two classical class I (BF1 and BF2) along with TAP1, TAP2, and tapasin (TAPBP) genes comprise the class I system, while two classical class II B (BLB1 and BLB2) and three DM (DMA, DMB1, and DMB2) genes comprise the class II system. Unlike typical mammals, all these chicken MHC genes are polymorphic, including those involved in peptide loading: TAP1, TAP2, tapasin (TAPBP), DMA, DMB1 and DMB2 [42,60,71–78]. Moreover, the chicken TAP genes are flanked by the two class I genes and the tapasin gene is located nearby, rather than these genes being separated by the class III region as in typical mammals.

The BF-BL region contains nonpolymorphic genes that are found in the MHC of typical mammals: the BRD2 (RING3) gene from the mammalian class II region and class III genes such as C4, tenascin, and steroid hydroxylase (CYP21) [58,59]. There are also genes that were unexpected: two CD1 genes (found in an MHC-paralogous region in mammals) [79–81], the centromere protein A (CENPA) gene (found on chromosome 2 in humans) [59], a BG gene (BG1, with some relation to butyrophilin-like genes, one of which is found between the class II and class III region in humans) [58,61] and two lectin-like cell surface molecules (BNK and Blec) that are most similar to the NK receptor gene NKR-P1 (KLRB1, CD161) and ligand LIT1/clr (found in the NKC, another paralogous region in mammals) [58,59,82]. Both the BG1 and BNK genes are highly polymorphic [38,78].

Overall, these data have been interpreted in two different ways. One view [47,58] is that the BF-BL region is the chicken MHC, since it contains the key genes found in the MHC of typical mammals along with genes found elsewhere in mammals, while the other genes of the B locus and Y locus are part of the MHC syntenic region, free to come and go in evolution, and thus keeping their names of BG, TRIM, and Y regions. The other view is that all genes in the B and Y loci are MHC genes since many of them are the homologs of genes found in the mammalian MHC, so these regions should be called MHC-B and MHC-Y [83,84]. Indeed, the view has expanded to include the other less well-characterized

regions that surround the B and Y loci, envisaging the whole of chromosome 16 as rich in potential immune genes [85].

As has been discussed over 20 years [5,47], a view of genes according to their function as the MHC leads to the BF-BL region being the chicken MHC, whereas a view of genes according to genomic location in one or another vertebrate has led to the idea of the whole chromosome being the MHC. The former view does not consider the regions outside the BF-BL region uninteresting or unimportant (far from it!), but just that they are not “the classical MHC.” If the latter view is considered appropriate, then any region with genes homologous to a gene found in the MHC of any vertebrate becomes part of the MHC: the paralogous regions in humans might be called MHC-1, MHC-6, MHC-9, and MHC-16 (based on their chromosomal location) even though they span as much as 70 Mbp each, and indeed the NKC might be called MHC-12. Perhaps taking these arguments on board, the proponents of MHC-B and MHC-Y have begun to call the BF-BL region “the core MHC” [59], the region containing classical class I and class II genes, which are discussed in the next section.

7.5 Classical and nonclassical major histocompatibility complex molecules

Most of what is known about MHC molecules was discovered in mammals, particularly humans and mice [3]. Classical class I and class II molecules generally have high levels of allelic polymorphism, high and wide tissue distribution, and bind peptides inside of cells to present to $\alpha\beta$ T cells. Nonclassical MHC molecules lack one or more of these canonical properties [13]. There are many nonclassical class I molecules, with only some encoded in the MHC, a wide range of sequence relationships to the classical molecules, and a wide range of functions from various immune responses to unrelated physiology [15,86]. There are only a few nonclassical class II molecules in typical mammals, of which DM and DO are involved in peptide loading of classical class II molecules [87].

Classical class I and class II molecules have similar extracellular domains and both present peptides on the surface of cells to $\alpha\beta$ T cells, but they differ in many important ways [10]. Classical class I molecules generally bind peptides derived from proteins located in the cytoplasm or nucleus, where viruses and a very few cytoplasmic bacteria replicate, and present these peptides to T cells bearing the CD8 coreceptor. CD8 T cells are generally cytotoxic lymphocytes (CTLs) that kill the infected cell, stopping viral replication. In contrast, classical class II molecules generally bind peptides derived from

proteins originating in intracellular vesicles that are in contact with the extracellular space and present peptides to T cells bearing the CD4 coreceptor. There are many different kinds of pathogens in the extracellular space, so depending on signals from innate immunity, different subsets of CD4 cells (such as Th1, Th2, Th17, and Treg) with appropriate kinds of responses are activated.

As mentioned earlier, MHC molecules can also be recognized by $\gamma\delta$ T cells and NK cells. Some $\gamma\delta$ T cells can recognize classical and some nonclassical class I molecules, as well as classical class II molecules [16]. NK cells bear a variety of NK receptors, some recognize the absence of class I molecules (by binding classical class I molecules and some nonclassical molecules with inhibitory receptors), and some activating receptors that sense the presence of some nonclassical class I molecules (for instance, those that are expressed after infection or stress) [88].

Both class I and class II molecules have four extracellular domains [10]: two membrane-proximal immunoglobulin-like domains with two domains on top that together form a platform of β -strands between two parallel α -helices. Class I molecules are composed of the class I heavy chain encoded in the MHC and the light chain β_2 -microglobulin (β_2m) that is encoded on a different chromosome, while class II molecules are composed of an α and a β chain typically encoded by A and B gene pairs in the MHC.

In classical MHC molecules [10], peptides can bind in the groove formed by the α -helices atop the β -sheet. Classical class I molecules bind relatively short peptides (typically 8–11mers) in which the free N- and C-termini are tightly bound by highly conserved residues, so-called anchor residues of the peptide are bound into deep pockets (typically only one or two closely related side chains from the second and last peptide position, P2 and P_c) and longer peptides bulge in the middle to be recognized by the TCR. In contrast, classical class II molecules generally bind longer peptides (15–30mers), bound along the length of a 9mer core by main chain interactions, with four or more anchor residues in less specific pockets (typically positions P1, P4, P7, and P9, each allowing four or more amino acids), and with the peptide hanging out the ends.

The bound peptides of classical class I and class II molecules differ in origin, processing and peptide-loading molecules, and recognizing immune cells [87]. Classical class I molecules bind peptides derived from proteins located in the cytoplasm or nucleus, with peptides produced by the proteasome (for which inducible components, certain PSMBs, optimize the cleavage), transported into the lumen of the endoplasmic reticulum (ER) by the TAPs, and loaded onto class I molecules with the help of the bespoke chaperone tapasin (or TAPBP), as part of the

peptide-loading complex (PLC) in the ER. The N-termini of the peptides are trimmed by the ER amino peptidase, a second step of quality control is provided by TAPBP-related protein (TAPBPR), and other more general proteins such as the oxidoreductase ERp57, the chaperones calnexin and calreticulin, and the glycosidase UGT1 are also involved.

In contrast, classical class II molecules bind peptides derived from proteins located in intracellular vesicles, which are also in contact with the extracellular space. Peptides are produced by a variety of general proteases, including cathepsins, and loaded onto the class II molecules with the aid of the bespoke chaperone DM. Invariant chain (Ii) binds nascent class II molecules in the ER, protecting the binding site and directing the class II molecules to multivesicular bodies (MVBs, also known as MIIC), where Ii is cleaved to produce class II invariant chain-associated peptide (CLIP) which can be exchanged for higher affinity peptides with the aid of DM, which itself is inhibited by DO in B cells and cortical thymic epithelial cells.

Classical and nonclassical MHC molecules are the ligands for TCRs and NK receptors, which are also quite different in organization and function. Both $\alpha\beta$ and $\gamma\delta$ T cells express receptors created by recombination and mutation in individual developing T cells in the thymus [89], resulting in TCRs with variable (V) regions that vary more-or-less randomly in sequence and therefore in molecular shape and character. Each individual will have a different set of classical MHC alleles, so $\alpha\beta$ thymocytes are positively selected by cortical epithelial cells for those clones that bind the particular MHC alleles present and then are negatively selected by medullary epithelial cells as well as by hemopoietic cells, retaining those clones that do not strongly bind the MHC alleles with self-peptides present; this central tolerance is the first of many mechanisms to limit self-reaction. In contrast, $\gamma\delta$ thymocytes develop in the thymus after activation by ligands on various cells. NK cells do not create new receptors by changes in DNA [17,88]; instead, haplotypes of NK receptor genes that vary in number, sequence, and character are inherited, but each NK cell clone expresses a different set of these receptors and is positively selected for recognition of the class I molecules present in the individual.

7.6 Chicken classical major histocompatibility complex molecules

The classical class I and class II B genes are among the candidates for the strong association of the BF-BL region with resistance and susceptibility to various infectious pathogens, including Rous sarcoma virus and MD. Extensive studies of the BF and BL genes and molecules

have given fundamental insights into the structure, function, and evolution of MHC molecules.

An early discovery about classical class I and class II molecules was that, despite there being two BF genes and two BLB genes, there is only one dominantly expressed molecule for each. The BF2 gene is at least 10 times better expressed than the BF1 gene at the level of RNA, protein, and antigenic peptide, at least partly due to differences in the promoter [42,75]. There are many BF2 alleles with widely divergent peptide-binding motifs, which are recognized by CTLs, while there are fewer BF1 molecule alleles, many of which have very similar peptide-binding sites, and which are recognized by NK cells [42,90,91]. Similarly, the BLB2 gene is highly expressed in spleen and blood compared to the BLB1 gene, but the BLB1 gene can be strongly expressed in the intestine (and may be better expressed elsewhere upon stimulation) [71,72,92].

The presence of single dominantly expressed classical MHC molecules could in principle explain the strong differential responses by the B locus, since a particular allele might find a protective peptide or not, leading to big differences between MHC haplotypes and thus strong associations with infectious disease (Fig. 7.2). In contrast, humans and other typical mammals express multigene families of well-expressed classical MHC molecules, with a good chance that one of the expressed molecules would confer protection, so that a mammalian MHC haplotype would confer more-or-less resistance to most pathogens,

which would read out as weak genetic associations (Fig. 7.2). Given the evidence for a single (or a dominantly expressed) classical class I gene in many, if not most, nonmammalian vertebrates [5,37,47], this finding suggested that outside of mammals, the MHC should determine strong associations with infectious disease. Evidence supporting this view has been found for various birds, amphibians, and fish [5,11,93,94], suggesting some change having happened on the lineage leading to (placental) mammals.

This notion that protective MHC alleles bind particular pathogen peptides found support from experiments with Rous sarcoma virus (which has only four viral genes), for which the BF2 molecules from two susceptibility haplotypes failed to bind the viral peptides tested, while a protective peptide was identified for a haplotype that confers resistance [42,43]. However, it was harder to explain why MD virus (MDV), an oncogenic herpesvirus with over 100 genes and a complex lifestyle [95,96], would not provide a protective peptide for even the most demanding MHC allele.

One potential answer to this conundrum was found by a detailed examination of chicken class I peptides and structures, which showed (Fig. 7.2) that there are BF2 alleles with very stringent peptide-binding requirements (“fastidious” from the B4/B13, B12, B15, and B19 haplotypes) that are well expressed at the cell surface, whereas other alleles with relaxed peptide-binding requirements (“promiscuous” from the B2, B14, and B21 haplotypes)

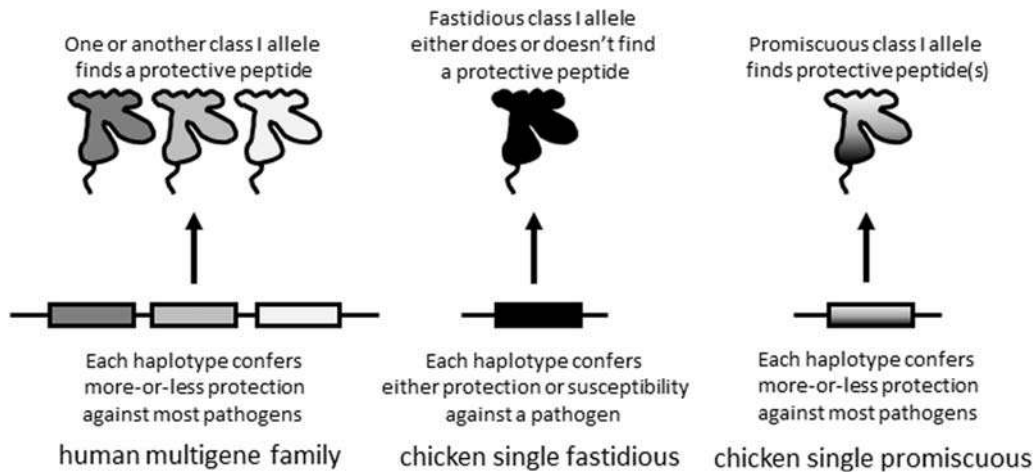


FIGURE 7.2 Relationship of class I molecules to genetic associations with resistance and susceptibility to infectious diseases. Left panel: Each of several fastidious class I molecules encoded by a human MHC haplotype has a chance to present a protective peptide, altogether conferring more-or-less resistance to most pathogens, which reads out as weak genetic associations (little difference between haplotypes). Middle panel: A single dominantly expressed class I molecule with a fastidious peptide motif may or may not present a peptide for protection from a particular pathogen, which reads out as strong genetic associations (big differences between haplotypes). Right panel: A single dominantly expressed class I molecule with a promiscuous peptide motif will have a good chance to present a protective peptide (just like a multigene family of human class I molecules), so comparison of two haplotypes with promiscuous class I molecules general will read out as a weak genetic association, whereas between haplotypes with promiscuous and fastidious class I molecules usually will read out as a strong genetic association. *Figure modified from Kaufman J. Generalists and specialists: a new view of how MHC class I molecules fight infectious pathogens. Trends Immunol 2018; 39: 367–379, available under Creative Commons CC BY license.*

are less well expressed [40–42,97,98]. These properties correlate with the reported hierarchy of MD resistance [34,99], with the poorly expressed promiscuous alleles expressed by the haplotypes that conferred resistance to MD. Moreover, several reports showed that promiscuous alleles confer stronger resistance to other chicken pathogens, such as Rous sarcoma virus (RSV), infectious bronchitis virus (IBV) and avian influenza virus (AIV) [100–102].

This hierarchy is a fundamental property of MHC molecules, which was strengthened by the findings for human class I alleles, although the extremes of the chicken molecules are more fastidious and more promiscuous than any human class I alleles [8,41]. Indeed, the typical fastidious chicken allele requires three anchor residues [42,97] rather than two by the typical human allele, while the promiscuous alleles can have broad hydrophobic pockets, remodel their binding sites, or allow the C-termini of their peptides to hang out of the groove to bind an astounding variety of peptides [40,41,103]. However, the strongest associations with infectious pathogens for humans, particularly for the progression of human immunodeficiency virus (HIV) infection to acquired immunodeficiency syndrome, were with particular fastidious alleles that bind HIV peptides that are difficult for the virus to change without loss of fitness [104–106].

This contrast between the situation in chickens and humans led to the generalist–specialist hypothesis, in which promiscuous alleles provide protection against a range of common pathogens, while fastidious alleles provide specialized protection against particular pathogens [8,42]. There are, in fact, examples of fastidious chicken alleles conferring protection to particular strains of RSV [107]. It has recently been reported that tapasin-independent HLA-B alleles (corresponding to promiscuous molecules) confer slower progression from HIV infection to AIDS, when elite controller alleles (corresponding to protective fastidious alleles) are removed from the analysis [108]. However, a bioinformatics and modeling study with predicted peptide repertoires of human class II alleles led to a different hypothesis that promiscuous molecules are selected in geographical regions with abundant and various extracellular pathogens [109].

The fact that commercial chickens have high frequencies of low-expressing BF2 molecules with promiscuous motifs (C. Tregaskes, N. Potts, R. Martin and J. Kaufman, unpublished) suggests that these alleles confer advantages in the field (as in the AIV experiments mentioned earlier [102]), but critical tests of these hypotheses are still required. The fact that many commercial flocks have relatively low diversity and that those MHC haplotypes present are mostly those with low-expression (and thus likely promiscuous) BF2 alleles may also account for the fact that many recent experiments of GWAS fail to find

significant associations with the B locus or with chromosome 16 [110–116].

Studies of the expression, peptides, and structures of chicken class II molecules are far less advanced, but already interesting results are being found. Analysis of the expression levels of cell surface for class II molecules on ex vivo cells from vaccinated and unvaccinated chickens has demonstrated an expression hierarchy for chicken class II molecules [117]. A structure for the dominantly expressed class II molecule BL2 from the B19 haplotype bound to a previously reported self-peptide looked largely as expected from mammals, with the canonical 9mer core bound along the length of the peptide [118]. However, an approach using in vitro MDV infection of bursal B cells in culture followed by immunopeptidomics has defined the peptide motifs for the BL1 and BL2 molecules of four haplotypes (B2, B15, B19, and B21) and found that the vast majority of peptides came from just a few MDV genes, with structural analysis showing that the BL2 gene from the B2 haplotype has a novel 10mer core for bound peptides [119]. The fact that these class II molecules, which are significantly more promiscuous than any class I molecule, bind so few peptides suggests that they could determine differential responses to infectious pathogens, and points to exciting research to be done.

7.7 Gene coevolution in the chicken major histocompatibility complex

It would seem counterintuitive that the presence of dominantly expressed classical class I and class II B genes means that individual chickens with some MHC haplotypes are susceptible to lethal infections, given that chickens would just need to upregulate the expression of the BF1 gene in order to be like a typical mammal, which expresses multigene families that confer more-or-less resistance to most infectious diseases. The genomic organization of the MHC in humans and chickens can explain this situation, with evidence across the nonmammalian vertebrates (and in one marsupial) being interpreted to support the organization in chickens being the primordial situation (as discussed in detail in [5,47]).

As described earlier, the human MHC has three polymorphic classical class I genes (HLA-A, B, and C) located in the class I region, while the genes that encode the inducible proteasome components (PSMBs or LMPs), the peptide transporter (TAP1 and TAP2), and the bespoke chaperone tapasin (TAPBP) are all monomorphic and located far away in the class II region. Enough recombination occurs that variants in these genes do not stay together during evolution, and so the antigen processing and peptide-loading genes have evolved to an average best fit for any class I allele that might arrive by

recombination. As an example, the monomorphic human and mouse TAP molecules translocate a wide variety of peptides, although there is some specificity, for example not pumping peptides with an acidic C-terminal residue [120].

In chickens, the two polymorphic classical class I genes (BF1 and BF2) flank the polymorphic TAP1 and TAP2 genes and are a short physical distance from the polymorphic TAPBP gene, since the class III region is outside the class I and class II regions [58]; the inducible PSMB genes have apparently been lost in chickens [121]. Moreover, the level of recombination within the BF-BL region is very low compared to the human MHC. As a result of traveling together as stable haplotypes, the TAP, TAPBP, and BF genes can coevolve, with unique combinations that are selected together for each haplotype. Thus the TAPs in a particular haplotype pump peptides that are well suited for the BF2 allele in that haplotype and, in general, would not supply peptide for a class I molecule with a different peptide-binding specificity, resulting in a single dominantly expressed class I molecule in each haplotype [47,76]. For instance, the TAP molecule from the B4 (also called B13) haplotype pumps peptides with three acidic residues (including the C-terminus), which fits no BF2 molecule examined except for the B4/B13 haplotype (Fig. 7.3) [76]. Thus it is expected that the BF1 gene is not strongly expressed (at least in part) because appropriate peptides are not pumped by most TAP alleles and, given that most BF1 alleles have very similar peptide-binding sites, these BF1 molecules must be very promiscuous to acquire at least some peptides from TAP alleles with a wide range of translocation specificities.

There is mechanistic evidence to support this concept of coevolution. In vitro assays using permeabilized cells of various haplotypes show that labeled peptides are

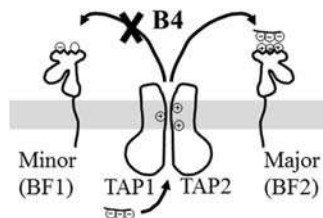


FIGURE 7.3 A single dominantly expressed class I gene can result from coevolution between TAP and BF2 genes. Only peptides with three negatively charged residues (minus signs inside circles, at positions 2, 5, and 8) are transported by the TAP from the B4 haplotype, presumably because of the three positively charged (plus signs inside circles) residues of the TAP1 molecule of the B4 haplotype. The peptides then bind to the dominantly expressed (major, BF2) class I molecule which has three positively charged (plus signs within circles) peptide-binding residues and not to the poorly expressed (minor, BF1) class I molecule which does not have such residues. *Figure modified from Kaufman J. The avian MHC. In: Schat KA., Kaspers B, Kaiser P., editors. Avian immunology. 2nd edition. Elsevier Ltd; 2013. p. 149–167.*

translocated only with the peptide motif of the BF2 molecule for that haplotype and that changes in the anchor residues affect the transport [76,122]. In chicken MHC haplotypes with fastidious alleles, the TAPs pump a more limited set of peptides than the BF2 molecule typically binds, while in haplotypes with promiscuous alleles, the TAPs pump peptides that the BF2 molecule will not bind [122]. There is also evidence that the maturation of a BF2 molecule works better in a cell of the same particular haplotype (presumably through tapasin in the PLC), although this result was not found by an in vitro assay with just tapasin and class I alleles [77].

However, some of the latest findings lead to conceptual problems with the idea that coevolution leads inexorably to a single dominantly expressed class I molecule. Although nowhere near as promiscuous as the monomorphic human TAP that pumps peptides for a multigene family of class I molecules, the B21 TAP (and by inference the TAPs of other chicken MHC haplotypes with promiscuous BF2 molecules) pumps a wide variety of peptides [122] which, in theory, should be able to supply peptides for more than one class I molecule. Experimental support for this idea comes from the fact that the expression level of a fastidious BF2 molecule from the B15 haplotype increases enormously when present as a heterozygote with the B21 haplotype, which has been interpreted as the B21 TAP supplying the B15 class I molecule with more peptides (and potentially contributing to heterozygote advantage) [122]. Thus one might expect that the presence of promiscuous TAPs should lead to multigene families of classical class I molecules, but no such MHC haplotypes in chickens have been found, for reasons that remain unclear. The fact that the classical class I genes for BF1 and BF2 are located in opposite transcriptional orientation means that extraordinary events would be required to expand the multigene family, unlike tandem class I genes in the same transcriptional orientation.

The class II system in chickens has superficial similarities with the class I system: two polymorphic class II B genes (BLB1 and BLB2) but only BLB2 widely and strongly expressed, both of which are located close to the bespoke chaperone genes (DMA, DMB1, and DMB2), which are all polymorphic [58,60,73,123]. The class II A gene (BLA) is a monomorphic DRA-like gene located 5 cM away from the BF-BL region (in a sense, like the light chain of class I molecules, β_2m , which is also not closely linked to the MHC) [124], while the invariant chain (Ii or CD74) gene is located on another chromosome [125]. Interestingly, both the class II B and chaperone genes are differentially expressed [92,126]: BLB2 and DMB2 are well expressed throughout the chicken, while BLB1 and DMB1 can be well expressed in the intestine, for reasons that are currently unknown. It would

be tempting to speculate that the DM1 heterodimer (DM α -DM β 1) is specific for the class II heterodimer BL1 (BL α -BL β 1), while the DM2 heterodimer is specific for BL2, but the evidence in mammals [127] is that most of the interactions are between the DM α and class II α chains, so the matter is yet to be understood. A variable number of apparently nonpolymorphic class II B genes are found in the Y region [57,128,129]; although very little is known, one possibility is that one or more may act like DO in mammals.

7.8 Other chicken genes important for the major histocompatibility complex

Although classical class I and class II genes are the key genes defining the MHC, other genes in B locus and elsewhere, some of them being highly polymorphic, could be contributing to various traits, including resistance to infectious disease [130].

A C4 gene was found in the BF-BL region by sequencing [58], but analysis of the C4 protein in the blood revealed that there were two C4 genes (Y. Palarasah, J. Kaufman and K. Skjød, unpublished). The sequence of the blood C4 protein was used to clone the cDNA and gene, which were then used to map the gene to chromosome 1. The extensive differences between the two genes included the single difference (histidine in MHC C4 and aspartic acid in non-MHC C4) responsible for the C4 reactivity to form covalent bonds with hydroxyl groups versus amino groups. This difference in specificity is found for the duplicated C4A and C4B genes found in humans and typical mammals, which differ in very few other amino acid positions. Analysis of the genomic regions surrounding the genes for chicken blood and MHC C4 molecules found comparable C4 genes in syntenic regions down to fish, although not in mammals. This led to a hypothesis that these two kinds of C4 genes were in fact very ancient, but that the C4 gene outside of the MHC had been lost in the lineage leading to mammals, which then had duplicated the single C4 gene in the MHC and then the two copies diverged to restore both functions. As work continued to understand the low expression of the MHC C4 gene in chickens, a bioinformatics analysis was published [131] which came to the same hypothesis about the evolutionary history of C4 genes. Currently, the reason for the differential expression between the two C4 genes and the possibility of polymorphism in either gene remains to be determined.

The genes for nonclassical CD1 molecules are found in an MHC-paralogous region in mammals (e.g., human chromosome 1), but at the edge of the BF-BL region in chickens [50,79–81]. The number of different CD1

isotypes (each from a different gene), the lipids that they bind and the cells that recognize them vary among mammals [132–135]. The two chicken CD1 genes are monomorphic as in mammals, but neither of them is closely related to any mammalian isotype, either in sequence [79–81] or in the structure of the lipid-binding sites, one of which is particularly simple [136,137]. Nothing is yet known about the cells that recognize these CD1 molecules nor is anything known about their roles in any disease (although in humans they are typically involved in the response to mycobacterial infections). The evidence that led to the presence of CD1 genes in the chicken MHC has been interpreted in different ways [50].

The nonclassical class I genes encoding YF molecules are all located in the Y region (also called the Rfp-Y or the MHC-Y region), but the number of expressed genes may vary from one to several dozen [57,138,139]. The one YF gene characterized in detail is apparently polymorphic, and both sequence and structure suggest that the binding site is hydrophobic [138,140]. The nature of the presented antigen, the cells that recognize the YF molecule(s), and the role of the Y region in infectious disease remain controversial [141–143], although there seems to be little doubt that YF genes (or at least some molecules encoded by the Y region) are recognized by immune cells [144,145] and a newly developed typing assay allows disease associations to be more easily discovered [63].

A single polymorphic BG1 gene is found at one edge of the BF-BL region, along with a single monomorphic BG0 gene on chromosome 2 and a multigene family of BG genes in the BG region [37,58–61,78,146]. Although the red blood cell BG antigens were largely responsible for the discovery of the B locus, they are expressed on a variety of cell types, with two broad categories of hemopoietic and tissue (probably mostly epithelial) genes determined at least in part by the promoters [53,55,61]. A conceptual concern is that the BG genes in the BG region are all arranged in tandem and in the same orientation, so that unequal crossing-over and deletion can lead to copy number variation (CNV) and can mix the tissue distribution conferred by the 5' end with functional features of the 3' end.

BG molecules are disulfide-linked dimeric proteins (either homo- or heterodimers) without glycosylation [54,61,147,148]. The extracellular V-like domain is similar to the large family of B7 costimulatory molecules, most closely related to mammalian butyrophilins [149], which are involved in negative costimulation of $\alpha\beta$ T cells and in determining the location in tissues as well as antigen recognition of $\gamma\delta$ T cells [150–152]. Despite the extracellular region being polymorphic, all the evidence to date suggests that it is differences in the cytoplasmic tails (which are generally long α -helical coiled coils) that are functionally important by sequence comparisons

between BG1 alleles and inferred alleles in the BG region [61,78,153].

There is no evidence reported for disease associations with the genes in the BG region, but one relatively conserved gene found in several haplotypes is the origin of a coiled coil that in intestinal epithelial cells displaces tropomyosin to regulate actin–myosin interaction [61,154,155]. However, there is genetic evidence to implicate the BG1 gene in response to Rous sarcomas and MD, specifically the cytoplasmic tail that in many BF-BL haplotypes has an immune-tyrosine inhibitory motif (ITIM) for signaling [36]. Given that many butyrophilins (and the two butyrophilin genes in chicken) have a 30.2 domain (also known as a PRY-SPRY domain) at their C-termini [151,156], it seems likely that various cytoplasmic proteins use the cytoplasmic tails of some BG genes as scaffolds.

Next to the BG1 gene at the edge of the BF-BL region is a pair of genes: the highly polymorphic BNK (later also called Blec2) and nearly monomorphic Blec (later called Blec1), most similar to the NK receptor gene NKPR1 (also known as NK1.1, KLRB1, and CD161) and its ligand (known as LLTI in humans, C1r in rodents and CLEC2D more generally) [38,58,82]. However, a BNK allele failed to engage with the Blec or class I molecules and instead responded to a trypsin-sensitive molecule on spleen cells [157]. One explanation is that each BNK allele reacts with one or more of the molecules encoded by genes similar to Blec that are found scattered in the BG region and the Y region [61,158].

Other candidates for NK receptors in chickens are chicken immunoglobulin-like receptors (ChIRs), almost all of which are located on a single microchromosome, chromosome 31 [159]. The mammalian equivalent, the leukocyte receptor complex (LRC), contains many genes of the same family: killer immunoglobulin-like receptor (KIR) genes expressed on NK cells and some nonconventional T cells, leukocyte immunoglobulin-like receptor (LILR) genes expressed on myeloid cells, and one receptor for antibodies (Fc α / μ R, or CD351). Some of these genes have ITIMs for inhibition, while others have a positively charged residue in the transmembrane region to interact with activating proteins bearing immune-tyrosine-activating motifs (ITAMs), and a few have both [160]. Similarly, the ChIR-A have positively charged residues in the transmembrane region, ChIR-B have ITIMs, and ChIR-AB have both. Many of the ChIR-AB molecules act as Fc receptors, binding the chicken antibody IgY [159]. It is expected that some or all of the ChIR-A and ChIR-B molecules are NK receptors, which is important to learn, since in mammals interactions between NK cells and class I molecules have enormous effects on immunity, autoimmunity, and reproduction [17]. Being able to type the NK microchromosome is the first step to test for such

potential epistasis [161]. The only other NK locus in mammals, the NKC, has only two lectin-like genes in chickens, one of which is like mammalian CD94 and the other encoding a molecule expressed on thrombocytes [162,163].

In between the BG1 gene and the BG region are various genes, most of which are part of the TRIM family [49,59]. TRIM genes are found scattered throughout mammalian genomes, with some coming and going from around the MHC of mammals [164]. TRIM genes have been implicated in a variety of functions, including as a restriction factor for HIV (TRIM5 α), an intracellular Fc receptor targeting proteins to the proteasome for degradation (TRIM21) and as regulators of cell-autonomous immunity [165–167]. So far, there is little known about any of these chicken genes, but it is likely that some of them will be involved in disease resistance [130].

7.9 Polymorphism and typing chicken major histocompatibility complex genes

Almost everything known about the biology of the B locus comes from a very few haplotypes originally discovered by serology, which mostly reflected the BG genes of the BG region, using birds related to commercial egg-laying poultry. The latest comprehensive analysis lists “standard B locus haplotypes” up to B29, although the chicken lines with some B haplotypes are no longer available [83]. Many different methods have been used to type the B locus and B locus genes, including restriction fragment length polymorphism and microsatellites [168]. Most recently, the B locus has been analyzed in many chicken populations using SNPs, showing a large number of haplotypes, lately described as BSNP haplotypes to differentiate them from haplotypes based originally on serology [169–173]. These haplotypes show many more differences in the TRIM region than in the BF-BL region, while the polymorphisms in BG genes have been difficult to understand [67], presumably because of CNV [61].

The analysis of sequence polymorphisms within B locus genes and cDNAs also was originally restricted to the standard haplotypes from egg-laying chickens [38,42,60,61,71–78,153,174], although extensive work with classical class I and class II B genes was carried out for some commercial broilers [175–178], Brazilian chickens [179], and red junglefowl [180]. Typing by reference strand-mediated conformation analysis (RSCA) followed by cloning and sequencing [180,181] has found new haplotypes and has shown that BLB1 and BLB2 genes can exchange exons in red junglefowl. Sequences over larger parts of the B locus have also been done [58–60,182] but are not yet practical for large numbers of haplotypes.

More recently, a polymerase chain reaction amplification followed by next-generation sequencing by MiSeq

(PCR-NGS) was developed, taking advantage of the short introns between exons 2 and 3 in chicken class I and class II B genes, and using a double barcoding system which allowed 1200 birds to be analyzed for BLB1, BLB2, BF1, and BF2 simultaneously. With a bespoke bioinformatics pipeline, this typing system was used to examine commercial broilers and egg-layers, fancy breeds, and African village chickens, discovering nearly 500 new BF and BLB alleles grouped in more than 200 so-called Bfbl haplotypes (C. Tregaskes, R. Martin and J. Kaufman, unpublished). An RSCA method has been developed for some genes in the ChIR region [161] and could easily be extended to BG genes. A short tandem repeat protocol for counting the upstream regions of YF genes has recently been described [63]. All of these methods may allow easier assignment of haplotypes and genes to particular traits, including disease resistance. However, the use of nomenclature based on haplotypes, last considered years ago [83], gives identical sequences different names, so newer versions are being considered [68].

7.10 Avian major histocompatibility complexes

The domestic chicken is only 1 of more than 9000 highly diverse species of birds [183]. The most ancient group comprises the flightless ratites (such as ostriches, rheas, cassowaries, and the like), with the galliform birds (such as chickens, turkeys, pheasants, quails, and the like) and the anseriform birds (waterbirds such as ducks, geese, and swans) considered early branches. Roughly half of bird species are passerines (songbirds and perching birds), with the remainder ranging from hummingbirds to parrots, eagles, and many more. The work on the MHC of turkeys and to a lesser extent other galliforms arises mainly for agriculture and sport, but the work on the MHC of other avian species has mostly been done by evolutionary biologists to understand the population biology and evolutionary ecology of diversity in wild populations.

The properties of the chicken MHC extend to the galliforms as far as has been examined. Turkeys have an MHC by emblematic functional assays [184], with an organization of the equivalent of the B locus much like chicken, including TRIM and BG genes [185–190], with expression of most genes [191] and with variation in class I and class II B genes [192–194]. Class I and II genes that segregate independently of the MHC are likely to be turkey equivalents of the Y locus, organized into two independently segregating regions [188,195]. Pheasants and grouse also have equivalents of both the B and Y locus, both with few genes [196–199] and with typing and sequencing being interpreted as different evolutionary histories for class I and class II B genes [199–201].

Evidence for BG genes was reported for pheasants [196]. Prairie chickens also have a compact B locus, but it includes a class II A gene, unlike in chickens where BLA is located 5 cM away from the BF-BL region [124,202], again with analysis of variation suggesting differences in selection between class I and class II B loci [203–205] and with loss in bottlenecked populations due to drift and loss of CNV [206]. The quail has the same kinds of genes as the chicken BF-BL region, but with several rounds of duplication of gene cassettes leading to more genes, with CNV for class II B genes [207–210]. Careful analysis of expression shows that there is a single dominantly expressed class I gene and a single dominantly expressed class II B gene, just as in chickens [210,211].

There is much less information for birds outside of galliforms, but there are hints that at least some properties of the chicken MHC apply, while others are different. Ducks have polymorphic TAP1 and TAP2 genes (but no tapasin gene) adjacent to five class I genes, of which the nearest is a classical gene always expressed at a high level and with high polymorphism (and with one structure determined), one classical gene is expressed well upon infection due to microRNA regulation, and three are non-classical [212–217]. There is a single classical class I gene reported for zebra finch (with many class II B genes, some of which are linked to the class I gene, but most interestingly the TAP genes are apparently on a different chromosome), a single class I gene for the Humboldt penguin, and, despite a large number of class I genes, a single dominantly expressed classical class I gene for three species of sparrows (Fig. 7.4) and for red-billed gulls

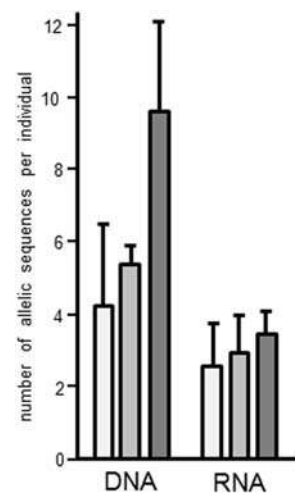


FIGURE 7.4 Three species of sparrows express only two to four transcripts from only two classical class I loci, despite having (many) more genes with apparently classical sequences. Figure modified from Drews A, Strandh M, Rberg L, Westerdahl H. Expression and phylogenetic analyses reveal paralogous lineages of putatively classical and non-classical MHC-I genes in three sparrow species (Passer). *BMC Evol Biol* 2017; 17: 152, available under Creative Commons CC BY license.

[218–222]. Similarly, there are few class II B genes in parrots, owls, raptors of many kinds, rails, coots, cranes as well as herons, egrets, and bitterns [223–234]. Five class II B genes, of which three are transcribed, were reported for the ratite named the little spotted kiwi [235], and up to four class II A/B gene pairs were reported for the Japanese crested ibis [236,237].

However, other species vary over a huge range in the number of class I and class II genes, often with CNV, and with evidence that different avian lineages have expanded one or the other [238–248]. Moreover, correlations with life history traits such as life span and migratory behavior have been reported, including the intriguing correlation between rainfall and number of class I alleles [247–251]. The complex nature of class II B genes is reported in the very basal passerines [252]. In contrast, helminth richness was correlated with expansion of class II B genes in non-passerines [253].

There can be many nonclassical class I genes in some birds, with clear separation into lineages in sparrows and blue tits considered by some to be reminiscent of B and Y regions in chickens [254–256], and with different functions inferred [245]. The presence of many class II B genes, such as in zebra finch [218], may also mean that there are nonclassical class II genes, with those described in the Hawaiian honeycreeper not the same radiation as the YLB genes in galliforms [257,258]. This difference between the nonclassical MHC molecules in galliforms and passerines fits the independent appearance and expansion of different nonclassical class I genes across all vertebrates [259]. However, it may be that different genes are the origin of even the classical MHC molecules in some avian species [260], in line with the “birth and death” model for mammalian class I genes [261].

The number of MHC alleles (generally amplifying and counting only exon 2 or exon 3 for class I genes, and exon 2 for class II genes) in these wild species is generally very high [220,233,238,240,241,254,262–269], even in inbred or bottlenecked species [270,271]. Even in island populations with low initial diversity, gene conversion can relatively rapidly restore diversity [272]. However, exceptions have been reported, such as falcons that have low intra- and interspecific variability despite having widespread distribution and despite the basal group of kestrels having high variability [273]. Also the endangered Humboldt penguin showed high levels of homozygosity for a single class I gene at the same time as significant diversity for a class II B gene [221,274].

The proposal based on galliform birds (mentioned earlier) that the selection pressures on class I and class II genes are different has also been reported for barn owls [275] and generalized to all birds, with the suggestion that passerines have stronger selection on class I genes, while other birds have stronger selection on class II genes

[276]. Moreover, the level of nucleotide divergence for class I genes in blue tits may be low, despite having a similar number of loci as other passerines [255].

At least some of the wider aspects of the B locus appear to be conserved in other birds, based on the genetic linkage of class I and BG genes in sandhill cranes [277] along with the presence of class II B genes in 16 species of cranes and related species [232]. In contrast, genome sequencing of the crested ibis found one contig as the TRIM region (described as a BG region); a second contig with a Col11A2 gene followed by one to four class II A/B gene pairs, then BRD2, DMA, DMB1, and DMB2, and then a single class I gene followed by TAP1, TAP2, and LTB4R1; and a third contig with tenascin and several class I genes [236,237]. These data were interpreted to suggest that the ibis MHC is closely related to the mammalian MHC, but the middle contig is organized like the BF-BL region of the chicken (except for the presence of a Col11A2 gene and multiple class II A/B gene pairs, and the lack of a tapasin gene and an obvious BF2 gene), and since the major contigs are not joined, the final contig might in fact represent the equivalent of a Y region. However, a gene order of Col11A2, two class II A–B gene pairs, BRD2, DMA, DMB1 and DMB2, class I, and TAP genes but then with several class I pseudogenes followed by a tenascin gene was reported for the Oriental stork, similar to the ibis and organized with some similarities to the chicken BF-BL region [278].

How can this diversity at all levels in so many different kinds of studies and in such a variety of species ever hope to be understood to formulate general principles? It seems likely from the existing information, albeit fragmented and incomplete, that the original birds had few classical and nonclassical MHC genes (Fig. 7.5), based on the fact that most bird species have low numbers [247]. It seems possible that a single classical class I gene or a single dominantly expressed classical class I gene (with less well-expressed classical-like genes, or ones that are inducible upon stimulation) may be widespread among the birds (although at least one exception has been forcibly argued [246]), and the same may be true for the class II system. The reason for such a focus on one locus may be due to gene coevolution as found in chickens, but perhaps it could be for other reasons, based on the separate genomic locations of class I and TAP genes in zebra finch and the apparent lack of a tapasin gene in ducks. Finally, it seems likely that nonclassical class I and/or class II genes expanded independently in a few avian lineages (but with large numbers of species) (Fig. 7.5) and that the functions of such genes will be of enormous interest. An excellent review summarizing aspects of this literature from the viewpoint of avian MHC evolutionary biologists recently has become available [279].

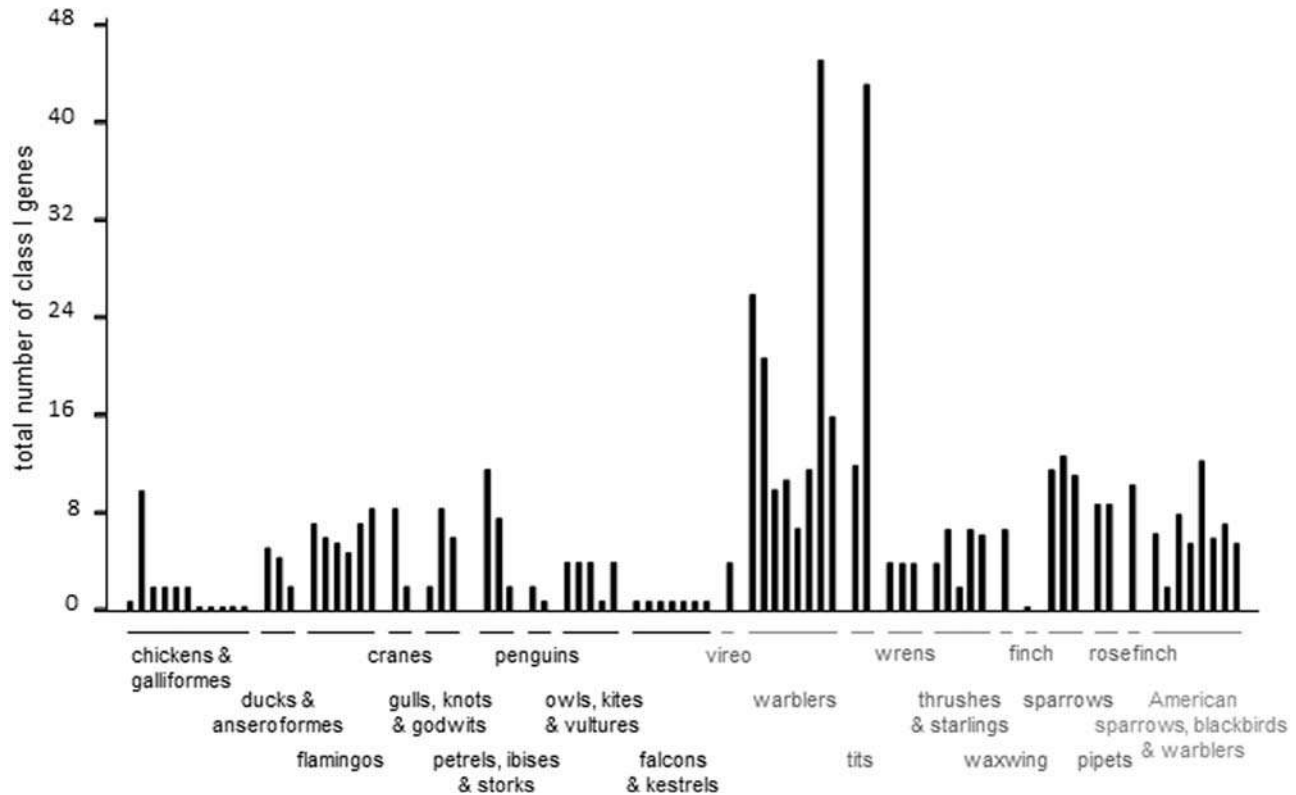


FIGURE 7.5 Most bird lineages have few class I genes, with large expansions in just a few clades. Bars indicate estimated total number of gene copies from extant species; passerine species in gray. Figure based on data from Minias P, Pikus E, Whittingham LA, Dunn PO. Evolution of copy number at the MHC varies across the avian tree of life. *Genome Biol Evol* 2019; 11: 17–28.

7.11 Immunity, disease resistance, and the major histocompatibility complex in wild birds

Given the importance of the MHC for immune responses in biomedicine and the strong associations of the chicken B locus with infectious disease, it was natural for evolutionary biologists to focus on immunity in wild bird populations, in particular asking population biology questions that cannot easily be approached in chickens. However, direct comparisons with results in chickens are difficult since evolutionary biologists have focused on eukaryotic parasites big enough to see easily (particularly avian malaria), whereas there are relatively few studies of parasite infections in chickens that until recently were mostly kept on concrete floors or in wire cages. Many studies of immunity have used various wild and captive populations of house sparrows, with varying numbers of reports for several warbler and tit species, as well a few other birds. Some studies have made use of MHC supertypes, in which alleles with similar amino acids in the peptide-binding site are grouped together [280,281], although it is now clear that different alleles within a supertype can differ enormously in peptide repertoire [282]. It should also

be stressed that functional associations with the alleles of a particular MHC gene do not necessarily imply causation, since the alleles of one gene may act as a marker for alleles of a nearby gene actually responsible for the trait.

One approach has been to look for evidence of selection at the population level. Selection of MHC alleles was suggested by much greater variation from year to year in frequencies of class I alleles compared to presumed neutral microsatellites in wild populations of great reed warblers [283]. One particular class I allele was associated with reduced nestling survival and smaller size while another was associated with larger size in an aviary population of house sparrows [284]. Over a 10-year study of Seychelles warblers, class I diversity correlated with juvenile survival, with one particular allele conferring a five-fold increase in life span [285]. In a wild great tit population, one class I supertype conferred higher survival rates, another conferred higher lifetime reproductive success, and a third conferred lower lifetime reproductive success, and with no evidence found for advantage of supertype diversity [286]. In the absence of disassortative mating, significant associations between class II B genes and fitness in female Magellanic penguins were interpreted to reflect differential pathogen resistance [287].

Variation at the MHC was associated with survival of the endangered prairie chickens after release [205]. Similarly, heterozygosity of class II B genes in the endangered Egyptian vulture was correlated with breeding success [288]. However, no evidence was found for an association of lifetime reproductive success with class II B genes in collared flycatchers [242] or for survival with class I genotype or diversity in the critically endangered Raso lark [289].

Sparrows immunized with several defined but complex antigens showed complicated effects associated with the MHC, with some effect of diversity of MHC class I alleles on response to a Newcastle disease viral vaccine [290–293]. Several studies showed local adaptation of sparrow populations with response to malarial parasites [265,294], with specific MHC class I alleles linked to either resistance or susceptibility of infection depending on the population [294,295], susceptibility explained by increased risk of infection with one malarial parasite coupled with decreased risk with a different malarial parasite in a particular population [296], and MHC class I diversity correlated with diversity of parasite strains [297].

A long-term study with great reed warblers found reduced infection by one malarial strain with increased heterozygosity of MHC class I alleles, as well as with one particular allele that was shown to affect the intensity of infection (quantitative resistance) [298,299]. Sedge warblers with greater numbers of MHC class I alleles (as supertypes) were found to have higher resistance to two strains of malarial parasite, with variation in MHC allele frequency between years correlated with number of infected carriers [300]. A particular MHC class II allele was associated with resistance to malaria infection in another warbler, the common yellowthroat [301], but numbers of malarial parasites correlated with the number of class I and class II alleles differently, depending on which population was being assessed [241,301–303].

A single class I allele was associated with quantitative resistance (as measured by intensity of infection) to a malarial parasite in blue tits but not with qualitative resistance (infection per se) [304], while in a wild population of great tits, one class I supertype conferred qualitative resistance to one malarial species while a different supertype conferred quantitative resistance to another malarial species [305]. A single class I allele in Berthelot's pipit was associated with malaria infection risk and was positively correlated with distance to the nearest poultry farm, a determinant of disease distribution in the study population, while another allele was negatively correlated [306].

Most of these studies with avian malaria involve typing for class I genes, but three studies have considered class II B genes. In addition to the class II allele associated with resistance in the common yellowthroat [301] mentioned earlier, greater numbers of class II B genes

correlated with lower prevalence of malarial parasites in collared flycatchers [242], and CNV of class II genes in nonpasserines was reported to be correlated with total helminth richness [253].

Only a few studies with microbes outside of malaria have been reported. A particular MHC haplotype was shown to confer recessive susceptibility to coccidiosis from *Eimeria* (an apicomplexan parasite like malaria) in an outbreak in a red junglefowl flock [307]. Plumage microbiota were reported to be less diverse in blue petrels with higher class II B diversity, with reduction of a symbiont of ectoparasites [308]. Such a finding might also explain the effect of the chicken MHC reported on an ectoparasite, the northern fowl mite [309], a finding otherwise difficult to understand. No correlation was found with MHC in an island population of parakeets devastated by the viral pathogen that causes beak and feather disease [310].

Despite the lack of access to original raw data and the general lack of follow-up that is characteristic of the evolutionary biology literature, overall these many reports agree that the MHC is involved in immunity and disease resistance, with evidence for both diversity and particular alleles being important. Whether the genes used to type the MHC are involved in these phenomena or simply act as markers for linked genes remains to be determined. Alleles of the single dominantly expressed classical class I molecule in sparrows may be responsible for the differential resistance of individual sparrows to avian malaria, but it is also possible that other class I, class II, or other genes in the MHC contribute to that. Moreover, the relative contribution of the MHC compared to genes in the rest of the genome also needs to be assessed. Study of human malaria over decades has found that immunity is complex [311]; in-depth studies of the avian malaria would be very helpful, along with more investigation of viral and bacterial pathogens.

7.12 Sexual selection and the major histocompatibility complex in wild birds

Given the long history of experiments suggesting that certain mammals choose mates dependent on olfactory cues determined by the MHC [20], various birds have been examined for reproductive behavior correlated with the MHC. An additional impetus was the observation that certain birds might use visual or auditory cues for territorial mating and reproductive success [312,313], properties which could be linked to MHC variation. Questions included whether the mate choice was to avoid inbreeding or to choose good genes, and whether such choice could be for maximal or optimally dissimilar MHC genes, or similar genes if they were beneficial.

One of the first reports claimed that MHC alleles correlated with spur length in male pheasants, a property known to be important for male viability, female mate choice, and offspring survival rate, which was interpreted to support the Hamilton and Zuk model that females discriminate secondary sexual characteristics on males to acquire good MHC genes for disease resistance and thus pass on disease resistance to their offspring [314]. Since then, the evidence in other birds (as in other vertebrates) has been variable and confused.

The most research has been carried out with various warblers. An initial study with great reed warblers found no evidence for MHC-based female mating preferences [315]. However, a 20-year study with more sophisticated tools found that, despite no difference in mean numbers, males with more class I alleles and females with fewer class I alleles were most successful in recruiting offspring, which was interpreted as an unresolved genetic sexual conflict [316]. Female sedge warblers were reported not to choose their pair male based on different class I alleles or on number of class I alleles, even in recently transplanted birds on islands with fewer constraints on resources, but to be more likely to have extra-pair males with higher numbers if the pair male had fewer class I alleles [317,318]. In contrast, a series of studies with common yellowthroats found that number of class II B alleles correlated with different sexual ornaments in two different populations, with females preferring males with larger black facial masks in Wisconsin but brighter yellow bibs in New York, but with the black facial masks being a target of male–male competition in New York. In the Wisconsin population, the number and similarity of class II B alleles between females, pair males, and extra-pair males did not determine the presence of offspring from extra-pair males [241,301,302].

Sparrows with similar numbers of MHC alleles formed mating pairs but were not more closely related by MHC allele sequences than by microsatellites; males with few MHC alleles or with no MHC allele in common with a female failed to form a pair [319]. In an island population of sparrows, females tended to mate with males that were similar in both MHC and microsatellites, and with even more similar extra-pair males, giving rise to less genetically diverse offspring than expected from random mating [320].

No evidence for MHC association with either mate selection or reproductive success was found for a wild population of great tits [321]. In contrast, male Leach's storm petrels chose females that differ at the class II B locus; heterozygote females were more successful in reproduction, with one class II B allele being disadvantageous in homozygotes [322]. In the crested ibis, females preferred MHC-heterozygous males that have darker nuptial plumage, while males preferred females that lack a

particular class II B allele which is disadvantageous for body mass [323]. Blue petrels mate for life, reciprocally choosing mates more dissimilar in class II than in class I alleles [324], apparently through odors, perhaps by influencing the feather microbiota [308]. Unexpectedly, in experimental tests, males approached odors of more dissimilar females, but females behaved in the opposite way [325]. Apparently sounds have not been so widely studied in this regard, but collared flycatchers with a disadvantageous class II B allele have birdsong with broader frequency range [326].

A cryptic mating choice was found for red junglefowl, with no mating preference based on the MHC but with males investing more sperm in MHC-dissimilar females, and females retaining sperm from MHC-dissimilar males, the latter associated with the minor classical class I locus BF1 in natural but not artificial mating [327,328].

Overall, there seems to be much variation in the associations between reproduction and MHC in birds, as has been concluded for vertebrates in general [21]. It seems most likely that mate choice is a part of the wider conflict between males and females over who controls the outcome of reproduction and therefore is likely to be under strong but idiosyncratic selection, potentially differing markedly in even relatively closely related species. The facts that cryptic sexual selection exists in red junglefowl [327,328] and that the classical class I molecule HLA-C in humans (functioning as an NK ligand) has such a profound effect on optimal placentation in humans, perhaps under opposing selection pressures by immunity and reproduction [17], are object lessons against overly simplified expectations.

7.13 Origin and evolution of the immune system

As extensively reviewed [5–7], results from investigating the chicken MHC have led to several insights relevant to studies of the origin and evolution of the adaptive and innate immune systems. The presence of NK receptor/ligand and CD1 genes in the chicken MHC suggested that ligands (such as MHC molecules), supporting genes (such as TAPs and tapasin), and receptors (including NK receptors, TCRs, and perhaps antibodies) originated in the same genetic locus, the primordial MHC. The concept of gene coevolution between chicken class I, TAP, and tapasin genes suggested a reason why the disparate genes of the primordial MHC had to coevolve in order to become a biological pathway, although once established the genes could relocate to other genomic regions unless there was a strong reason for them to stay together (such as the coevolution of polymorphism between TAP and BF2 genes). The locations of chicken class II B, TAP, class I,

and C4 genes suggested that the class III region was originally outside of the class I and class II regions. The similar organizations found for the MHCs of the frog *Xenopus* and the marsupial American opossum suggested an inversion in the lineage leading to placental mammals that led to the class III region separating the TAP and tapasin genes from the class I genes, breaking the coevolutionary relationships and resulting in monomorphic “average best fits” in mammalian peptide-loading genes.

More recently, new insights have arisen from the discovery of a hierarchy of chicken class I alleles from low-expressing promiscuous molecules to high-expressing fastidious molecules, with the promiscuous class I molecules served by promiscuous TAP molecules and the fastidious class I molecules served by fastidious TAP molecules [8,40–42,122]. The existence of a similar hierarchy of human class I molecules suggests that at least some of these phenomena are general [8,41].

Similarly, position 86 of human classical class molecules is a tyrosine that ensures that peptide C-termini are tightly bound, whereas the equivalent position in human class II molecules allows the C-terminus of the peptide to hang out of the peptide-binding groove. In contrast to mammals, chicken classical class I molecules were discovered to have an arginine in this position, in common with all other nonmammalian vertebrates, that allows peptides to hang out of the groove at the C-terminus [103,329,330].

These two sets of discoveries, that chicken class I molecules can be promiscuous and that they can allow peptides to hang out of the groove, bring the properties of chicken class I molecules much closer to the properties of mammalian class II molecules. Given that class I and class II molecules were predicted long ago and then found to be similar in structure, and were predicted to have a common origin, these results could be interpreted as support for the model that class II molecules are primordial [5,329,331], a model for which there is increasing evidence [332].

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Introduction to the avian innate immune system; properties, effects, and integration with other parts of the immune system

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Traditionally, the innate immune system was viewed as a “nonspecific” scavenger system that fights invading pathogens immediately after they enter the body and was defined by phagocytosis and lytic functions. However, this view has changed to include specificity (pattern recognition), first line of defense, wound healing, and immune modulation, with greater recognition of the interplay between different parts of the immune system. The innate immune system involves a range of soluble and cell-associated cascades and many of the processes are evolutionarily ancient, emerging in some of the first multicellular organisms. This chapter will consider different sections of the innate immune system, including soluble factors as well as those associated with various cells of the innate immune system. However, it is important to consider the relationship between the molecules and processes described in each of the subchapters as well as the fundamental role for innate immune mechanisms in stimulating the adaptive immune response. Put simply, without innate immune processes, infections would rapidly overwhelm the host and adaptive immune processes would not be initiated. Hence, innate immunity is much more than a first line of defense, and we hope this is well illustrated by the following sections. Many of the processes and cell types associated with innate immunity are present in a wide array of organisms and a great deal is similar to that which might be described in biomedical texts. However, there are key differences between birds and mammals and the following sections will illustrate some of these. As with many other aspects of the avian

immunology, most research has been carried out with the chicken and although less is known about the innate systems of other avian species, some of these can be key in determining the resistance or susceptibility of different taxa to infections such as avian influenza.

Innate immunity is expressed in a variety of guises, from the initial response to physical and chemical attacks by invading microorganisms to the coordinated recruitment and actions of a series of specialized cell populations. Broadly speaking, there are innate mechanisms that operate as a constitutive barrier function, including the nature of epithelial surfaces and the molecules that coat them, such as mucus and antimicrobial chemical components (e.g., lysozyme). There are also inducible biochemical responses, including alterations in mucus composition and increased secretion of constitutive components as well as other antimicrobial compounds (e.g., defensins). The complement system represents an inducible cascade that liberates both antimicrobial elements (e.g., the C9 membrane attack complex) and elements that attract and modulate the cellular immune system (e.g., C3a and C5a). The cellular components of the innate immune system range from the action of specialized epithelial cells to the activity of more classically defined immune cell populations, such as macrophages, granulocytes (polymorphonuclear cells), thrombocytes, and natural killer (NK) cell populations.

Intact physical barriers protect the host against pathogen colonization and invasion. This is best illustrated after their breakdown, which can result in lesions in the skin,

the airways, and other mucosal surfaces with increased risk of opportunistic infection. Cannibalism, observed in all kinds of rearing systems, is a very obvious prelude to infections. Ectoparasites such as the red dust mite or infections with helminths can also break down the natural barriers. Although not of host origin, the normal flora present on body surfaces helps to prevent colonization by pathogens. This is the basis for prophylaxis designated as “probiotics” which is much used by the poultry industry and many of these do not just work by competitive exclusion, they enhance the immune system. Other barrier mechanisms such as ciliary movement in the airways, fatty acids on the skin, peristaltic movement of the intestine, the gastric acidic pH, and secretion of mucus and antimicrobial peptides cumulatively serve as highly effective barriers to infection. However, when these barriers are breached, the host needs to rapidly respond to such challenges by triggering a diverse array of immunological cascades. Some of these responses can lead to changes in barrier function, including enhanced production of antimicrobial compounds and changes in mucus structure at the body surfaces. The ability to change these barrier functions is clearly a response to infection.

Perhaps one of the most important questions is how the diverse sets of immune responses are triggered and the answer to this “when to fire” question is remarkably simple. The body contains a group of sensors (receptors) that detect molecules associated with microbes or damaged self (pathology). The concept of pattern recognition and the recognition of conserved structures from pathogens was first proposed by Charles Janeway in 1988 and this triggering system is key to the initiation of most innate immune processes. Collectively these receptors are called pattern recognition receptors (PRR) and [Chapter 8.6](#) considers the biology of avian PRRs. However, in this general introduction, it is important to indicate that these PRRs are present in secretions, tissue fluids, as well as at the cell surface and intracellularly. The PRRs offer specificity to the innate immune system and integration of signals from diverse PRRs initiates and guides all downstream immune responses. The PRRs are evolutionarily ancient and most organisms contain between 100 and 200 PRR genes within the genome.

Classically, innate responses are considered important in the earliest phases of microbial invasion, limiting the spread of the pathogen until adaptive responses, mediated by B and T cells, become mobilized to clear the infection. In this sense, one should consider why innate responses are more rapid than adaptive responses; the nature of the pathogen recognition elements is fundamental to this distinction. Innate pathogen recognition is mediated by the PRRs that are encoded in the germline DNA. This contrasts with the receptors on B and T cells [B-cell receptor (BCR) and T-cell receptor (TCR), respectively] which are

formed by rearrangement of receptor gene elements, a process which provides an immense diversity of subtly different receptors. As a consequence, innate receptors have less diverse specificity for invading microorganism but are more abundant throughout the body than each adaptive receptor. For example, in the cellular innate immune system, 100 or so different PRRs are expressed on a lot of different cells (i.e., high frequency), with specific patterns of distribution according to cell type. Hence, each PRR is usually expressed on millions of cells. In contrast, any single TCR or BCR is found on a very small population of cells derived from a specific clone which was selected during B- or T-cell development (see [Chapters 4–6](#)). With T cells, the number of clones in the naïve (unexposed) population has been estimated in the region of 10–100 cells in an overall pool of about 100 million. Many of the characteristics that define innate and adaptive immunity are a consequence of the diversity and frequency of receptors used to sense the invading pathogens. Where the frequency of the pathogen detection receptor is high, the response can be very rapid but the ability to differentiate between pathogens (the diversity of specificity) is low. Since each BCR and TCR is expressed on a single clone of cells, the speed of the response is governed by the necessity to increase the number of any particular receptor by cell division, resulting in a slower response to primary challenge but characterized by an immense diversity of potential specificity.

Another characteristic that differentiates innate and adaptive responses is immunological memory. While the latter responds more efficiently to second exposure, the timing and magnitude of the innate response is not so influenced by preexposure and simply reflects the immediate stimulus (e.g., numbers of microorganisms). The capacity of adaptive cell populations to respond faster to second exposure is achieved in two ways: by altering the activation requirements for specific (previously activated) clones of cells and by increasing the frequency of the specific cells in the memory population, thereby increasing the frequency of specific TCRs and BCRs. In contrast, the frequency of receptor expression in innate cell populations is already high and the retention of a proportion of previously exposed cells does not alter the specificity of the repertoire for pathogen recognition. Hence, the genetic conformation of the receptors—germline or rearranged—along with the frequency and diversity of these receptors is the basis for many differences in the biology of innate and adaptive immunity. However, differences are sometimes not clear cut; for example, exposure to a particular stimulus can alter the numbers of NK cells expressing a particular distribution of receptors thereby introducing bias in the NK cell pool and affording a degree of increased responsiveness to recently encountered pathogens. This feature of the response will change the rate of

NK cell responsiveness to a previously encountered pathogen and could be viewed as a low-level “memory,” but the duration of such changes is less prolonged than with B or T cells and could be regarded as increased preparedness for challenge with a current pathogen. Similarly, there are some references in the literature to “innate immune memory” when referring to cells, such as macrophages, having different behavior after they have been primed by a stimulus. Many of these changes are relatively short term and represent changes in the transcriptome and/or the methylation state of genes and rather than this being true memory, it is best referred to as “trained” innate immunity.

Although often considered separately, as in this book, the innate and adaptive responses are highly integrated. Innate immune systems are very ancient and found in any microbe, animal or plant that is challenged by infections. In most cases the innate system functions in isolation from the adaptive immune system which emerged at the base of the vertebrate lineage. However, with vertebrates, the innate and adaptive immune components act in concert to combat pathogens and are highly integrated. The earliest pathogen recognition events that occur in the body lead to recruitment and enhancement of innate responses, as well as activation of the adaptive immune system. Indeed, without innate immune activation the adaptive immune system would not become activated. The local innate response at the site of infection also serves to recruit cells of the developing adaptive (and innate) immune response by virtue of chemotactic molecules (e.g., some complement components and chemokines) and alteration of receptors on endothelial cells lining the vessels.

Innate activation of a variety of cell subsets can increase their capacity to interact with T cells, the most important of these are the dendritic cell (DC) subsets. These cells are reviewed in [Chapter 8.1](#), but we should point out here that DCs play a unique integrating role in the immune system—these being the only cells that can activate naïve T-cell subsets by virtue of their high levels of major histocompatibility (MHC) molecules and costimulatory activity (e.g., expressing CD80/86). The DCs not only activate naïve T cells but are also primary players in directing the type of T-cell response which develops using a variety of mechanisms, including secretion of cytokines such as interleukin (IL)-10, IL-12, and IL-18 (see [Chapter 9](#) for a full review of cytokines and chemokines). Immature DCs reside in the tissues where they constantly sample their environment; exposure to pathogen components and triggering of PRR leads to DC activation and migration to areas where they can contact naïve T cells (reviewed in [Chapter 2](#)). During the migration phase, DCs mature, manifesting as a reduction in phagocytic capacity and increased expression of MHC

molecules and surface costimulatory molecules. Depending upon the spectrum of pathogen molecules activating the DC (by interaction with PRR) discussed in [Chapter 8.6](#) during the earliest phases of activation, DCs mature differently, providing the capacity to differentially influence downstream T-cell responses. DCs represent an innate cell subset that is a master driver of the adaptive response.

The innate immune system is not only involved in initiating and directing adaptive responses but is often used as an effector arm of the adaptive response; hence, it is inappropriate to delineate innate- and adaptive-mediated killing of invading pathogens in all but the very initial phases of the infection. Examples of such interactions are many and varied; here we highlight a few to illustrate the importance of “innate effectors” in expression of adaptive immunity. Of these interactions, perhaps, the easiest to comprehend is that the classical pathway of complement activation involves initiation via antibody the adaptive effector molecule secreted by B cells. In this circumstance, an “innate pathway” is being initiated and targeted by a clear component of the adaptive immune system. In terms of cellular interactions, the interaction between the T cell and macrophage is an excellent example of adaptive influence on a classical innate cell population. The macrophage is firmly placed within the spectrum of innate cell types and performs a variety of functions, including phagocytosis and production of antimicrobial compounds such as the reactive oxygen intermediates and nitric oxide (see [Chapter 8.1](#)). T-cell responses often involve the production of interferon gamma (IFN- γ), a cytokine that has the ability to activate macrophages, increasing their capacity to phagocytose and kill invading microorganisms. In this circumstance, the macrophage can be viewed as one of the effector arms of the T-cell response. Similarly, a range of phagocytic cells, including macrophages and polymorphonuclear cells, express Fc receptors that bind immunoglobulin (produced by B cells) to enhance recognition and engulfment of foreign pathogen-derived material. In such cases, the phagocytic cells of the innate system act within the context of antibody- and B cell-mediated immunity. A third example of the integration of adaptive and innate responses includes the influence of T cells on specialized epithelial cell subsets such as goblet cells (mucus producing), paneth cells, and intermediate cells in the gut, altering their numbers, inducing secretion of products (e.g., defensins), and altering the chemical composition of mucus. The list of interactions between the parts of the immune system considered innate and adaptive is huge and it is best to consider these systems as part of an integrated whole rather than as separate entities.

In this introduction, we have tried to explain the organization of the innate immune system in terms of

structural, chemical, and cellular components, some of which are expressed constitutively, while others are inducible. We have also compared and contrasted receptor-based constraints on the innate and adaptive immune systems to offer a clear discrimination between these systems. Finally, we highlighted the complexity of

the interactions between the innate and adaptive immune systems to illustrate the level of intercommunication and integration that underpins immunity *in vivo*. Notwithstanding this, it is impractical to deliver the details within different areas of innate immunity without separating these into comprehensible segments.

Macrophages and dendritic cells

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8.1.1 Introduction

8.1.1.1 Antigen presentation

Antigen presentation is the mechanism by which the antigenic environment is sampled and information imparted to the effector arms of the adaptive immune system, B and T lymphocytes. Depending on the precise context, antigen presentation can result in either activation or tolerization of lymphocytes, respective examples are the response to a pathogen challenge or tolerance to self-antigen. An antigen is subjected to either endogenous or exogenous processing, and the resulting peptides are expressed on the surface of the antigen-presenting cell (APC) bound to either major histocompatibility (MHC) class I or class II molecules (see Chapter 7). The type of MHC molecule involved with presentation not only reflects the source of the antigen but has the primary role in determining the ensuing immune response to it.

Almost all cells express class I heterodimer molecules on their outer surface and use the specialized MHC peptide-binding cleft to express peptides derived from endogenous antigens, sampled from the cytoplasm or nucleus. The avian MHC region and the proteins it encodes are described in detail in Chapter 7. Endogenous processing degrades the host's own proteins, tumor antigens if a cell has been neoplastically transformed, or antigens derived from pathogens, which replicate within the cytoplasm or nucleus. These proteins are degraded to peptides by the proteasome, and the peptides are translocated to the endoplasmic reticulum (ER), where they become bound to MHC class I molecules. The peptide stabilizes the MHC molecular complex, which is transported to the cell surface via the Golgi apparatus. If an MHC molecule bearing a peptide derived from a foreign or transformed antigen is expressed on the cell surface, the antigen–MHC complex is recognized by a T-cell receptor (TCR) on a CD8 + cell.

The MHC/TCR interaction induces the CD8 + cell to proliferate and becomes an armed antigen-specific cytotoxic T lymphocyte (CTL). Activated CTL can kill other cells presenting the same antigen on their surface in the context of MHC class I. In this way, intracellular pathogens or transformed cells are killed, thereby limiting their further replication or spread of the pathogen.

Exogenous antigens can be endocytosed by specialized APC such as macrophages or taken up by conventional dendritic cells (cDCs) and B cells. The cell surface of macrophages and cDCs are designed to maximize the interaction with the environment and potential pathogens. Phagocytosis is a deliberate, receptor-driven process based on recognition of the pathogen and engagement with it by receptors on cell surface. Multiple phagocytic receptors are activated during the recognition of pathogens, including PRRs (pathogen recognition receptors), opsonic receptors (immunoglobulin or complement receptors), and apoptotic receptors (phosphatidylserine receptors). Macropinocytosis is not driven by receptor–ligand interactions but is the process of fluid engulfing, plus any fluid-phase cargo and a large part of plasma membrane with its resident transmembrane molecules, particles, or pathogens attached to them on the external face of the plasma membrane [1,2]. These exogenous antigens reside within endosomes. Also included here are antigens derived from pathogens, such as *Salmonella*, which invade APC and reside within intracellular vesicles. The endosome fuses with a lysosome containing acid proteases to form a phagolysosome; this results in the digestion of the exogenous antigens into antigenic peptides. Peptide-containing phagolysosomes fuse with other vesicles which contain the α and β chains of the MHC class II molecule, in the process replacing a stabilizing invariant chain, so that the antigen peptide can be transported to, and presented on, the surface of the cell in the context of the MHC class II molecule. Antigen–MHC

complexes are recognized on the cell surface by the TCR of CD4 + T cells, which then activate other immune effector cells. B cells presenting antigen using MHC class II molecules are induced to secrete antigen-specific antibody by interaction with activated CD4 + Th2 cells of the same specificity. Macrophages harboring intravesicular bacteria or parasites can be activated and induced to kill them by interaction with Th1 cells. When APCs are not infected, they can acquire exogenous antigens from the infectious pathogen and present them on MHC class I molecules in a process known as cross-presentation. DCs are considered the most relevant cross-presenting cells, and in humans and mice the cDC1 are potent cross-presenting cells *in vivo*. Two main pathways for cross-presentation have been described—the cytosolic and the vacuolar. In the cytosolic pathway, internalized antigens are transported from the endosomal compartment into the cytosol to be degraded by the proteasome. The peptides are transported back into the ER or endosomes where they can be loaded onto MHC class I molecules. In the vacuolar pathway, antigen processing and loading onto MHC class I molecules takes place in the endo/lysosomal compartments [3,4]. Cross-dressing and gap-junction-mediated peptide transfer are distinct mechanisms of cross-presentation. Cross-dressing involves the transfer of loaded MHC class I molecules from a donor cell to a cDC that is mediated through cell–cell contact and does not require intracellular antigen [5]. This mechanism has been shown to induce memory CD8 + T cells response after viral infection [6]. The transport of preprocessed peptides can occur via direct cell–cell contact, mediated by gap junctions. These antigen-derived peptides then enter the normal MHC class I presentation pathway [6,7]. These alternative pathways of cross-presentation do not require the donor cells to be an APC offering the possibility that peptides and loaded MHCI can be obtained directly from infected cells. During infection, cross-presentation ensures that viral infections can generate both CTL and antibody responses, irrespective of their site of replication. Costimulation, discussed in Chapter 4 in the context of B cells, is essential for the generation of an immune response. T cells recognizing antigen on the surface of a cDC—in the context of the MHC molecule and costimulation—are induced to proliferate and differentiate into effector T cells. T cells recognizing the antigen–MHC complex in the absence of a “second signal” become anergic, even in the face of further stimulation by DCs. Most of these antigen presentation mechanisms are expected to exist in the chicken but still await experimental confirmation.

8.1.1.2 Dendritic cells

In the early 1970s, Ralph Steinman and Zanvil Cohn described a rare cell type in murine spleen cell

preparations which could be distinguished from macrophages and other leukocytes [8]. They proposed the name “dendritic cells” (from the Greek δένδριτης = “tree like”) based on cellular morphology. Later in that decade, Steinman and colleagues reported that these DCs were required for the stimulation of T lymphocytes in mixed lymphocyte reactions (MLRs) [9]. Their potency suggested that only DCs could efficiently present antigens to naive lymphocytes. While it was early recognized that these DCs were part of the mononuclear phagocyte system (MPS), which also includes monocytes and macrophages, the distinction between and classification of these cell types based on functionality and surface markers has been controversial. More recently, it has been recognized that Steinman’s original DCs are a distinct cell lineage that can be differentiated from related MPS cells on the basis of ontogeny [10] (Fig. 8.1.1). These cells are now referred to as “classical” or “conventional” dendritic cells to distinguish them from other cell lineages, such as monocyte-derived DCs or bone marrow-derived DCs. The cytokine Flt3L, acting through the receptor Flt3, drives the generation of cDCs, whereas macrophages and monocytes lack Flt3 expression.

The existence of an equivalent-specialist APC in birds has long been speculated, but until recently evidence was mostly circumstantial. Of the three types of mammalian professional APC, B cells, macrophages, and DCs, the first can be excluded as being necessary for antigen presentation since bursectomized birds, lacking B cells, can mount normal T-cell responses [11]. It is possible that avian macrophages are sufficient to mount normal immune responses. However, it is only with (1) the recent advent of recombinant cytokines, (2) the development of cell isolation protocols, (3) the generation of DC-relevant antibodies, and (4) the cell subset-specific gene expression analysis that studies on avian DCs as a distinct population have been possible. Although we are only now attaining an understanding of avian DC biology, it is already apparent that many of the characteristics of mammalian DCs—a prerequisite to their unique function—are replicated in whole or in part in birds. Mammalian cDCs consist of two subsets: cDC1 and cDC2 [12–14]. Each cDC subset exhibits functional specialization [15]. The cDC1 subset is specialized for cross-presentation of antigens and induction on Th1 responses, whereas the cDC2 subset is specialized for the induction of Th2 and Th17 immune responses. Using transcriptomic approaches, several groups have identified a single chicken cDC subset, which is equivalent to the mammalian Flt3 + XCR1 + cDC1 subset [16,17]. XCR1 + cDCs are functionally specialized for antigen cross-presentation and the generation of CD8 + cytotoxic T-cell responses [18]. A single cDC subset in chickens implies that either chicken XCR1 + cDCs exhibit a wider range of functions than their

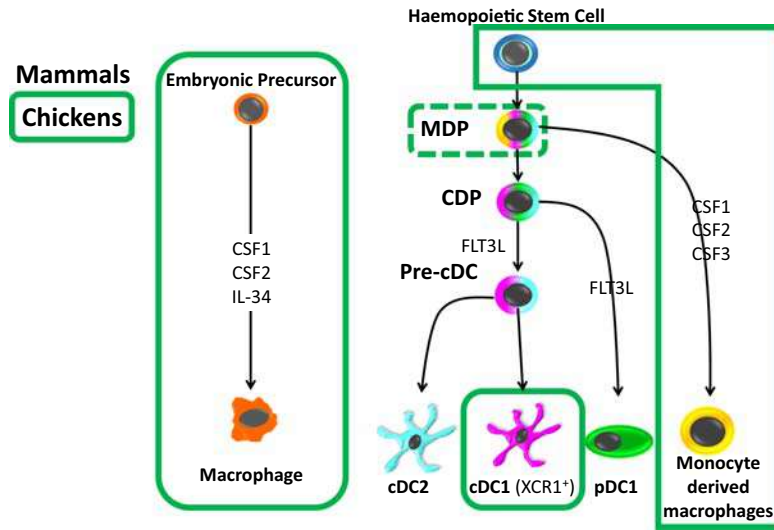


FIGURE 8.1.1 The relationship of mammalian and chicken mononuclear phagocytic system cell subsets based on ontogeny. This overview is largely based on mammalian models, with known chicken cell subsets shown in green boxes. The precursor(s) of chicken mononuclear phagocytic system (MPS) cells are largely unknown. However, a putative chicken monocyte-DC progenitor (MDP) cell is shown in a dashed green box. As such, the exact relationship of various chicken cell subsets is largely speculative and highlights that the development of chicken MPS cell populations are not precisely equivalent to their mammalian counterparts. For example, if chickens do contain a single cDC population, then logically there cannot be a common DC precursor (CDP) equivalent population. This arrangement also highlights the lack of knowledge concerning the origin of chicken specific MPS cell subsets, such as BSDC, FDC, TIM4⁺, and MRC1L-B⁺ tissue macrophage subsets. For example, are MRC1L-B⁺ tissue macrophages derived from embryonic and/or HSC progenitors? Key growth factors (CSF1–3, IL-34, and FLT3L) relevant to different classes of MPS cells are also shown. *CSF1*, colony-stimulating factor 1; *CSF2*, colony-stimulating factor 2 (also known as GM-CSF); *CSF3*, colony-stimulating factor 3; *IL-34*, interleukin-34; *FLT3L*, FMS-like tyrosine kinase 3 ligand; *pDC*, plasmacytoid dendritic cell; *cDC*, conventional dendritic cell.

mammalian counterpart or that the cDC2 functions are performed by cells other than cDCs in the chicken. The rapid development in avian genome editing, the development of cell lineage-specific knockout chickens, and the implementation of novel technologies such as single-cell RNASeq will hopefully elucidate the roles of chicken cDCs, and other APC in the near future.

Although much of the molecular machinery which allows APC to present antigen has been described for the chicken (see Chapter 7), this is not of itself sufficient to define an immune surveillance system. The acts of antigen uptake, processing, and presentation place different requirements on the APC, resulting in a change of state from a so-called “immature” phenotype, which is efficient at antigen capture, to the “mature” phenotype that is optimal for antigen presentation. Furthermore, antigen capture may take place at a site which is anatomically distant from where antigen presentation to lymphocytes occurs; DCs must therefore also have, or acquire, the ability to selectively migrate. These issues are discussed later.

8.1.1.3 Macrophages

Macrophages represent a heterogeneous group of cells which are found throughout the body in both vertebrates

and invertebrates. The term *macrophage* was introduced in 1884 by the developmental embryologist Ilya Metchnikoff to describe leukocytes which are capable of ingesting and destroying foreign substances, including microorganisms [2]. The macrophage group constitutes a broad array of MPS cell types consisting of classical macrophages, brain microglial cells, liver Kupffer cells, epidermal Langerhans cells, and bone osteoclasts. These cells play a fundamental role in tissue development and homeostasis, innate and acquired immunity, inflammation, and immunopathology [19]. Members of the colony-stimulating factor (CSF) superfamily are involved in mammalian myelopoiesis. CSF1 receptor (CSF1R)-mediated signaling is necessary for the differentiation and survival of cells of the MPS, especially macrophages. CSF1R belongs to the type III protein tyrosine kinase receptor family and binding of its ligands, CSF1 or IL-34, induces homodimerization of the receptor and subsequent activation of receptor signaling [20]. CSF1R/CSF1/IL-34 are functionally conserved in birds and seem to be conserved across all vertebrate species [21]. Recently, structural predictions and evolutionary analysis using a large collection of avian genomic sequences suggest that at least in birds the CSF1–CSF1R interaction may be subjected to pathogen selective pressure [22].

The genomic sequence of CSF1R contains a highly conserved regulatory element, Fms-intronic regulatory element (FIRE) [23,24]. The FIRE segment along with the genomic DNA containing the CSF1R promoter is sufficient to drive the expression of a reporter gene, such as enhanced green fluorescent protein (eGFP), specifically in all macrophage-lineage cells in mice [24]. The FIRE segment is highly conserved in avian species and was used along with the chicken CSF1R promoter to generate CSF1R-reporter transgenic chickens to visualize and study the chicken MPS in more detail [25]. A majority of cells of the chicken MPS system express the CSF1R-transgene with low levels of transgene expression by heterophils and no expression in Kupffer cells, discussed below [17,26]. Major progress has been made in the field of mammalian macrophage biology by definition of their ontogeny, heterogeneity, structure, and function in many tissues and during pathological states [19]. By comparison, knowledge of avian macrophages is more limited, but progress has been made in the generation of new antibodies, the CSF1R-reporter transgenic chickens, and next-generation sequencing techniques. Here, we will give an overview of the recent progress in avian macrophage and monocyte biology.

Chicken monocytes constitute approximately 5%–10% of peripheral blood leukocytes with absolute numbers in the range of 1.5×10^3 cells per 1 μ L of blood, but this number may vary considerably between different chicken lines [27–30]. In humans, monocytes are not a homogeneous population, rather, several monocyte subsets can be discriminated using cell surface markers: classical (CD14 + /CD16 –), nonclassical (CD14 + /CD16 + +), and intermediate (CD14 + /CD16 +) subpopulations. Each subpopulation expresses different innate immune recognition receptors and mediates distinct functions [31]. These differences become even more heterogeneous as monocytes differentiate into tissue-resident macrophages and monocyte-derived DCs under inflammatory conditions [32]. Chicken blood monocytes uniformly express CD45, MRC1L-B, recognized by the mAb KUL01 [27], CSF1R, and MHC class II [17,26]. These cells divide into two separate populations based on the expression of the apoptotic cell plasma membrane phosphatidylserine receptor, T-cell immunoglobulin, and mucin domain-containing 4 (TIM4) which is not expressed on human or mouse monocytes [17]. RNA-seq analysis of these two monocyte subpopulations demonstrates the enrichment of the non-classical monocyte-related genes by the Tim4 + /CSF1R-transgene + cells. Whether these cells represent monocyte progenitors that may replace tissue-resident macrophages or resident scavenger cells of the blood is still to be determined. The Tim4-/CSF1R-transgene + cells express, CD14, MyD88, and TLR4 and TLR2a, in common with mouse monocytes [17]. Ex vivo monocytes from chickens

of different MHC haplotypes differ in their ability to phagocytose *Salmonella typhimurium* but not *Salmonella infantis* [29], while in whole blood infection assays, monocytes can kill *Staphylococcus aureus* but have limited capacity to control *Candida albicans* infection [30]. Many studies have examined the function of monocytes using monocyte-derived macrophages by enrichment via monocyte adherence to plastic. Several studies show that monocyte-derived macrophages respond to Toll-like receptor (TLR) agonists [33–36], *Toxoplasmosis gondii* [37,38], avian leukosis virus (ALV) [39], *Eimeria tenella* [38,40], *Salmonella pullorum* [41], and *S. typhimurium* [42].

In mammals, circulatory monocytes constituting the primary source of replenishment of all resident tissue macrophages throughout the lifetime of the animal is a redundant concept as evidence shows that macrophages develop prenatally from embryonic precursors that in a homeostatic environment are self-maintained by self-renewal [43]. These prenatal macrophages express a core macrophage cell program which is diversified following their tissue colonization by expression of tissue-specific transcriptional regulators, these include microglial cells, red pulp macrophages, and Kupffer cells [44]. After birth, bone marrow-derived blood monocytes replenish resident macrophages with high turnover, such as the intestine, or are recruited following injury, infection, and inflammation [45,46]. Interembryonic transplantation of HH16 bone marrow progenitors into HH16 chick embryos, before the onset of definitive hematopoiesis, can produce long-lived tissue-resident macrophages, whereas macrophages derived from yolk sac donors are not retained into the adult bird [47]. In chickens, MRC1L-B (KUL01) expression has been the defining feature in studying tissue-resident macrophages in the liver, spleen, intestine, brain [48,49], and cochlea [50]. Recently, Hu et al. demonstrated that MRC1L-B (KUL01) is not a universal marker for chicken macrophages and that not all cells of the MPS are labeled with the reporter gene in the CSF1R-reporter transgenic chickens [17]. Previous studies had shown mAb KUL01 positive staining of large macrophage-like cells in the chicken liver and were proposed as Kupffer cells [48]. In contrast, Hu et al. described the staining of small round cells in the liver with mAb KUL01 that also express the CSF1R-transgene, while CSF1R-transgene-/TIM4 + cells are stellate and irregular, lining the walls of hepatic sinusoids consistent with their identity as Kupffer cells. Transcriptome analysis of these cells further indicated their Kupffer cell lineage and using more sensitive flow cytometric analysis Kupffer cells were shown to stain for KUL01, albeit at lower levels than other mononuclear phagocyte populations [17].

With the generation of the CSF1R-reporter transgenic animals, the visualization and analysis of tissue-resident macrophages, such as in the feather pulp, muscle, intestine, brain, air sac, and lung, has become more accessible

in chickens [17,25,51]. As discussed, in the liver in addition to bona fide Kupffer cells, two subpopulations have been described based on *CSF1R*-transgene expression, Tim4 + /*CSF1R*-transgene⁻/CD45 + /MHC class II + cells and Tim4 + /*CSF1R*-transgene + /CD45 + /MHC class II + cells. Interestingly, both liver subpopulations express similar levels of *CSF1R* mRNA indicating that the *CSF1R* expression is more tightly controlled in chicken Kupffer cells and not all cells of the chicken MPS are labeled with the transgene driven by *CSF1R* genetic elements use to generate the *CSF1R*-transgenic chickens [51].

Through transcriptome and flow cytometric analysis, several tissue-resident macrophage subpopulations have been described in chickens. In the spleen, comparative gene expression profiling shows that MRC1L-B + macrophages are enriched for mammalian monocyte/macrophage-associated genes, while MRC1LB-/putative CD11c + cells resemble cDC [16]. Recently, two subpopulations of splenic macrophages were defined based on MHC class II and MRC1L-B expression. The MRC1L-B^{lo}/MHC class II^{hi} subpopulation expresses higher levels of MHC class I and CD80 molecules in contrast to the MRC1L-B^{hi}/MHC class II^{lo} subpopulation which has a higher capacity to phagocytose dead cells [52]. Defining the transcriptome of white and red pulp macrophages and whether cells resembling mammalian marginal zone macrophages are present in the chicken spleen is still to be determined (see Chapter 2).

While characterizing the distribution and ontogeny of CD45 + HSC, Dora et al. noted the presence of ramified CD45 + cells, characteristic of chicken macrophages, in the gut mesenchyme [53]. Hindgut grafting shows that the cells are of hematopoietic origin and are defined after hatching as CD45 + /MHC class II + /*CSF1R* + /chB6 + cells, similar to microglia cells. The enteric nervous system macrophages are in the muscular layer and myenteric ganglia and were named intraganglionic macrophages which represent a novel chicken myeloid cell population [53].

Inhibiting CSF-1 signaling through *CSF1R* by neutralizing CSF1 using antichickens CSF1 mAb (ROS-AV183) decreases the number of *CSF1R*-transgene expressing cells in the bursa and cecal tonsils, decreases chB6 + B cells in both blood and spleen, and completely ablates Tim4 + Kupffer cells. However, the neutralization of CSF1 has no effect on blood monocytes in 1-week-old chicks suggesting that chicken monocytes, similar to murine monocytes, may not depend on exogenous CSF1 for their survival [54]. During disease, rapid increases in MRC1L-B + macrophages have been demonstrated in the intestinal response to *E. tenella* [55] or *Salmonella enterica* infection [56]. This is also evident in the lung after individual or mixed avian pathogenic *Escherichia coli* (APEC) and infectious bronchitis virus (IBV)

infections [57] or low and highly pathogenic avian influenza virus (AIV) infections [58–60] and in the bursa and spleen after infectious bursal disease virus (IBDV) infection [61]. Currently, it is unknown if the MRC1LB + macrophage increase in tissues postinfection is related to inflammatory induced monocyte recruitment or if tissue macrophages undergo rapid in situ proliferation upon infection or both. Further functional characterization of the inflammatory chemokine–chemokine receptor network and cell tracing experiments are required to address the role of inflammation in monocyte/macrophage recruitment and self-renewal in chicken tissues.

Macrophages are key modulators and effector cells and their response to antigen influences the other arms of the immune system. Dichotomy has been proposed for macrophage activation: classic versus alternative, also called M1 and M2. This polarity of macrophages is derived from their ability to produce or antagonize prototypic inflammatory responses. Defining the function of chicken macrophages and how they provide primary host defense and maintain tissue homeostasis is critical to developing immunological strategies to improve immune response to pathogens. With the generation of *CSF1R*-transgenic chickens, the extent of the MPS will be elucidated. In addition, the development of mAb specific for cell types in parallel with single-cell RNA-seq will contribute to defining the ontogeny, dichotomy, and function of tissue-resident macrophages in chickens.

Finally, microglial cells are brain resident macrophages that are seeded by yolk sac-derived cells during development. These cells are involved in many physiological and pathological brain processes. Chicken microglia cells have similar morphology as mammalian microglia cells and express a core gene program that is conserved across a number of species throughout evolution [62].

8.1.1.4 Development of myeloid cells

Members of the CSF superfamily are involved in mammalian myelopoiesis, namely the generation of monocytes, macrophages, DC, and polymorphonuclear phagocytes (e.g., neutrophils and eosinophils). The family has three canonical members, namely macrophage (M)-CSF (or CSF1), granulocyte (G)-CSF (or CSF3), and GM-CSF (or CSF2). Originally, these cytokines were defined as growth factors capable of inducing myeloid colony formation from bone marrow precursors in vitro. Across these three CSFs, the entire landscape of phagocytes can potentially be controlled.

In mammalian myelopoiesis, the long-held view that circulatory monocytes constitute the primary source of replenishment of tissue-resident macrophages throughout life is no longer applicable. Monocytes differentiate in the bone marrow in response to CSF2 and CSF1, among others [63]. The majority of tissue-resident macrophages are thought to be self-maintained by self-renewal and

derived from progenitors that seed peripheral tissues in the early embryo [64]. These prenatal macrophages express a core macrophage cell program which is diversified following their tissue colonization [44]. The exact contribution of a particular macrophage population from monocytic or early embryonic precursors varies from tissue to tissue and is largely unknown for chicken tissue macrophage populations. Tissue-resident macrophage populations are dependent on CSF1 and to a lesser extent CSF3. This is evident for chicken Kupffer cells and subpopulations of resident tissue mononuclear phagocytes in the bursa and cecal tonsils that were depleted or decreased following CSF1 inhibition [54]. Like monocytes, cDC development (cDCpoiesis) largely occurs in the bone marrow, is dependent on Flt3L, and involves a developmental cascade of bone marrow-resident hematopoietic stem cell-derived precursor and progenitor cells [14]. In the steady state, mammalian cDC populations in peripheral tissues are maintained by pre-cDC or cDC entering tissues from the blood and dividing locally [14,65].

In the chicken, myelopoiesis has been studied with the help of avian retroviruses which express the v-myc oncogene (e.g., MC29) and induce myelocytomatosis in vivo or the v-myb oncogene (e.g., E26 or avian myeloblastosis virus, AMV) which induces myeloblastosis. These and other ALVs have also been used to investigate hematopoiesis in the chicken system. A detailed description of this system can be found elsewhere [66]. Chicken myeloid cells transformed by v-myc or v-myb require a growth factor, secreted by activated macrophages and termed *chicken myelomonocytic growth factor* (cMGF) [67] for proliferation. cMGF was the first chicken cytokine to be cloned and characterized at the molecular and functional level and shown to be related to mammalian CSF3 and interleukin (IL)-6 [68]. Binding of cMGF to myeloid progenitor cells causes rapid STAT5 phosphorylation followed by proliferation and further differentiation [69] in vitro systems. In vivo, overexpression of cMGF by a recombinant fowlpox virus significantly increased the number of circulating monocytes and their activation status [70], confirming the functional relevance of this cytokine. Initial analysis of the chicken genome failed to identify a mammalian homolog to this cytokine in the published genome sequence [71] which was later shown to be due to a large sequence gap at the predicted location [72]. Using a primer walking strategy and extensive analysis of the coding region, it was confirmed that MGF is identical with mammalian CSF3, and it was suggested that the cytokine be renamed accordingly.

Earlier, chicken GM-CSF (CSF2) was cloned, shown to drive the proliferation of bone marrow cells [73], and used for the in vitro differentiation of myelomonocytic stem cells into macrophages and DCs (for more details see Section 8.3.1). The third member of the CSF group,

CSF1, was identified from sequence data derived from the zebra finch genome leading to the cloning of chicken CSF1 [47]. Bone marrow cells cultured in the presence of recombinant chicken CSF1 differentiate into pure macrophage cultures within 6 days. The same effect was achieved with recombinant chicken IL-34. Both cytokines bind to the CSF1R, albeit at distinct regions. Interestingly, this feature seems to be conserved across all vertebrate species [47]. With the availability of all three avian members of the CSF group (CSF1, CSF2, and CSF3), myelopoiesis can now be studied in more detail in vitro as well as in vivo systems [74].

Research on transcription factors regulating avian myeloid cell differentiation is still highly limited compared with our knowledge of the mammalian system. Many transcription factors involved in the development of monocyte, macrophage, and cDC lineages are conserved in chickens and cell type identity in chickens has been defined by molecular fingerprinting through gene expression profiling [16,17]. In addition to STAT5, *MafB* was identified, using the v-myb transformed chicken monoblastic cell line BM2 [75], as another regulatory factor in monocyte/macrophage differentiation in the chicken [76,77]. Cap Analysis of Gene Expression (CAGE) has also contributed to the profiling of genome-wide set of transcription start sites critical for differentiation and maturation from pregastrulation to hatching. CAGE can identify stage-specific expression profiles and enriched transcription factors responsible for lineage commitment and their corresponding regulatory modules [78].

Embryonic macrophages do not follow the same pathway as described previously for postnatal monocyte/macrophage development, and therefore, represent an independent lineage. Studies with yolk sac chimeras (see Chapter 3) have shown that primitive hematopoietic cells arise from yolk sac mesoderm during early development, whereas definitive blood cells are derived from intraembryonic precursors [79]. In the yolk sac of embryos incubated for 2.5–4.5 days, cells with macrophage-like morphology have been found, and these increase in number during development, showing phagocytic activity in areas of cell death [17,25,80,81]. It has been suggested that these cells play a crucial role in the removal of apoptotic cells during development and are homologs of mammalian fetal macrophages [82].

8.1.1.5 Sources of avian macrophages and dendritic cells

Several sources for avian macrophages have been described, the most important ones being peritoneal

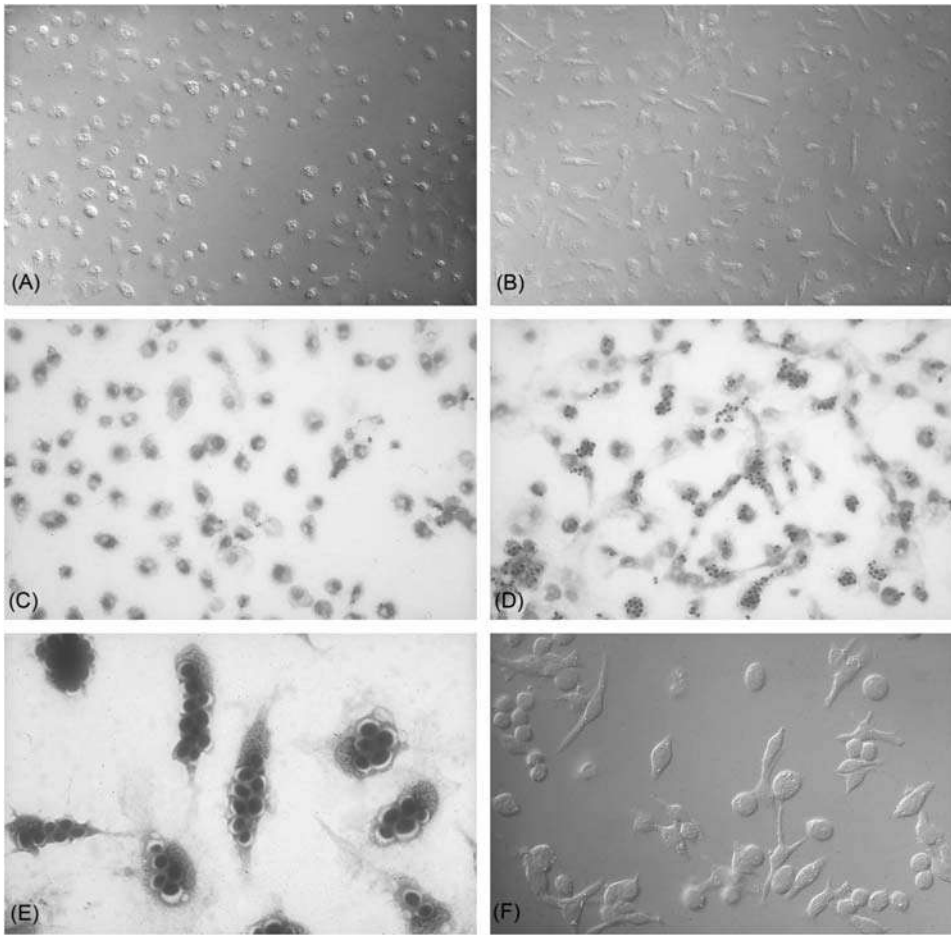


FIGURE 8.1.2 (A) Differential interference contrast image of monocyte-derived macrophages; 72 h culture on a microscope glass slide, nonactivated cells. (B) Differential interference contrast image of monocyte-derived macrophages; 48-h culture on a microscope glass slide, activated with IFN- γ for 24 h. (C) Pappenheim staining of monocyte-derived macrophages; 48-h culture on a microscope glass slide, nonactivated cells. (D) Pappenheim staining of monocyte-derived macrophages; 48-h culture on a microscope glass slide, activated with IFN- γ for 24 h; phagocytosis of yeast cells. (E) Pappenheim staining of monocyte-derived macrophages; 48-h culture on a microscope glass slide, activated with IFN- γ for 24 h; phagocytosis of yeast cells; high-power magnification. (F) Differential interference contrast image of HD11 cell line.

exudate cells and monocyte-derived macrophages. In mice and rats, macrophages are constitutively present on the serosal membranes of the peritoneal cavity, and peritoneal lavage yields significant numbers of nonactivated resting macrophages [83]. In contrast, these cells are essentially absent in the chicken, and macrophages have to be recruited from the circulation into the body cavity by inflammatory stimuli prior to the lavage procedure [84,85]. Comparison of different stimulation protocols revealed that a single injection of a 3% Sephadex G-40 suspension in saline gives optimal results and primarily attracts cells with macrophage morphology. Repeated Sephadex injections increase the number of lymphocytes in the samples, whereas the injection of starch primarily elicits heterophils [86]. Sephadex-elicited cells strongly adhere to tissue culture plastic and show macrophage morphology and characteristic macrophage activities such as phagocytosis and bacterial lysis [87,88]. Comparable results were obtained for turkeys [89] and quail [86], suggesting that this procedure can be applied to most avian species. It should be noted that Sephadex-elicited macrophages are functionally activated cells [90] and may respond differently to cells obtained from other sources (Fig. 8.1.2).

As an alternative technique, mononuclear cells have been isolated from different lymphoid organs, bone marrow, blood, or tissue by adherence to glass or tissue culture plastic surfaces. Carrell and Ebeling [91] were the first to describe the generation of pure macrophage cultures from chicken blood, a system later used to demonstrate the formation of epithelioid and giant cells [92] and the differentiation of monocytes to macrophages [93]. The adherence of chicken monocytes is slow and requires one to several days of culture rather than hours, as routinely used for the selection of human monocytes and macrophages [94]. Importantly, short-term cultures of peripheral blood mononuclear cells separated by density-gradient centrifugation (Ficoll-Paque) give rise to nearly pure thrombocyte preparations after removal of nonadherent cells [95]. The nucleated avian thrombocytes attach to both glass and plastic surfaces within 30 minutes [96] but die at 48–72 hours or are actively phagocytosed by adherent macrophages in the cultures [93]. Thus a protocol has been established in which monocyte-derived macrophages can be selected from peripheral blood by adherence to glass or plastic for at least 48 hours [97]. Since these cells adhere firmly, nonadherent and dead

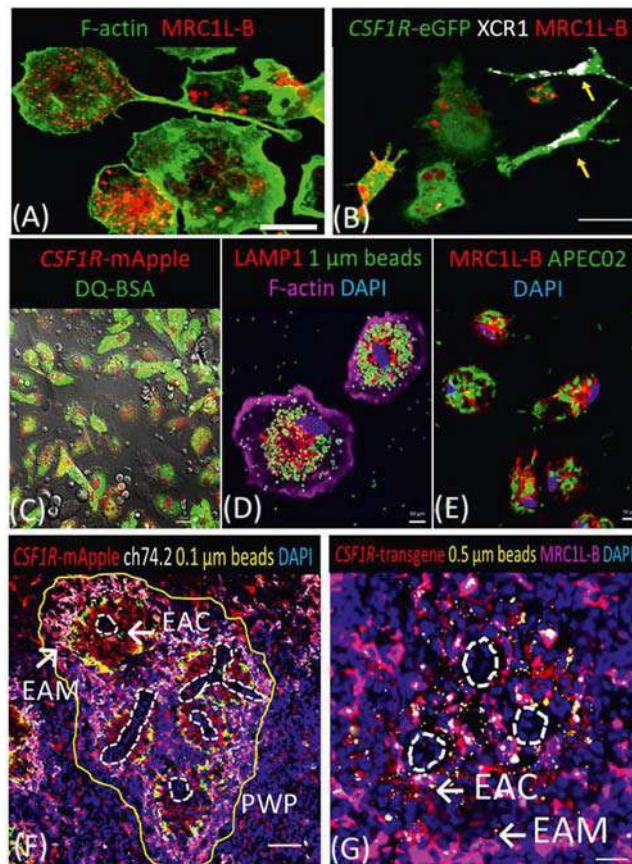


FIGURE 8.1.3 (A) Image of an overnight culture of adherent splenic cells, PFA fixed. Macrophages have intracellular punctate staining with the macrophage marker MRC1L-B (red, F-actin visualized with Phalloidin green). (B) A live image 1-hour poststaining of an overnight culture of splenic adherent cells from a CSF1R-eGFP transgenic reporter chicken. Cells were stained for cDC marker XCR1 (white) and MRC1L-B (red). Macrophages have internalized MRC1L-B, which is confined to intracellular vesicles. Yellow arrows highlight the two XCR1 + cDC. Scale bar = 10 μ m. (C) Day 3 culture of CSF1R-mApple chicken (red) lung adherent cells were incubated with DQ-BSA. After 3 h, the hydrolysis of the DQ-BSA complex (green) has taken place in the intracellular vesicles. (D) Day 3 culture of splenic adherent cells incubated with 1 μ m fluorescent latex beads (green). Three hours posttreatment, numerous beads are phagocytosed and colocalize with LAMP1 + intracellular vesicles (red). Nuclei are visualized with DAPI (blue and F-actin magenta) (E) Day 2 lung adherent cells incubated for 1 h with APEC02-GFP (green) detected in MRC1L-B + macrophages (red), nuclei (blue). (F) CSF1R-mApple reporter chickens were intravenously injected with 0.1 μ m fluorescent latex beads. Fifteen minutes postinjection, beads (yellow) are located in a ring between the CSF1R-mApple + ch74.2- ellipsoid-associated cells (EAC-red) and the CSF1R-mApple+ ch74.2 + ellipsoid-associated macrophages (EAM-magenta). Scale bar = 50 μ m (G) CSF1R-eGFP reporter chickens were intravenously injected with 0.5 μ m fluorescent latex beads (white). Three hours postinjection, large deposits of beads are located in the CSF1R-eGFP+ MRC1L-B + EAM (yellow) and few beads observed in the CSF1R-eGFP + MRC1L-B- EAC (green).

Credit Kate Sutton and Adam Balic.

cells can easily be removed by vigorously washing the culture dishes. Alternatively, slow spin centrifugation ($62 \times g$) will separate the majority of thrombocytes from the lymphocytes but a similar 48 hour adherence is still recommended to increase the purity of macrophages. In contrast to Sephadex-elicited macrophages, these adherent cells display a nonactivated phenotype with comparatively weak phagocytosis of nonopsonized particles and low-level MHC class II antigen expression [98,99]. These cells may therefore provide a preferential source to study macrophage differentiation and activation (Fig. 8.1.3).

Now a more widely used technology to generate avian macrophage cultures takes advantage of the identification of

two essential growth and differentiation factors for macrophages [21,47]. Suspensions of bone marrow cells cultured in the presence of recombinant chicken CSF1 (M-CSF) or IL-34 develop into confluent bone marrow-derived macrophage (BMDM) cultures within 6 days, whereas cells do not survive in the absence of these cytokines [54]. This effect is mediated through binding of CSF1 or IL-34 to the CSF1R. The culture conditions of BMDM can now be controlled by using recombinant CSF1 or IL-34 but the growth of macrophages from bone marrow cells was already described in the 1970s by Graf who used chicken serum as likely source of CSF1. These bone marrow-derived cell cultures were exploited to study host–virus interactions [59,99,100].

To further elucidate the functional properties of these BMDMs, it will be important to compare the macrophage activities and response patterns between the different macrophage culture systems to select appropriate experimental settings. BMDMs from male and female birds retain an intrinsic sex identity that includes differential expression of interferon (IFN)-responsive autosomal genes [101].

In 2009 Wu et al. described functional bone marrow-derived DC (chBMDC) in the chicken for the first time [102]. Chicken bone marrow cells were cultured in the presence of recombinant chicken GM-CSF and recombinant chicken IL-4 for 7 days. The cells showed the typical morphology of DCs, with surface phenotype of MHC class II + (high), putative CD11c + (high), CD40 + (moderate), CD1.1 + (moderate), CD86 + (low), CD83 – , and DEC-205 – . Upon maturation with lipopolysaccharide (LPS) or CD40L, surface expression of CD40, CD1.1, CD86, CD83, and DEC205 was increased. Receptor-mediated uptake and macropinocytosis were assessed by the uptake of FITC-dextran and fluorescent beads, respectively, and both decreased after stimulation. Nonactivated chBMDC stimulated both allogeneic and syngeneic PBL to proliferate in a MLR. However, LPS- or CD40L-stimulated chBMDCs were more effective T-cell stimulators in MLR than nonstimulated chBMDC. Cultured chBMDC could be matured to a Th1-promoting phenotype by LPS or CD40L stimulation, as determined by mRNA expression levels of Th1 and Th2 cytokines.

Resident tissue macrophages and dendritic cells have been isolated for functional studies. These cells are usually selected from cell suspensions obtained by tissue dissociation through adherence, as described for monocyte-derived macrophages [97,103,104] or through discontinuous density-gradient centrifugation as described for mammals. The extraction of populations of macrophages and DCs from chicken spleen [104], lung [105,106], liver [17], and brain [62] that appear to be functional has also been reported.

8.1.1.6 Avian myeloid cell lines

Myeloid cell lines have been of great value for studies on lineage differentiation and avian macrophage function. As discussed earlier, myeloblast- and macrophage-like cell lines can be obtained by retroviral transformation in vitro and in vivo. By using the v-myc-containing AMV MC29, Beug and colleagues [107] developed a macrophage-like cell line initially described as LSCC-HD (MC/MA1), which was subsequently renamed HD11 [67] (Fig. 8.1.2F). In the original report, it was shown that these cells are slightly adherent, express Fc receptors, actively phagocytose bacteria and react with a macrophage-specific antiserum; however, it was also shown that they lack markers of other hematopoietic cell lineages [107]. Since then, HD11 cells have been used by

numerous investigators to study avian macrophage biology, and this has led to their improved characterization.

The BM2 myeloblastoid cell line was established by transformation with AMV containing the v-myc oncogene [108]. This nonproducer cell line displays blastoid morphology and expresses a thus far uncharacterized Fc receptor but lacks Fc receptor-mediated phagocytosis. Upon stimulation with LPS, BM2 cells acquire a phenotype more closely related to macrophages. Additional treatment of these cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) induces terminal differentiation to mature macrophages, an effect not reversed by TPA removal [109]. BM2 cells have subsequently been used to investigate the differentiation of monocytes, macrophages [75], and osteoclasts [110].

The third avian macrophage-like cell line was obtained from a chicken which developed splenomegaly after infection with the JM/102W strain of Marek's disease virus (MDV). This cell line, called MQ-NCSU, phagocytosed sheep red blood cells (SRBC) and bacteria and expressed Fc receptors, high levels of MHC class II antigens, and transferrin receptors. In addition, these cells react with the monoclonal antibody (mAb) K1, which is specific for monocytes/macrophages and thrombocytes [111] and they display the morphological characteristics of malignantly transformed mononuclear phagocytes. Thus far, the transforming agent has not been described for this cell line. It should be noted, however, that this cell line also stains with a polyclonal anti-reticuloendotheliosis virus (REV) serum (Calnek and Schat, unpublished observations) suggesting that the cell line is actually transformed by the *v-rel* oncogene of REV.

More recently, another mononuclear cell line, named HTC, displaying monocyte/macrophage morphology and functional characteristics of macrophages was established from cells growing out of a primary chicken heterophil culture. This cell line, termed HTC, was positive for MDV and ALV using PCR analysis and produced ALV antigen detected by enzyme-linked immunoassay. This suggests that the cells were most likely transformed by ALV [112].

Lawson and colleagues established the turkey macrophage cell line IAH30 by transformation of peripheral blood leukocytes with the ALV strain 966 [113]. This cell line reacted with mAb against chicken β_2 -microglobulin and chicken monocyte/macrophage/thrombocyte marker (recognized by the mAb K1) and secreted nitric oxide upon IFN- γ stimulation, but it did not express MRC1L-B recognized by the mAb KUL01.

8.1.1.7 Cell surface markers for avian myeloid cells

Avian myeloid cells possess a variety of cell surface molecules that can be shared by other cell types and although there are a lot of overlapping markers, some are distinct for resident tissue macrophages in chickens. The first mAb

reacting with myeloid cells of the chicken was developed by immunizing mice with AMV- and MC29-transformed chicken myeloblasts and macrophages. Four antibodies were selected that were specific for myeloid cells [114]. Mab 51-2 has subsequently been used for the identification of myeloid cells by others [115]. Using Sephadex-elicited abdominal exudate macrophages as immunogen, Trembicki et al. established the mAb CMTD-1 and CMTD-2, which reacted with mature macrophages but did not react with blood monocytes or myeloid precursors [116]. These two mAb are thought to detect a carbohydrate epitope on inflammatory macrophages [89]. The mAb CVI-ChNL-68.1 specifically reacts with surface antigen on monocytes, macrophages, and interdigitating cells in tissue sections, and stains the surface of ~10% of splenocytes and ~16% of peritoneal cavity cells [117]. A more restricted reaction pattern has been found for the mAbs CVI-ChNL-74.2 and CVI-ChNL-74.3, with the former recognizing a subpopulation of macrophages in lymphoid tissues, while the latter reacts with an unknown cytoplasmic (granular) antigen in follicular dendritic cells (FDCs) [118]. The mAb K1, selected from a number of antibodies obtained after immunizing mice with chicken spleen cell suspensions, was originally described as monocyte/macrophage specific [119]. Subsequently, it was shown that this antibody reacts with a heterodimeric cell surface protein shared by monocytes/macrophages and thrombocytes [120]. This mAb has been used in flow cytometry and immunohistology to identify monocytes and macrophages [120,121] and to discriminate lymphocytes from thrombocytes in peripheral blood leukocyte preparations obtained by density-gradient centrifugation [120–122]. Although mAb K1 can be used to detect thrombocytes, these cells can be stained more specifically with mAb 11C3, which reacts with the thrombocyte-restricted integrin gpIIb/IIIa [123], mAb23C6 binding to the CD51/CD61 heterodimer [124] or mAbs reacting with chicken CLEC-2 [125] or TREM-B1 [126]. The mAb 8F2 was proposed to target “putative” chicken CD11c. This mAb stains the cell surface of monocytes/macrophages and chBMDC and also other cells types, such as chicken NK cells and heterophils [126], bursal secretory dendritic cells [127], and has been used by several groups to analyze cDC and macrophages in the spleen [16,128] and the lung [51]. Recently, Jansen and colleagues demonstrated the binding of mAb 5C7, which recognizes putative CD11 family members b or c, on chBMDC [129].

A cell lineage-specific mAb, designated KUL01, for chicken MPS cells was developed by Mast et al. [48]. This antibody has proved to be a valuable tool, both for flow cytometry and immunohistology to study tissue-resident macrophages and blood monocytes [16,17,130–134]. In tissue sections, KUL01 stains macrophages and interdigitating cells, as well as cells resembling mammalian epidermal Langerhans cells [48]. Using

a proteomic approach, Butter and colleagues succeeded in identifying the KUL01 antigen as the chicken MRC1L-B and hence KUL01 + macrophages should be named MRC1L-B + macrophages [135]. Thus KUL01 is the first monocyte/macrophage lineage-specific mAb for which the antigen has been defined at the molecular level (Fig. 8.1.3A). An antibody raised against chicken DEC205 (CD205 or Ly75) shows that this C-type lectin receptor is expressed on DC [104] and thymus cortical epithelial cells [136]. The mAb ROS-AV170 was specifically raised against chicken CSF1R and detects blood monocytes, while staining of freshly isolated splenocytes and bone marrow cells requires cell permeabilization due to receptor internalization and degradation upon ligand interaction [137]. This mAb is useful for the analysis of the heterogeneity of chBMDC cultures grown with recombinant chicken IL-4 and GM-CSF [129], blood monocytes, and antigen-sampling M cells [26,137]. Recently, two mAbs against chicken CSF1 were developed, ROS-AV181 and 183, with AV183 having neutralizing effects [54]. Using these antibody, Wu et al. were able to show that chBMDM grown with chicken CSF1 express CSF1 at their cell surface which is not observed in murine chBMDM [54]. MAb against chicken Tim4, a receptor involved in apoptotic cell recognition, can detect tissue-resident macrophages, a subpopulation of blood monocytes, and yolk sac-derived macrophages [17].

Although the number of myeloid-specific mAb is limited, several antigens have been identified which are simultaneously expressed on monocyte/macrophages, cDC, and on other leukocytes. A range of mAb was used to demonstrate MHC class II antigen expression on monocytes and macrophages, including mAb C1a-1 [138], 21-1A6 [139], P2M11 [120], and 2G11 [113]. MHC class II antigen expression was shown for myeloid cells of chickens [133,141], quail, and turkeys [113]. In addition, monocytes/macrophages were found to be MHC class I positive [140] to express CD40 [130], CD44 [112] and CD45 [112,119], and the IL-15 receptor alpha chain [141]. Attempts to demonstrate CD4 expression on chicken monocytes, analogous to its expression on human monocytes, have failed [48]. Many monocytes/macrophage and DC-associated genes have been studied in the chicken, such as CD14 [142], CD83 [104,143], CD200R [144], CD205 (DEC205) [104], CD208 (DC-LAMP) [145], TNFRSF11A [146], and galectin-8 [147] molecules; all of which show expression on myeloid cells in addition to other cell subsets.

Membrane-bound C-type lectins can be analyzed by their intrinsic nature to bind to glycans without the need for mAb production. For example, avian influenza glycans, *N*-acetylglucosamine (GlcNAc), and *N*-acetylgalactosamine (GalNAc) interact with Ca^{2+} -dependent C-type lectins expressed by chBMDC [148]. Recently, a

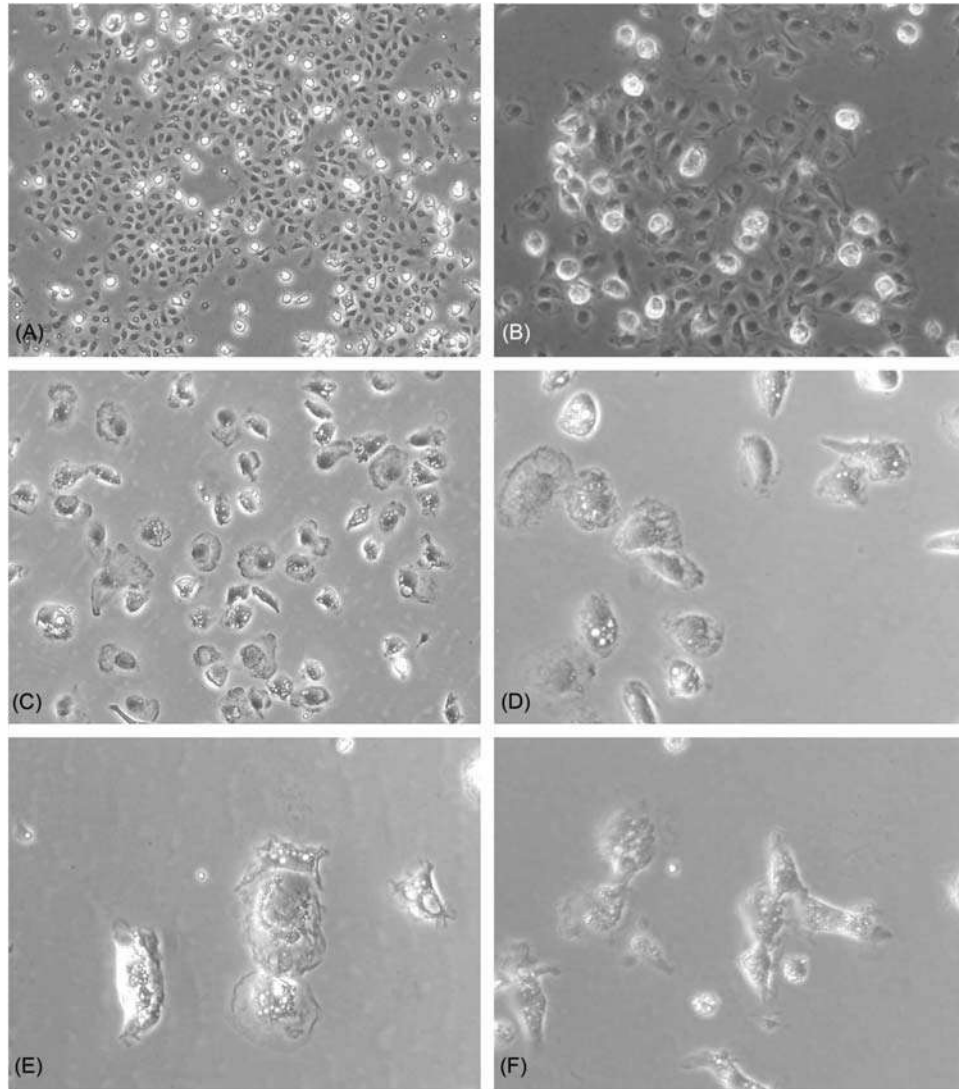


FIGURE 8.1.4 (A) Phase contrast image of monocyte-derived macrophages showing an overnight culture of Ficoll-separated peripheral blood mononuclear cells after removal of non-adherent cells. Small gray cells in the background represent firmly adherent thrombocytes; bright round cells are loosely adherent monocytes. (B) Same culture as in (A) at higher magnification. (C) Phase contrast image of monocyte-derived macrophages showing a 72-h culture of non-activated cells. (D) Phase contrast image of monocyte-derived macrophages showing a 72-h culture of IFN-activated cells. (E) Same culture as in (D) at higher magnification. (F) Phase contrast image of monocyte-derived macrophages showing 72-h culture of LPS-activated cells; same magnification as in part (E).

thorough analysis of the chicken genome for C-type lectins demonstrated that chickens have a vastly different repertoire compared to mammals sharing only a few orthologues [149]. This group also shows that chBMDC can increase their phagocytosis capacity after treatment with the β -1-6-(D)-glucan, pustulan which targets Dectin-1 in mammals but the orthologue of Dectin-1 has not been identified in the chicken genome [150]. Chicken DC-SIGN was annotated based on comparison with the human homolog [151] and its expression was measured by qRT-PCR in relation to AIV glycan recognition [148]. However, a thorough synteny, reciprocal BLASTp search,

and phylogenetic analysis demonstrate that annotated chicken DC-SIGN is in fact chicken hepatic lectin that is highly expressed in the liver [149]. Comparative gene profiling analysis between chicken and mammalian monocytes/macrophages and cDC1 have shown the conservation of a number of key surface genes, such as *FLT3*, *XCRI*, *CADMI*, *MARCO*, *TLR4*, and *TLR2A* [16,106]. The generation of mAb to these gene products or the development of transgenic animals along with defining the pattern-associated molecular targets (PAMP) for chicken C-type lectins will advance our understanding of avian macrophage and DC biology.

8.1.1.8 Characterization of macrophages and DC in tissue sections

Both mAbs and *CSF1R*-reporter transgenic animals have been used to analyze macrophages and to a limited extent cDC in a range of tissues and in a variety of in situ pathological settings, during ontogeny and organ development. A general discussion on the distribution and morphological features of these tissue macrophages can be found in Chapter 2, and more specific discussions are provided for the mucosal tissues in Chapters 11.1–11.3. Most of these studies have used the previously described mAbs CVI-ChNL-68.1 [119], CVI-ChNL-74.2, and CVI-ChNL-74.3 [118] as well as mAb KUL01, whereas recent studies have used a combination of these mAb with Tim4, CSF1R, or with the *CSF1R*-reporter transgenic birds to identify macrophages and cDCs in situ.

In spleen sections, CVI-ChNL-68.1 shows a specific reaction with red pulp macrophages, interdigitating cells, and monocytes, whereas CVI-ChNL-74.2 identifies red pulp macrophages and a splenic macrophage population called ellipsoid-associated macrophages that reside at the interface between periellipsoidal white pulp and red pulp, a region equivalent to the mammalian marginal zone [118,152]. The ellipsoidal-associated macrophages also express MRC1L-B and TIM4 [17]. A highly specific staining pattern was found for CVI-ChNL-74.3, which reacts with FDCs of the germinal centers [118]. These cells lack CD45 expression [153] and are positive for staining with mAb S-100 [154] or through specific binding of IgY [51,127] and immune complexes postimmunization [152–156]. These cells were also found to express high levels of the *CSF1R*-transgene [25,51]. In spleen and lung sections, TIM4⁺ macrophages outline the germinal centers which are structurally similar to mammalian metallophilic macrophages. TIM4⁺ macrophages are also intermingled with B cells inside germinal centers where they engulf B-cell debris which are equivalent to tingible-body macrophages [17,51].

Using the *CSF1R*-reporter transgenic animals, macrophages were found to first appear at ED2 (HH13) and are located in regions, such as the embryo leg bud, where high levels of programmed cell death take place. These cells are abundant for intracellular phagolysosomes where the expression of Tim4 co-localizes, while *CSF1R*-transgene⁺ macrophages residing outside these regions lack phagolysosomes and express Tim4 at the cell surface [17,25]. Staining of tissue macrophages by mAb KUL01 was initially described for spleen, gut, liver, skin, and brain [48] and subsequently by numerous studies investigating the mucosa-associated lymphoid tissue immune response to pathogenic challenge [55–60]. In brain sections, ramified microglial cells can be distinguished by their expression of CD45, MRC1L-B and CSF1R and chB6 [25,48,53,157].

Feather-pulp macrophages can also be defined based on CD45 and *CSF1R*-transgene expression [25].

Chicken Langerhans-like cells were first described as adenosine triphosphate positive and MHC class II⁺ [158,159]. More recent work demonstrated that these cells express the CD45 antigen and have a cytoplasm rich in vimentin-intermediate filaments [160]. These workers also described subpopulations based on MHC class II and CD3 expression: MHC class II⁺/CD3[−], MHC class II[−]/CD3[−], or MHC class II[−]/CD3⁺, with the first two cell types representing Langerhans cells. The MHC class II⁺ Langerhans cells also express MRC1L-B and CSF1R [127]. Other workers have described morphological DCs in various tissues [158,161–163].

Characterization of cDCs in tissues is still limited. Comparative transcriptome analysis of MRC1L-B/MHC class II⁺/CD11c (8F2)⁺ splenocytes shows these cells to be enriched for cDC1-related gene signature. However, as MHC class II and mAb 8F2 bind multiple cell types, making identification of cDC in tissue sections difficult. DEC205⁺ and CD83⁺ cells have been described in the spleen, thymus, bursa, and lung tissues [104]. Balic et al. have generated specific reagents, such as mAb FLT3 and fluorescently conjugated XCL1 which binds to XCR1 expressing cells, that allow for the discrimination between cDC and macrophages in chickens which in future will progress the field of chicken DC biology [164] (Fig. 8.1.4B).

8.1.1.9 Functional properties of chicken macrophages

Macrophages display a series of functional characteristics which allow them to perform their multiple tasks in tissue homeostasis, pathogen recognition and destruction, modulation of innate immune responses, and activation of the adaptive immune system.

8.1.1.10 Macrophage migration

To develop into resident tissue macrophages under normal physiological conditions or during inflammation, monocytes must leave the circulation and migrate to an appropriate location in response to chemotactic signals. The molecules and signals involved in this process are largely undefined for avian macrophages, but it may be assumed that they are similar to those described for their murine and human counterparts, even though the number of inflammatory chemokine receptors encoded in the chicken genome is limited in comparison with the mammalian system [71,74]. Histological studies have, however, demonstrated the adhesion of macrophages to the blood vessel wall and their active migration to sites of infection and inflammation. Dietert and coworkers used Sephadex particles to induce

an inflammatory environment in the abdominal cavity of chickens and turkeys and were able to describe the kinetics of monocyte attraction and their functional maturation to inflammatory macrophages [116,165–167]. In this system, macrophages are rapidly and selectively recruited to the inflammatory site and make up more than 80% and 95% of the adherent cell population in the abdominal exudate samples after 4 and 72 hours, respectively. Since resident macrophages are largely absent on the lung surface (see Chapter 11.2) and in the abdominal cavity, rapid influx of phagocytes into sites of infection may be essential for the efficient control of pathogens in birds [168]. The response of chicken monocytes to chemotactic signals and its inhibition was first shown by classical migration inhibition assays [169] and subsequently in vitro experiments with the synthetic signal peptide f-MLP [170]. Chicken macrophage migration inhibition factor was recently cloned, and its functional properties were described. Like its mammalian orthologue, this cytokine potently inhibited monocyte migration in vitro assays [171]. Migration assays on ex vivo MRC1L-B^{hi}/MHCII^{lo} cells show their CCR5-dependent migratory pattern [52]. Beyond that, information regarding the expression of chemokine receptors and chemokines is lacking, and efforts are needed to identify these interacting molecules in macrophages and DCs in the future. The recent description of the chicken chemokine and chemokine receptor families [71,172] (see Chapter 9) should enable cloning of these genes and the development of experimental tools, such as recombinant chemokines and chemokine receptor-specific mAbs.

8.1.1.11 Phagocytosis

Phagocytosis is perhaps the best known and, in evolutionary terms, most conserved function of macrophages that have been used to identify these cells in situ and to discriminate them from other leukocytes in mixed-cell preparations. Phagocytosis of particles and microorganisms is mediated by specific cell surface receptors which have been characterized in great detail for mammals [173].

The earliest macrophages appear in the yolk sac at HH13 [80] which was confirmed in *CSF1R*-reporter transgenic chickens. These cells were confined to the lumen of primitive blood vessels [25]. In chicken embryos, macrophages with phagocytic activity have been observed as early as embryonic incubation day (EID) 12 in the liver and EID 16 in the spleen [174]. Elicited macrophages can be obtained in day-old chicks and turkey poults [89] demonstrating that this part of the innate immune system is functional at hatch. Cultured monocyte-derived macrophages, Sephadex-elicited macrophages, tissue-derived macrophages, and cells of the macrophage cell lines HD11, MQ-NCSU, and HTC spontaneously phagocytose SRBC, fluorescent microbeads, bacteria, or other particulate matter to different

degrees [107,111,112,165]. (Fig. 8.1.2D and E). Although elicited macrophages harvested early after inflammatory stimulation of the abdominal cavity are efficient at phagocytosis, this can be significantly increased if cells are collected at a later time, indicating that macrophages undergo functional maturation in response to inflammatory stimuli [165]. Interestingly, phagocytosis of nonopsonized particles is restricted to a subpopulation of these cells, reflecting the functional heterogeneity not only of primary cell cultures [89] but also of macrophage cell lines [111,112] and resident tissue macrophages. In cultures of elicited macrophages, the number of phagocytic cells can be increased from 50% to more than 90% by opsonization with antibodies. This effect correlates with the expression level of Fc receptors, as analyzed by the SRBC-rosette assay [89].

Phagocytosis by primary macrophages has also been demonstrated with a range of bacterial species, including *S. enterica*, [90,175], *E. coli* [176,177,181], *Campylobacter jejuni* [87], and *Pasteurella multocida* [178], and the fungal pathogen *C. albicans* [179]. It is influenced by different immunomodulators [180,181]. Opsonization clearly increases phagocytotic activity in vitro for both bacteria [87] and fungi [90,179]. Although a primary task of macrophages is to take up bacteria, many studies have not controlled for the fact that the bacteria also actively invade cells and claim phagocytosis without measuring uptake of killed bacteria [181].

Binding of microorganisms prior to phagocytosis requires receptor-mediated recognition. Macrophages are equipped with a range of receptor systems such as scavenger receptors (SR), complement receptors, Fc receptors, C-type lectins, and mannose receptors mediating opsonic and nonopsonic recognition [173,186]. Functional studies strongly indicate the presence of some of these molecules on primary avian macrophages and macrophage cell lines. SRBC opsonized with anti-SRBC serum from chickens or quail strongly adhere to cultured macrophages and are more efficiently phagocytosed than nonopsonized erythrocytes [107,111,182]. The observation that *C. albicans* adheres to macrophages and is rapidly phagocytosed led to the functional characterization of the chicken mannose receptor and its ultrastructural localization on the cell surface [103]. Mining of genomic databases identified many protein homologs to mammalian SR, and functional assays using SR ligands demonstrated their relevance in avian macrophage biology [183]. MAb with specificity for members of these receptor families are lacking, with the exception of DEC205 [104], MRC1L-B (recognized by mAb KUL01, as described above), and the FcR CHIR-AB1, which is strongly expressed on monocytes and macrophages [184].

Detection and uptake of microorganisms induce the activation of effector mechanisms which, in most cases, leads to pathogen destruction. As already mentioned, during phagocytosis particles are internalized into phagosomes which subsequently fuse with lysosomes to form a

phagolysosome. Lysosomes contain a variety of antimicrobial proteins and enzymes, such as acid phosphatase and β -glucuronidase; some of these have also been described for avian macrophages [185,186]. Histochemical demonstration of nonspecific esterase activity has been used to identify macrophages in tissue sections [48,118,187,188]. Lysozyme expressed by chicken macrophages has attracted some attention since it is progressively activated during macrophage differentiation and has therefore been used as a stage-specific marker. Enzyme expression is low in myeloblasts but high in mature macrophages [189,195] where it is expressed constitutively [190]. Furthermore, LPS stimulation of HD11 cells leads to lysozyme gene activation, which may play a role in the antibacterial response of macrophages [191,192].

The clearance of dying cells by macrophages, a process called efferocytosis, is an integral component of tissue hemostasis, resolving inflammation and restoring function of damaged and infected tissues. This process has profound immunomodulatory effects, such as limiting the release of intracellular damage-associated molecular patterns that drive inflammation. During development, programmed cell death plays an important role in embryo development, contributing to the appropriate formation of various organs and structures. In the *CSF1R*-transgenic chickens, macrophages were visualized in areas of programmed cell death such as the interdigit regions of embryo leg buds where they express lysosomes, suggesting their phagocytic function [25]. In posthatch animals, TIM4 Kupffer cells and TIM4 bursal cells play a role in the detection and clearance of apoptotic cells in the steady state [17].

8.1.1.12 Respiratory burst activity

Release of reactive oxygen and nitrogen intermediates has been recognized as an important microbicidal mechanism of activated macrophages. Neutrophils (see Chapter 8.2) and macrophages reduce oxygen to superoxide, a reaction catalyzed by the NADPH oxidase. O_2^- further reacts with itself to form H_2O_2 , from which highly reactive oxidants such as HOCl can be formed [193,194]. Stimulation of elicited chicken macrophages with phorbol ester has been shown to induce the release of high levels of superoxide [166,195,202], an effect also found with HD11 [166,196,202], MQ-NCSU [196,197], and HTC cells [112,197]. Respiratory burst activity can also be induced with phorbol myristic acid, zymosan A, a calcium ionophore [198], and different *Salmonella* serotypes [199]. Respiratory burst activity to *Salmonella gallinarum* is genetically determined and corresponds to a higher rate of elimination of intracellular bacteria [200]. In contrast, a significant impairment of phorbol myristic acid-induced respiratory burst activity was described after infection of HD11 cells with five serovars of *Salmonella* [201].

8.1.1.13 Nitric oxide production: a readout system for avian macrophage activation

In 1987 nitric oxide (NO) was identified as a highly potent antimicrobial effector molecule secreted by activated macrophages [202]. Subsequent work showed that NO is generated from L-arginine, which is converted to L-citrulline and NO by the action of nitric oxide synthase (NOS). NOS exists in three isoforms: the constitutively expressed neuronal NOS (nNOS), the endothelial isoform (eNOS), and the inducible enzyme (iNOS), which is now referred to as NOS II. The constitutive isoform is produced in low amounts and functions as a physiological signaling molecule involved in neurotransmission and regulation of the vascular tone. In contrast, macrophages produce high amounts of NO in response to inflammatory stimuli and cytokines, by upregulation of NOS II activity. Studies using NOS II-deficient mouse models have clearly demonstrated the antimicrobial potential of this highly reactive radical [203]. NO released by macrophages is rapidly converted to NO_2^- and NO_3^- at a ratio of 3:2, making direct measurement of NO technically demanding. In contrast, NO-derived NO_2^- can easily be quantified using the so-called Griess reaction [204], a method widely applied in avian macrophage research.

Dietert and colleagues were the first to demonstrate NO production by chicken macrophages [205]. Stimulation of Sephadex-elicited macrophages or HD11 cells by LPS led to the secretion of maximal NO_2^- levels into the cell culture medium within 24 hours. This response was shown to be L-arginine-dependent, since its depletion completely prevented NO_2^- accumulation, and NOS II activity could be inhibited by L-arginine analogs [205]. The chicken is unable to synthesize L-arginine from ornithine because of a lack of carbamoyl phosphate synthase I and ornithine transcarbamoylase activity in the urea cycle [206,207]. Therefore this metabolic pathway depends on the nutritional supply of L-arginine—an assumption that has been confirmed by the demonstration of impaired NO formation by macrophages from birds kept on an L-arginine-restricted diet [208]. In addition, tryptophan was identified as an important amino acid which, through degradation, influences NO production by the de novo synthesis of nicotinamide adenine dinucleotide [209]. As in mammals, chicken NOS II requires the cofactor tetrahydrobiopterin [210] which is synthesized from GTP via a well-defined enzymatic pathway [211]. NOS II activity and tetrahydrobiopterin synthesis are induced in a coordinated way in chicken macrophages in response to stimulation with LPS or IFN- γ [212]. Molecular cloning of chicken NOS II revealed 66%–70% protein sequence identity with mouse and human NOS II and led to the identification of several conserved transcription factor binding sites. The importance of the nuclear factor-kappa B (NF- κ B) pathway for NOS II

induction by LPS has been clearly demonstrated using NF- κ B inhibitors [213]. This work has subsequently been confirmed by others and extended to demonstrate a common signaling pathway for LPS and CpG oligodeoxynucleotide (ODN)-mediated induction of NOS II involving activated protein kinase C and mitogen-activated protein kinases. A distinctive feature of CpG ODN-mediated NO production is the requirement for clathrin-dependent endocytosis and endosomal maturation, consistent with observations of mammalian macrophages [214].

Because of the simplicity of NO₂⁻ quantification, measurement of NOS II activity in chicken macrophages has become a frequently used method for the analysis of avian macrophage activation. Based on this assay, chicken IFN- γ was identified by functional expression cloning and shown to be the major macrophage-activating factor secreted by mitogen-stimulated lymphocytes [215,216], while chicken IFN- α was finally identified as a true type I IFN with little or no macrophage-activating effects on its own [216,217]. However, chicken IFN- γ and IFN- α potentiate NO secretion by HD11 cells synergistically [218]. Several sources of recombinant IFN- γ have been used successfully to activate chicken macrophages, including *E. coli*- and COS cell-derived IFN- γ [215,216] as well as IFN- γ expressed by the baculovirus system [219]. Similarly, turkey BMDMs do not respond to type I IFN or LPS alone but produce NO if activated by a combination of these factors [220], while turkey IFN- γ is a potent macrophage-activating factor on its own [113]. It should be noted that the latter experiment was performed with a transformed turkey cell line, which may not respond to cytokine treatment in the same way that primary macrophages respond. Nevertheless, these studies have identified IFN- γ as a potent activator of avian macrophages, suggesting an important role for this cytokine in macrophage-mediated protection from pathogenic microorganisms. NO production by chicken macrophages is also induced by IL-4 alone but inhibited by the same cytokine if combined with different TLR agonists (i.e., LPS, LTA, PGN, CpG, or polyI:C), while IL-18 and IL-10 displayed no effect on NOS II activity [221,222].

Monocyte-derived macrophages infected with AVL-J increase their NO production up to 36 hours postinfection [39]. Higher NO concentration is produced in monocyte-derived macrophages infected with *S. gallinarum* G9 compared to *S. typhimurium* 4/74 and *Salmonella* Dublin 3246 infected cells, regardless of whether the bacteria were opsonized or not prior to infection [223]. NO is also important in the control of MDV. The induction of NO by chicken embryonic fibroblast (CEF) stimulated with chIFN- γ and LPS inhibits virus replication [224]. In the same study, higher NO production was demonstrated in CEF-derived from MDV-resistant chicken lines compared to susceptible

chicken lines. Although the mechanisms of inhibition are still to be determined, this study demonstrates that NO interferes with viral replication and is linked to susceptible and resistance to MDV infection [224].

NO secretion has also been used as a readout system for the identification of PAMPs with macrophage-activating activity. From the initial studies on NO biology in chickens, it became clear that LPS is a potent inducer of NO production [205] and NOS II transcription [213]. Interestingly, repeated stimulation of chicken macrophages with LPS in vitro or in vivo induced a complete block of NO production [225]. This macrophage response has already been demonstrated in mammals and is known as endotoxin tolerance. Comparison of LPS derived from different Gram-negative bacteria revealed only moderate [226,227] or no [175] differences in their NO-inducing activities. On the other hand, macrophages obtained from chickens with different genetic backgrounds exhibit significantly different NOS II expression activities in response to LPS, thus defining hyper- and hyporesponder lines [228,229]. This observation has been attributed in part to differences in cell surface expression levels of the LPS receptor, TLR4, using antihuman TLR4 antibodies [226]. However, this interpretation needs further verification with the chicken TLR4-specific tools still to be developed. PAMPs from *C. jejuni* induced significant upregulation of iNOS gene expression but only the outer membrane protein induced the highest NO production in macrophage cell line [230].

Bacterial DNA contains sequence motifs termed CpG ODN, which have immunostimulatory properties in mammals [231]. CpG ODN also induces NOS II activity in chicken macrophage cell lines [232,233,240] and primary chicken macrophage cultures [33,234]. Both the optimal stimulatory sequence motif ODN 2006 for human cells and the optimal mouse ODN 1826 activate chicken HD11 cells, but ODN 1826 appears to be less potent [232,240]. Comparison of several CpG ODN identified the GTCGTT motif as the most active NO inducer [234]. It is interesting to note that while CpG ODN-mediated activation of macrophages and other chicken immune cells [235] has been shown independently in several studies, the mammalian CpG ODN receptor, TLR9, is not present in the conserved syntenic locus in the chicken genome [236]. Functional studies employing reporter gene assays and expression of recombinant chicken TLR helped to describe ligand specificity of chicken TLR3 and TLR7 [237]. The same approach finally identified TLR21 as the CpG DNA receptor in chickens [238,239].

Besides LPS and CpG ODN, lipoteichoic acid, a TLR2 agonist, induced moderate amounts of NO in monocyte-derived macrophages from 3-day-old chicks. In contrast, the tested agonists of TLR2, TLR3, TLR5, TLR7 (Pam3CSK4, polyinositol cytosine, *S. typhimurium*

flagellin and loxoribine, respectively) showed very weak or no stimulating properties [33]. In addition to cytokines and TLR agonists, CD40 ligand-mediated activation of macrophages also leads to NO production [130].

8.1.1.14 Cytokine response of avian macrophages

Macrophages residing in the tissues are the first cells to encounter pathogens. Detection of microorganisms through PRR not only triggers the uptake and destruction of the invaders but induces the secretion of signaling molecules which help to activate additional arms of the immune defense system. These molecules include a range of cytokines and chemokines which act locally but also on a systemic level (details of chicken cytokines and chemokines can be found in Chapter 9).

Early studies addressing cytokine secretion by avian macrophages relied on biological assays for cytokine detection and indicated the release of IL-1 β [240], IL-6 [241], and tumor necrosis factor (TNF)- α -like factor [242,243] in response to inflammatory stimuli. However, a more detailed characterization of the macrophage cytokine response was not achieved until the first avian inflammatory cytokines had been cloned. It is now well established that activation of chicken macrophage cell lines and primary macrophages with different PAMPs induce the release of IL-1 β [244,245] and IL-6 [112,246,247]. Interestingly, attempts to identify an avian homolog of TNF- α in the chicken genome has been problematic for many years despite the repeated demonstration of a TNF-like activity secreted by macrophages [243,248] and the upregulation of a related family member, TL1a, in the spleen in response to LPS treatment. Finally, chicken TNF- α was identified in 2018. It shares 45% sequence similarity with its mammalian counterpart [249]. Comparing chicken TNF- α with other avian species identified the hooded crow (*Corvus cornix*) TNF- α in an 80 kb contig that contained nine additional genes, the orthologs of which lie close to human TNF- α in the MHC locus, suggesting several genes of the MHC complex, including TNF- α , were displaced to a new location in the avian ancestor [249]. Recently, using fluorescent in situ hybridization, TNF- α was located on chromosome 16q [250]. A strong induction of TNF- α mRNA levels occurs in monocyte- and splenic-derived macrophages stimulated with LPS at early time-points of 2–4 hours [249]. Meta-analysis of RNA-seq datasets shows TNF mRNA is induced 59-, 60- and twofold in the chBMDM, chBMDC, and heterophils, respectively, after challenge with LPS for 24 hours. Their cognate receptors, TNFR1 and TNFR2, mRNA expression were unaffected [250]. Recombinant TL1a exhibits cytotoxic activity against the mouse L292 cell line and chicken fibroblasts [251]. In LPS-induced

TNF- α factor treated HD11 cells, TL1a mRNA expression is increased after 24 hours [252]. Another TNF family member, RANKL, has been shown to enhance the mRNA expression levels of proinflammatory cytokines in LPS-stimulated chBMDC [146].

In addition to proinflammatory cytokines, LPS-activated HD11 cells and monocyte-derived macrophages express the antiinflammatory cytokine IL-10 and may thereby exert multiple immunoregulatory effects during infections [253]. RT-PCR and quantitative RT-PCR techniques are increasingly used to investigate the cytokine and chemokine responses of avian macrophages to whole microorganisms and PAMPs. HD11 cells infected with *C. jejuni* responded with significantly increased mRNA levels for IL-1 β and IL-6, and for the two IL-8-like chemokines, 9E3/CEF4 (CXCLi2) and K60 (CXCLi1) [247]. Similarly, increased expression of pro-inflammatory cytokines (IL-1 β and IL-6) and chemokines (CCLi2 and CXCLi1) was observed in macrophages infected with either *S. gallinarum* or *typhimurium*. In this study, significant differences in cytokine expression level and pattern were observed between macrophages derived from resistant and susceptible chicken lines. This included differences in IL-18 mRNA abundance, which may in part explain the resistant phenotype since IL-18 is involved in the induction of IFN- γ , a critical cytokine in the control of *Salmonella* infections [254]. Chicken BMDM cultures derived from MDV-susceptible and resistant birds also observed a significant decrease in IL-18 mRNA expression levels in the susceptible chicken cells after MDV infection [255]. RNA-seq analysis showed that MDV-susceptible chBMDMs upregulate chemokine receptors, interleukins, and TNF superfamily genes, while MDV-resistant cells downregulate TLR, JNK-STAT signaling pathways. This may be a strategy of host cells to induce cells death and thus control MDV infection.

Comparison of macrophage response to different *Salmonella* serovars revealed clear differences in cytokine and chemokine expression patterns [256], the molecular basis of which is still unknown. Analysis of host-specific responses to *C. jejuni* shows that chicken and human macrophages react in a similar fashion to infection, producing proinflammatory cytokines, IL-1 β , and IL-6, which are unaffected by the lack of the capsule or O-methyl phosphoramidate virulence factors which modify the polysaccharide capsule of *C. jejuni* which is vital for colonization and tissue invasion [257]. Numerous studies have analyzed host–pathogen interactions in a diverse range of bacterial [258,259] or viral [260–263] infections demonstrating the induction of pro-inflammatory cytokines by chicken macrophages and their role in the host response to infection.

The induction of IL-1 β , IL-6, IL-18, and chemokine expression seems to be a uniform response pattern of

chicken macrophages to microbial stimuli since it was observed not only in response to bacteria but also after viral [131] and parasitic [264] infection of macrophage cultures. A macrophage-specific complementary (c)DNA microarray has been constructed and used to compare the response of monocyte-derived macrophages to *E. coli* and LPS. Significant changes in the expression levels have been observed for 981 and 243 genes in response to *E. coli* and LPS, respectively, clearly demonstrating the magnitude of macrophage reactions and the problems associated with data analysis and interpretation [265]. This array has also been used to study the macrophage response to *Eimeria* parasites in more detail and has led to the identification of an extended list of chicken cytokines that are differentially regulated after infection with different *Eimeria* species [254,264]. Microarray analyses have been carried out to investigate the chicken macrophage response to LPS [267], *Salmonella enteritidis* [267], and *Mycoplasma synoviae* [258]. These studies reveal the kinetics, immunomodulation, and complexity of chicken macrophage responses to bacterial antigens and organisms.

With the rapid progress in RNA-sequencing technology, more studies were conducted that investigated macrophage response patterns to infection. RNA-seq analysis of monocyte-derived macrophages infected with different serovars of *Salmonella* showed *S. gallinarum* infection induces over 1100 differentially expressed genes (DEGs) compared to the responses of *S. typhimurium* (625 DEGs) and *Salmonella dublin* (656 DEGs) infection providing new insights into the interaction between *Salmonella* and the host and increase understanding of *S. gallinarum* host specificity [223]. Recently, Albers et al. analyzed the transcriptome of APEC infected lung macrophages/DC and found that although APEC infection induced a strong pro-inflammatory response, many immune-related pathways were repressed in APEC02 infected cells compared to APEC01 infected cells [181]. Multiplex qRT-PCR platforms have been developed to evaluate transcription of up to 96 chicken immune response genes. This system can be used to analyze the complex interplay between the avian immune cells and pathogens by including pathogen-specific probes [268]. As progress is being made in developing mAb, transgenic chickens, and sequencing technologies, the functional consequences of antigen-macrophage interactions will be resolved in greater detail.

Macrophages have also been studied intensively in mammals in the context of host–microbiota interaction and the effect of microbiota-derived metabolites on macrophage function and differentiation [269]. Fermentation of dietary fibers by the commensal microbiota generates short-chain fatty acids such as acetate, propionate, and butyrate. The latter received particular attention in chickens since butyrate is added to the diet to improve gut

health under commercial conditions. Butyrate induced the antimicrobial activity of macrophages through upregulation of host defense peptides, while at the same time, barely triggers pro-inflammatory cytokine expression [270] in monocytes/macrophages and HD11 cells. Efforts are underway to identify new components inducing antimicrobial peptides as alternatives to antibiotics [271].

8.1.2 Functional properties of chicken antigen-presenting cells

8.1.2.1 Maturation from antigen sampling to antigen presenting

There is an ongoing debate as to the relative importance of monocytes, macrophages, and cDCs as specialized APC depending on the choice of definitions and surface markers. Chicken cDCs have been identified at a gene transcription level [16,17,106], but as yet there are no markers that definitively identify these cells. Functional studies have not been reported quantifying the relative antigen-presenting capacity of different chicken APC subsets. Nevertheless, as this is a rapidly developing field of research and cell lineage-specific markers and functional studies are likely to be published in the near future. As such it should be stressed that the term “dendritic cell” includes a number of ontogenically distinct (and potentially functionally distinct) chicken myeloid subsets of cell lineages cell, such as cDCs, chBMDC, monocyte-derived DCs (MoDCs), FDCs, bursal secretory dendritic cells (BSDCs), etc. The development and function of these cell lineages is largely unknown, but here we present the current state-of-the-art.

When generated in vitro from bone marrow hematopoietic precursors or peripheral blood monocytes with different cytokine or cytokine combinations, immature DCs and mature DCs are defined in mammals by their distinct morphology, phenotype, and function, which mirrors the development in vivo.

Wu et al. described the efficient generation of chicken bone marrow-derived DCs from bone marrow precursors. Bone marrow cells were cultured in the presence of recombinant chicken GM-CSF and IL-4 for 7 days [102]. The cultured cells had the morphology of typical mammalian BMDC. Most of the nonstimulated cultured cells showed high surface expression of MHC class II and putative CD11c, moderate or low levels of costimulatory molecules, and lacked surface expression of CD83 or DEC205. The stimulated cells actively endocytosed FITC-dextran and phagocytosed fluorescent latex beads but were poor stimulators of PBL in MLR. Based on these criteria, the cells were defined as immature chBMDC.

Immature chBMDC can be induced in vitro to mature chBMDC with many different stimuli [102]. In the same

study, LPS and chicken CD40L were used to stimulate chBMDC to investigate if they drive the maturation of immature chBMDC. LPS- and CD40L-stimulated chBMDC had elevated surface expression levels of costimulatory molecules (CD40 and CD86), CD83, and DEC205, compared to nonstimulated chBMDCs. In mammalian species, these criteria are used to characterize mature BMDCs. As in mammals, chicken IL-21 blocks the maturation of chBMDC [272].

Receptor-mediated endocytosis of dextran depends on the expression of the mannose receptor, which in mammals is expressed at high levels on immature DCs and is low or absent on mature DCs [102]. In this study, LPS- or CD40L-stimulated mature chBMDCs showed a significant decrease in their capability to uptake FITC-dextran compared with that of immature nonstimulated chBMDCs. Immature chBMDCs stain at high levels with the mAb KUL01 [146], which recognizes the chicken mannose receptor (see earlier). LPS- or CD40L-stimulated mature chBMDCs lacked phagocytotic capacity as measured by uptake of fluorescent microbeads. Together, these results suggest that, upon maturation, chBMDCs lose the ability to phagocytose but maintain a reduced capacity for endocytosis.

In vitro, mammalian DCs (including cDCs, BMDCs, and MoDCs) can induce a MLR [273–275]. The most striking difference between DCs and macrophages is that only DCs are effective stimulators in syngeneic MLR. In vivo, DCs require between 10^3 and 10^6 less antigen than other APCs to drive T-cell proliferation [275]. In chickens, nonstimulated chBMDC can induce both allogeneic and syngeneic MLRs. After LPS or CD40L stimulation, chBMDC dramatically enhanced their allostimulatory capacity. This demonstrates that the chBMDCs internalized and processed antigen for presentation to T cells in vitro and ultimately makes the strongest case that immunostimulatory chBMDCs were indeed produced by this method. LPS and CD40L were therefore shown to be strong inducers of functional maturation of chBMDC [102]. Splenic-derived conventional DC also induce significantly higher mRNA levels of IFN- γ compared to splenic-derived macrophages in allogeneic MLR demonstrating that similar to mice, chicken cDCs are effective T-cell stimulators [16].

Polarized Th1 and Th2 reactions occur in the chicken in response to challenge with intracellular and extracellular pathogens, respectively [276]. ChBMDC matured with LPS or CD40L have a Th1-promoting phenotype, as determined by increased high-level expression of IL-12 α and IFN- γ mRNA and decreased levels of IL-4 mRNA. A number of groups have shown that exposure of chBMDC to various pathogens or pathogen-derived molecules modulated inflammatory cytokine and MHC class II gene expression including, Newcastle disease virus [277], ALV [278], IBDV [279], LPS [280], and CpG oligonucleotides

[281]. Lamont et al. [282] showed that chBMDC from Leghorn and Fayoumi chickens exhibit differences in the expression of the immune-related genes CCL4, CCL5, CD40, GM-CSF, IFN- γ , IL-10, IL-12 β , IL-1 β , IL-6, IL-8, and iNOS when exposed to the same levels of LPS.

Vervelde et al. [283] investigated the role of chBMDC in response to AIV. AIV infects chBMDC in vitro, but there were differences in replication between low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) viruses; viral RNA levels of the former did not increase with time, whereas levels did increase for HPAI. The HPAI viruses, but not the LPAI viruses, induced strong inflammatory responses in the infected DCs, leading to the hypothesis that HPAI infection may deregulate the immune response through overactivation of DC. These data show that not only can chBMDC be used to study immune responses to different pathogens but can also be used to detect innate differences in immune response between different chicken breeds or between different strains of the same pathogen.

More recently, Dalgaard et al. [150] showed that exposure to the β -1-6-glucan, pustulan upregulated MHC class II expression and inflammatory cytokine expression in chBMDC. Furthermore, pustulan pulsed chBMDC promoted ex vivo CD4 $^+$ but not CD8 $^+$ T-cell recall response to IBV, indicating that this approach can enhance the efficacy of MHC class II antigen presentation, but not the antigen cross-presentation via MHC class I.

The potential for DC to drive protective immune responses has been tested in the context of *Eimeria tenella* infection [284]. It is not clear if these “DCs” are cDC, another cell population entirely, or a mixture of myeloid APCs due to the lack of definitive cDC markers in the chicken. Nevertheless, intestinal *Eimeria* Ag-positive, CD45 $^+$ DCs were isolated from infected chickens and loaded ex vivo with parasite antigen (Ag). Chickens were then vaccinated intramuscularly with either Ag-pulsed DC or Ag-pulsed DC-derived exosomes and subsequently challenged with *E. tenella*. Both vaccines gave increased immune responses and protected against challenge [284].

8.1.2.2 Migration

The epidermal DCs described by Igyarto et al. [160] can be mobilized from the epidermis by hapten treatment of the epidermis in vivo. This resulted in a decrease in the number of the Langerhans cells in the dermis and the concurrent appearance of hapten-positive cells in the so-called dermal lymphoid nodules. This suggests that these dermal nodules maintain some regional immunological function similar to mammalian lymph nodes.

The location of antigen presentation in avian species which lack mammalian-like lymph nodes remains to be fully elucidated. Although the spleen has a role in

mounting responses to antigens administered intravenously, responses to antigens given locally appear to be initiated elsewhere [285]. Early work described the presence of lymphoid nodules in the chicken [285,286] and subsequent studies have identified a number of novel lymphoid structures and aggregates, including the lung (also see Chapter 11.2) [287–290]. Recently, a large number of cells with a cDC1 transcriptome were identified in the chicken liver. In the mouse liver, cDC1 make up a small proportion of cells [291] suggesting the chicken liver might be an important site of Ag recognition and presentation in birds [17]. Their role in mounting immune responses remains to be determined.

In mammals, the CC chemokine receptors 6 and 7 (CCR6 and CCR7) are important for the control of the trafficking of cDCs. CCR6 is expressed primarily on immature DCs in the periphery and plays a role in the recruitment of immature DCs to sites of potential antigen entry. DC maturation involves downregulation of CCR6 but upregulation of CCR7. Both chicken CCR6 and CCR7 were expressed at the mRNA level in immature chBMDC, as measured by qRT-PCR [292]. After chBMDC maturation, following stimulation with LPS or CD40L, expression levels of CCR6 mRNA were downregulated, whereas those of CCR7 were upregulated, suggesting that these two chemokine receptors play a similar role in the trafficking of chicken DCs as they do in mammals.

8.1.2.3 Other nonmyeloid antigen-presenting cells

Murine FDCs were first observed in 1965 when Mitchell and Abbott identified labeled *Salmonella* Adelaide in the lymph node. These cells were described as having fine processes that trapped antigen at the surface which is why they were believed to be a phagocytic cell [293]. In later studies it became evident that mammalian FDC lacked MHC class II expression, a molecule expressed at high levels by conventional, hematopoietic DCs and bind processed and unprocessed antigens that associate with their dendritic processes [51] from which the name, FDC, was derived [294]. To date, few studies have been performed on chicken FDC. Similar to mammalian FDC, chicken FDC exhibit numerous filiform and convoluted dendritic processes with electron-dense granules surrounding the nucleus and are located in the germinal centers [295]. FDC express antigen-antibody complexes (known as immune complexes, IC) at their cell surface and have the unique capacity to bind and retain native antigen for long periods of time. Isolated chicken FDC express complement factors C3 and B, and adhesion molecules, VCAM-1, ICAM-1 [295] while in situ they were found to express CSF1R, vimentin and bind to IgY [25,127]. Contrasting

studies have shown chicken FDC to express MHC class II in the spleen [127] while other studies have described their lack of MHC class II expression in the cecal tonsils and the lung [51,295]. Chicken FDC can trap antigens for presentation to B cells [153,296,297].

Based on lineage tracing experiments and their resistance to irradiation, FDC in mammals are understood to develop from mesenchymal stem cells [298,299], while their origin is unclear in chickens. Bursectomized-irradiated chickens lack germinal centers which can be formed in the spleen and cecal tonsils when bursectomized and intact chickens are engrafted with donor bursas [300,301]. These experiments provide evidence that FDC development is a bursa- or possibly a B cell-dependent process as seen in mammals [302].

A highly elongated shape cell with short dendrite-like processes and the presence of cytoplasmic secretory granules was identified in the chicken bursa and based on these features was named BSDC [303]; also see Chapter 2). Immature BSDCs are defined by cytoplasmic granules localized around the cytocentrum, a region close to the nucleus involved in microtubule organization, while in mature BSDC granules are situated beneath the cell membrane of dendritic processes [303]. BSDCs have a similar phenotype to FDC, expressing complement factor/or Fc receptors, vimentin, CVI-ChNL-74.3, MHC class II [304], CSF1R, and CD11c [127]. A general discussion on the distribution and morphological features of BSDC through bursal development can be found in Chapter 2. Chick-quail celom grafting chimeras, parabiosis and yolk sac chimera studies have shown that BSDC originate from CD45 + yolk sac cells [53,127].

8.1.3 Concluding remarks

Avian macrophage biology has been studied in much detail in vitro, utilizing transformed cell lines or primary macrophage cultures. The obvious limitations of cell culture techniques should be kept in mind when interpreting and comparing results. Cell lines are convenient tools for cell biology studies. However, they represent only a single stage of differentiation within the myelomonocytic lineage, and cell lines representing a range of differentiation states are not available. Furthermore, transformation has a significant impact on the functional properties of such cells, and results obtained from studies with cell lines may not properly reflect macrophage function. On the other hand, primary macrophage cultures are more likely to be heterogeneous, and isolation of these cells should lead to their activation and differentiation. Growing out macrophages from bone marrow with recombinant chicken CSF-1 may provide a more homogeneous population that is unlikely to be activated, as this method reflects the normal generation of macrophages in the body during homeostasis.

Finally, the efficacy of monocyte and macrophage isolation and cellular responses may be different when using different chicken lines, as demonstrated in the *Salmonella* model [254]. As the repertoire of readout systems for macrophage and DC function increases, with progress in gene identification and gene expression technology, our understanding of the role of these cells in the avian immune system, and whether they represent distinct cell types or if DCs are just a specialized subset of macrophages, will improve. Importantly, in future, new tools and techniques will need to be developed that will enable specific identification of macrophage and DC subsets in situ, and functional studies will need to be done in vivo by depletion, transgene technologies or gene-silencing approaches. Recent advances in the production of tools for the identification and generation of avian macrophages and DCs presage a rapid expansion of knowledge in this crucial area of avian immunobiology.

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Avian granulocytes

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Chickens have a reduced repertoire of polymorphonuclear cells with eosinophils and basophils being effectively replaced by a single cell type, the heterophil, which is the predominant cell responding to innate inflammatory reactions in the chicken. Lacking functional eosinophils [1] and IgE, chickens lack components of the antihelminthic worm and allergic responses that are controlled by Th2 cytokines in mammals and thus may have reduced requirement for this class of cytokines.

Mast cells: A high density of mast cells in the thymus of chicken embryos suggests their possible role in thymic development [2]. Using histochemical techniques and light microscopy, mast cells have been identified in lymphoid organs (spleen, thymus, and bursa of Fabricius) of chickens from hatch through 120 days posthatch [3]. The number of mast cells appears to be age-related, but their role in the lymphoid tissues is still undetermined.

Heterophils: Heterophils are the most numerous granulocytic leukocyte in the peripheral blood characterized by a multilobes nucleus and intracellular granules [4,5]. As effector cells, heterophils rapidly (within minutes) migrate from the peripheral blood to the site of infection/injury [6,7]. Heterophils are specialized white blood cells, which are the primary components of innate immunity. Considered the avian equivalent to mammalian neutrophils, heterophils respond to similar microbial stimuli, exhibit chemotaxis, phagocytize microbial pathogens, and produce cytokines and metabolites to maintain immune homeostasis. Heterophils are involved in all roles played by the innate immune response ranging from inflammation and defense against pathogens to wound healing and tissue remodeling. As the “first responders” to bacterial infection and tissue injury, heterophils initiate the acute inflammatory response initially by recognizing and killing bacterial pathogens through the release of an arsenal of antimicrobial mediators including oxygen radicals, proteolytic enzymes, and antimicrobial peptides (AMPs) and then initiate injury repair by the activation of intracellular

cell signaling cascade resulting in the expression of effector molecules including chemokines and cytokines [8–11].

Heterophils initiate the innate immune response by recognizing conserved molecular signatures shared and produced by a large group of microbes and called microbial-associated molecular patterns (MAMPs). Heterophil recognition of these MAMPs is mediated by transmembrane or cytoplasmic proteins called pattern recognition receptors (PRRs) [12]. Innate immune recognition of MAMPs initiates a signal transduction cascade that leads to the transcription of immune response genes and the production of various effector molecules [11].

8.2.1 Functional activities of heterophils

Age-related differences in heterophil function have been noted in neonatal chickens and turkeys [13,14]. These functional deficiencies, including phagocytosis, oxidative burst, and degranulation, last up to 14 days posthatch in chickens [14] and 21 days in turkeys [13]. However, we then demonstrated that heterophils from neonatal chickens can respond to cytokine modulation [chicken interleukin-2 (IL-2) and chicken interferon-gamma (IFN- γ)] with enhanced functional competence [15,16].

The host defense peptide, cathelicidin-2 (CATH-2), was constitutively expressed exclusively only in heterophils beginning on day 18 of embryogenesis and decreased after hatching in the spleen, bursa, small intestine, and lung [17]. However, posthatch hematopoiesis begins in the bone marrow and a very high number of heterophils are then found in the circulating blood. The authors suggest that the presence of CATH-2 in heterophils “is strategically located to respond to infectious stimuli in early embryonic and early post-hatch life” [17].

Phagocytosis. Heterophils circulate in the blood where they are the first cells to respond to tissue injury and infection [6]. At the site, heterophils function to contain

and clear injurious stimuli. Phagocytosis is an active, receptor-mediated internalization of pathogens. Initially, receptors expressed on the phagocytes recognize ligands on the surface of the pathogen. Heterophils express specific but redundant receptors such as Fc receptors that bind antibodies and complement receptors (CRs) that bind complement components, which mediate opsonization-dependent phagocytosis, while receptors such as dectin-1, scavenger receptors, and mannose receptors mediate non-opsonic phagocytosis [18]. Recognition of the ligand by the receptor initiates a series of signal transduction pathways that induce the remodeling of the actin cytoskeleton to form membrane extensions that surround the pathogen. Once phagocytized, pathogens are entrapped within a membrane-bound vacuole called the phagosome that triggers the onset of fusion of cytoplasmic granules with the phagosome resulting in the killing of entrapped pathogens via microbiocidal substances produced by the generation of an oxidative burst or degranulation and liberation of antimicrobial products.

Oxidative burst. Functionally, heterophils are similar to the mammalian neutrophil, but heterophils lack specific proteolytic enzymes, including myeloperoxidase, catalase, and alkaline phosphatase, normally found in neutrophils [19–22]. Heterophils have a limited oxidative burst ability because they lack a hydrogen peroxide (H_2O_2) disposal system (myeloperoxidase and catalase-mediated); thereby, relying solely on a glutathione peroxidase disposal system [19,23–25]. However, despite their lack of myeloperoxidase heterophils do undergo oxidative burst in response to numerous stimuli [15,26–28] generating O_2^- and subsequently H_2O_2 . Other reactive oxygen species, like halide derivatives, are not generated and the amount of H_2O_2 produced is much lower than that produced by neutrophils [29,30].

Degranulation. Due to their lack of myeloperoxidase, heterophils rely on nonoxidative mechanisms for antimicrobial activity. Heterophils kill bacteria with the release of proteins from intracellular granules in a process referred to as degranulation. The released granules contain molecules involved in inflammation and innate host defenses including metalloproteinase enzymes, β -glucuronidase, lysozyme, acid phosphatase, phosphorylase, acid α -glucosidases, and AMPs [31–33]. Of the 14-known chicken β -defensins, only gallinacin-1 and -2 have been identified in heterophil granules [34–36]. Further, cathelicidin-1 and -2 are also present in heterophil granules and are released when the cells are stimulated [37]. A recent study using liquid chromatography and mass spectrophotometry analysis of heterophils from chickens injected with *Salmonella* showed that these cells specifically expressed a number of granular proteins including the antimicrobial proteins lysozyme-1 and cathelicidin-1, -2, and -3 that were released following the activation of a number of endoplasmic reticulum proteins [38].

In chicken heterophils, direct evidence of degranulation has been reported by measuring β -glucuronidase release in an in vitro assay system [15,39,40]. Administration of Toll-like receptor (TLR) agonists unmethylated CpG oligonucleotides (CpG ODN), peptidoglycan, lipopolysaccharide (LPS), flagellin (FLG), and lipoteichoic acid (LTA) have all been shown to induce heterophil degranulation [32,41,42]. Likewise, opsonized and nonopsonized bacteria induce degranulation of β -glucuronidase [15,39]. Degranulation of heterophils was significantly inhibited by an inhibitor of p38 mitogen-activated protein kinase (MAPK) and phosphoinositol-3 kinase (PI-3K) signaling pathways [16,39].

Antimicrobial peptides. Defensins are cysteine-rich, antimicrobial peptides found in plants, insects, birds, and mammals and have two main functions. The first is to bind microbial cell membranes and form pores leading to the death of the microbe. The second function of defensins is to chemo-attract cells of the innate immune system with roles in the microbial attack including macrophages and mast cells [42]. Defensins are species-specific with only the β -defensin isoform is found in birds [42]. Beta-defensins are either constitutively expressed in granules of heterophils [34,35,43] or inducible in epithelial cells in response to signaling from PPRs [1].

Extracellular traps. Like neutrophils, heterophils generate extracellular traps (HETs) [44,45], which contributes to the overall microbiocidal mechanisms of these immune cells. HETs serve as a “deathtrap” [45] and allow the heterophil to capture and kill pathogenic microbes outside the cell. The first description of HETs found that stimulation in vitro with either hydrogen peroxide or phorbol myristate acetate induced the production of HETs that contained DNA, elastase, and histone-DNA complexes [44,46]. Further studies have shown that infective laryngotracheitis virus-induced HETs in the infected trachea of chickens [47]. The in vitro exposure of heterophils to two different serovars of *Salmonella enterica* (serovar Enteritidis and Infantis) generated HETs, whereas in vivo neither microbe induced HETs in the cecum [44]. Ochratoxin A, a toxin produced by *Aspergillus* and *Penicillium* species, induced a reactive oxygen-dependent formation of HETs that was mediated by the activation of both the p38 and ERK MAPK signaling pathways [48]. Most recently, it was shown that in vitro exposure of *Eimeria tenella* sporozoites to heterophils prompted the formation of HETs composed of histone- and elastin-DNA complexes that were regulated by p38 and Rac1 signaling pathways [49]. However, the authors were not able to determine how this killing mechanism may function in vivo during a coccidial infection.

8.2.2 Receptors

Pattern recognition receptors. Heterophils mediate the innate immune response by recognizing conserved molecular signatures shared and produced by a large group of

microbes and called MAMPs or danger signals in the environment, that is, danger-associated molecular patterns (DAMPs). Heterophil recognition of these MAMPs/DAMPs is mediated by transmembrane or cytoplasmic proteins called PRRs [12]. Innate immune recognition of MAMPs initiates a signal transduction cascade that leads to the transcription of immune response genes through nuclear factor- κ B (NF- κ B) and the production of various effector molecules (see below and Chapter 8.6).

TLRs. TLRs consist of a leucine-rich repeat domain that specifically binds to individual MAMPs, a transmembrane region, and a cytoplasmic Toll/IL-1 receptor domain [50]. According to Keestra and colleagues [51], the chicken homologs of the human TLRs are TLR1 type 1, TLR1 type 2, TLR2 type 1, TLR2 type 2, TLR3, TLR4, TLR5, TLR7, as well two that are unique for chickens, TLR15, and TLR21 [52–57] (see Chapter 8.6). Heterophils constitutively express 8 of the 10 known chicken TLRs which, when stimulated with specific TLR agonists, functionally activate heterophil oxidative burst and degranulation and induce the upregulation of expression of pro-inflammatory cytokines (IL1 β , IL6) and chemokines (CXCLi2, CCLi4) [9,28,41]. Moreover, heterophils binding of TLR4 and TLR5 by LPS and FLG activate the small GTPase Ras family thereby inducing differential signaling cascades to activate the downstream extracellular signal-regulated kinase mitogen activated protein kinase (ERK MAP kinase) [56]. The differential activation of the small GTPases may play a role in inducing the effector functions of heterophils stimulated by LPS and FLG, with the LPS/Ras pathway inhibiting apoptosis and inducing the respiratory burst and the FLG/Rap1 pathway mediating cell adhesion, phagocytosis, and degranulation. In chickens, TLR activation is followed by increased transcription of cytokines, including IL-1 β and IL-6, and chemokines, including CXCLi2, CXCLi1, and CCLi4 [9,41,57]. Additionally, stimulation of heterophils with LTA, which is found in the cell walls of gram-positive bacteria, activates TLR2 and drives an increased oxidative burst in heterophils [27].

The two chicken-specific TLRs, TLR15, and TLR21 have been identified and characterized. TLR21 is the functional homolog of TLR9 in humans that recognizes bacterial CpG DNA [58]. TLR21 activation by CpG oligodeoxynucleotide induces heterophil degranulation in vitro [32] and in vivo primed heterophils degranulation and oxidative burst [59,60].

Stimulation of chicken heterophils with the TLR agonists FLG (TLR5) and LPS (TLR4) induced the activation of all three members of the MAPK family, ERK, c-Jun, and p38 that then induced the translocation and activation of the transcription factors NF- κ B and AP-1 resulting in the increased expression of IL-6 and CXCLi2 [9,41,57].

C-type lectin receptors (CLRs). Nerren and Kogut [61] were the first to report that the CLR, dectin-1, the primary PRR for exogenous β -glucan, a component of fungal and bacterial cell walls, respond to a Dectin-1-specific agonist by significantly increased production of an oxidative burst. Further, we have shown that administration of β -glucan as a feed additive resulted in increased phagocytosis, oxidative burst, and bactericidal capacity of chicken heterophils, resulting in significantly reduced organ invasion by *Salmonella enteritidis* [62].

8.2.3 Other innate immune receptors

Triggering receptors expressed on myeloid. A class of innate receptors called the triggering receptors expressed on myeloid cells (TREM) have been found in the chicken genome and consists of one activating gene (TREM-A1) and two inhibitory genes (TREM-B1 and TREM-B2). The effect of TREM-A1 receptor ligation using a specific agonistic antibody on chicken heterophil functions has been described [63]. Activation via TREM-A1 induces a significant increase in phagocytosis of *S. enteritidis*, a rapid degranulation, and a dramatic upregulation in gene expression of the pro-inflammatory cytokine, IL-6, and the inflammatory chemokine, CXCLi2. However, we found no direct TREM-A1 stimulation of the heterophil oxidative burst [63]. However, avian TREM-A1 ligation synergizes with the activation of TLR4 ligand, LPS. In addition, the synergistic activity of LPS and TREM-A1 resulted in increased production of an oxidative burst in heterophils demonstrating that TREM-A1 in chickens acts in synergy with TLR to induced differential function activation of the heterophil.

Complement and Fc receptors. Phagocytosis is a multistep process involving particle attachment to the cell membrane and ingestion of the particle. Phagocytosis is triggered by the binding of microbes through either receptor for opsonized particles or nonspecific glycosylated receptors that recognize lectins on target microbes [64,65]. Phagocytic efficiency is dependent on these opsonins bound to the surface of the particle. In serum, the main opsonins are IgY and complement fragments which, when attached to a microbe, can be recognized by specific surface receptors on the phagocytic cell. These specific receptors are the CRs that recognize complement fragments and the Fc receptor which recognizes the carboxyl terminus of an antibody molecule [15,64,65]. The interactions between the surface-bound opsonins and the specific receptors initiate a cascade of biochemical reactions in the phagocyte resulting in the activation of cellular defense mechanisms, ingestion of the ligand, and killing of the pathogen [15].

Heterophil Fc receptors cross-linking with IgY-bacterial complexes induces both an oxidative burst and

degranulation [15]. Interestingly, the signaling pathways activated by the Fc receptor-mediated functions were different with the oxidative burst being a pertussis toxin-sensitive, Ca^{2+} -dependent, G protein-, and protein kinase C-dependent protein phosphorylation event [15]. However, Fc receptor activation of heterophil degranulation activated Syk tyrosine kinase that stimulates the downstream phosphorylation of p38 MAPK, phospholipase C, and phosphatidylinositol-3 kinase pathways [66].

CRs recognize complement fragments in serum that bind to surface structures on bacterial pathogens [65]. Initial studies looking at CR stimulation of an oxidative burst and degranulation by chicken heterophils were regulated by distinct signal transduction pathways. Specifically, engaging CR on chicken heterophils activated the proximal tyrosine kinases (*Src* and *Syk*) and stimulated downstream phosphorylation of phospholipase C, phospholipase D, phosphoinositol 3'-kinase, and JNK MAPK to specifically induce degranulation [39]. The induction of the oxidative burst by the stimulation of CR was mediated by a protein kinase C-dependent, tyrosine kinase-dependent phosphorylation pathway [15].

Scavenger receptors. The scavenger receptor superfamily includes eight different subclasses of functionally and structurally divergent cell surface and secreted proteins involved in innate immunity especially their ability to bind a range of extremely diverse ligands, including both Gram-positive and Gram-negative bacteria, a variety of polyanions including the bacterial cell wall components LPS and LTA, DNA, and RNA ODN, environmental particles, endogenous and modified host-derived molecules, and apoptotic cells [67,68]. SR-class A ligands [fucoidan, poly(I), and poly(G)] had no stimulatory effect on functional response (oxidative burst, degranulation) in heterophils [69]. However, low-density lipoprotein, an SR-class B ligand stimulated an oxidative burst in heterophils [69].

Cytokines and cytokine receptors. rChIL-2 had no effect on the functional activities of heterophils from day-of-hatch chickens but significantly increased the phagocytosis and bactericidal activity of heterophils from 7- and 14-day-old chickens. rChIL-2 induced no direct stimulation of the respiratory burst by heterophils but primed heterophils from 7- and 14-day-old birds for an enhanced respiratory burst in response to phorbol ester stimulation. Lastly, rChIL-2 had neither direct nor priming effects on heterophil degranulation [70].

Heterophils exposed to opsonized and nonopsonized *S. enteritidis* in the presence of IFN- γ exhibited enhanced the innate response, manifested by the increased transcription of both pro-inflammatory cytokine genes (IL-1 β , IL-6, IL-8) and enhanced acquired immune response observed by the increased transcription of Th1 cytokine genes (IL-18, IFN- γ) [8,41]. However, recombinant

chicken IL-6 had no effect on the functional activity of chicken heterophils [71].

8.2.4 Genetic effects on heterophil genotype and phenotype

It has repeatedly been shown over the last 15 years that there are substantial differences in heterophil innate immune responses between lines of chickens, both inbred and commercial, that differ in their resistance to multiple bacterial and parasitic infections. Two commercial lines, resistance to *Salmonella*, *Campylobacter*, *Enterococcus*, and *Eimeria* infections, were correlated with increased heterophil functionality and increased constitutive (and inducible) levels of mRNA expression for pro-inflammatory cytokines (IL-1 β and IL-6) and chemokines [72–78]. The role of heterophils as an early responder to multiple enteric pathogens makes this cell a viable genomic and/or phenotypic marker for the selection of poultry for immune efficiency and robustness [76].

Further proof that heterophil function and gene expression are under genetic control was shown by Redmond and colleagues [46,78,79]. Genetically dissimilar breeds of chickens displayed heterophil functional phenotypes that varied depending on the breed/cross of chickens [46,79]. Comparison of heterophils between three distinct breeds of chickens that included Leghorns (egg production), broilers (meat production), and the Fayoumi (ornamental) produced birds whose heterophils are significantly different with respect to cytokine and chemokine mRNA expression in response to exposure to *S. enteritidis* [80,81]. Heterophils from the commercial lines exhibited no changes in expression of pro- and anti-inflammatory cytokine mRNA, whereas the heterophils from the nonselection pressured Fayoumi line increased expression of all the cytokine mRNA [80]. These results suggest that the heterophils (innate immune responsiveness) from the birds selected for production traits appear to be less responsive to pathogens such as *Salmonella*.

Similar genetic differences in heterophil responsiveness were found in cells isolated from a commercial turkey line when compared to those isolated from the wild-type Rio Grande turkey line [82]. Heterophils from the wild-type line responder to stimulation with greater oxidative burst and degranulation activity than heterophils from the commercial line suggesting again that selection for performance resulted in decreased heterophil function (innate responsiveness). Further studies demonstrated that the differential functional responsiveness of heterophils between the commercial and wild-type turkeys was the result of the decreased phosphorylation of the ERK and p38 MAPK signaling pathways that are major transducers of immune functions and mediators of cytokine

production [81]. Lastly, the commercial turkey heterophils exhibited very little functional responsiveness to stimulation with multiple TLR agonists.

8.2.5 Heterophil isolation

Chicken heterophils are isolated from the peripheral blood as originally described [6] a technology subsequently altered to improve isolation through the years [8,9,26–28,41,56,57,63]. Briefly, peripheral blood is collected and pooled, mixed with 1% methylcellulose (1:1, v/v), and centrifuged at $25 \times g$ for 15 minutes. This procedure is vital to remove the nucleated red blood cells found in chickens. The supernatant is then diluted with Ca^{2+} - and Mg^{2+} -free Hanks balanced salt solution and carefully layered onto a discontinuous Histopaque gradient (specific gravity 1.077/1.119) and centrifuged at $250 \times g$ for 60 minutes. The heterophils, located predominately below the Histopaque 1.077/1.119 interface, are collected, washed, and resuspended in RPMI-1640. We routinely collect about 25% of the bottom 1.119 layer to maximize heterophil numbers. However, this also results in the collection of a number of mononuclear cells that can be removed, if needed, by a 1 hour plastic adherence in tissue culture flasks in a 5% CO_2 incubator at 41°C . The heterophils will not adhere to the tissue culture plastic. Heterophils isolated are typically >90% pure and >95% viable.

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Thrombocyte functions in the avian immune system

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8.3.1 Introduction

Thrombocytes, or platelets, are cells found in the blood of animals, including mammals and birds. They are present in high numbers and only erythrocytes outnumber thrombocytes in the blood. In mammals, platelets are enucleated, while in avian species these cells have nuclei [1]. In any animal, the primary function of thrombocytes or platelets is to stop bleeding in order to keep blood inside of blood vessels after damage; however, mammalian platelets induce thrombosis more quickly than avian thrombocytes [2]. Additionally, avian thrombocytes lack the ability to form three-dimensional shear-resistant clots in arterial vessels [3]. The development of these cells differs in mammals and birds; large megakaryocytes give rise to mammalian platelets, while in birds, thrombocytes are formed from stem cell precursor thromboblats [4]. However, transcription factors shared between avian and mammalian cells that are committed to the platelet/thrombocyte lineage, such as GATA-1, Ets, Myb, and Jun have been referenced in the literature, additionally, chicken thrombopoietin has been identified and found to perform similar functions to thrombopoietin in mammals, by inducing thrombocyte development [5].

In chickens, thrombocytes are the most common white blood cell in circulation, having been shown to make up 78% of circulating peripheral blood mononuclear cells [6]. In addition to providing hemostasis in chickens, various other functions of these cells have been elucidated with transcriptome analysis. Major molecular functions represented by the thrombocyte transcriptome include catalysis, transporter functions, and transcriptional activation, while the biological involvements of thrombocytes are highlighted by metabolic, developmental, and regulatory processes [7]. Chicken thrombocytes can be cultured,

although initial attempts found that apoptosis occurred after 24 hours; similar results have also been observed in ostrich thrombocytes after 48 hours of culture conditions [8]. Combatting rapid cell death in culture conditions has been investigated, and soluble products from lymphocytes have been shown to prolong the life of these cells in culture [9].

In recent years, research has focused on the immunological properties of thrombocytes and platelets. The roles of mammalian platelets in the immune system are understood [10] and research continues to further uncover the mechanisms and functions of platelets in immune responses. For avian thrombocytes, immunological functions also have been uncovered, including roles in inflammation and tissue repair [11,12], cytokine production, phagocytosis, and innate and adaptive immune responses. The following is a detailed discussion of avian thrombocytes, including their physical structure and surface marker expression, the role of thrombocytes in innate responses and the formation of adaptive immune responses, and infection of thrombocytes with microorganisms.

8.3.2 Avian thrombocyte structure

8.3.2.1 Physical characteristics

Thrombocytes in avian species can be differentiated from other blood cells when examined microscopically (Fig. 8.3.1). Compared to erythrocytes, thrombocytes are smaller and have an increased nucleus to cytoplasm ratio with a more rounded nucleus [13]. Differentiation of thrombocytes and lymphocytes is more difficult morphologically, although thrombocytes are normally smaller with clear cytoplasm and usually contain vacuoles [4].

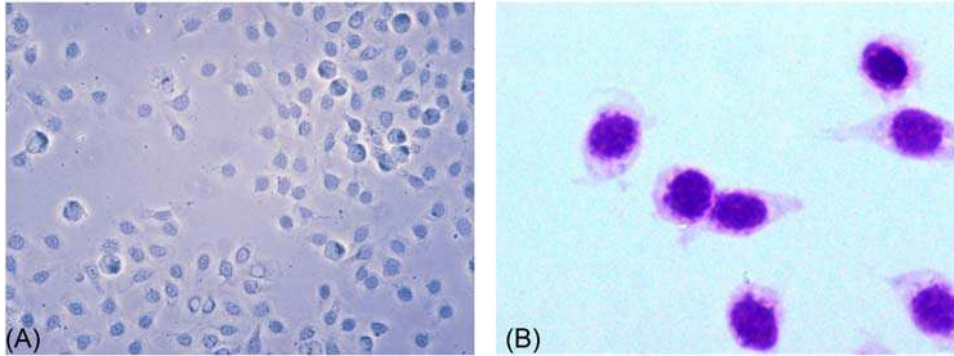


FIGURE 8.3.1 Images of chicken thrombocytes. (A) FicolI-separated blood mononuclear cells were adhered to glass surface for 12 h and nonadherent cells were removed by intensive washing. For comparison with adherent macrophages, see Figure 8.1.3. (B) Duck thrombocytes were sorted by flow cytometry and stained with Diff-Quick staining. Courtesy of Sonja Härtle, Faculty of Veterinary Medicine, LMU Munich.

Generally, thrombocytes can be described as small ovular, rounded, or rectangular cells with large dense nuclei, often containing vacuoles. The granules of chicken thrombocytes have also been described to resemble “dense bodies” found in mammalian cells [14]. Specific centrifugation parameters cannot isolate thrombocytes from chicken blood, as they are present in the buffy coat of centrifuged chicken blood along with other leukocytes. Additionally, based on their physical similarities, flow cytometry-based separation of thrombocytes and other leukocytes is difficult, therefore the characterization of specific protein structures on thrombocytes has been crucial for research purposes.

8.3.2.2 Surface protein expression

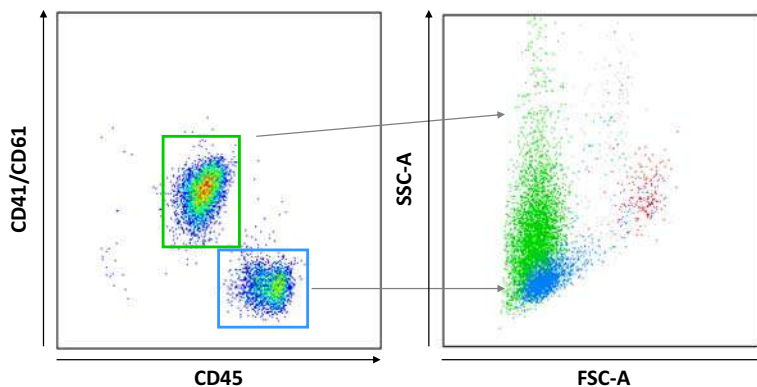
Beyond physical characteristics, many avian thrombocyte surface markers have been identified, providing a greater ability to isolate and experiment with these cells. In addition, the development of antibodies that recognize thrombocytes via sometimes unidentified proteins has also been a subject of research. Most of this work has focused on chicken thrombocytes. A summary of surface proteins and cross-reactive antibodies for chicken thrombocytes is provided in Table 8.3.1. Initial research on B-G antigens, which are part of the chicken major histocompatibility complex (MHC) gene complex, and B cell development revealed that B-G antigen RNA and proteins are present in thrombocytes [15]. Studies that attempt to identify antibodies specific for thrombocytes typically are carried out by immunization of mice with various chicken cells followed by production of hybridomas and subsequent screening of monoclonal antibodies. For example, an antibody, 2C12, which recognizes chicken thrombocytes and Marek’s disease lymphoma cells was produced after immunization of mice with a Marek’s disease virus-transformed kidney cell line [24]. Subsequently, an antibody shown to recognize cells of myeloid lineage designated K1 was found to bind macrophages and thrombocytes and has since been routinely used to detect chicken thrombocytes or to produce pure lymphocyte

populations by removing K1 positive cells [25]. This group of researchers suggested the antibody might recognize an adhesion molecule, although the specific molecule recognized by K1 is still undetermined. Although K1 recognizes thrombocytes and macrophages, other markers and antibodies have been developed that are exclusive to macrophages or monocytes, such as the KUL01 antibody [29] and colony-stimulating factor 1 receptor [30], making it possible to select specific populations of cells. An antibody, 11C3, that was shown to recognize the avian homolog of the mammalian fibrinogen receptor (CD41/CD61) was generated and was also shown to recognize chicken thrombocytes in addition to thromboblats [16]. HUKT, a monoclonal antibody, was generated after immunizing mice with chicken thrombocyte-rich preparations and was found to bind to thrombocytes in addition to cells of the thrombocyte lineage [26]. Additionally, cells bound by HUKT were shown to express low levels of CD45 and lacked CD3 and Bu-1, indicating these cells were not T or B cells. Indeed, several groups have since found thrombocytes to express low levels of CD45 compared to lymphocytes [17,31]. Flow cytometry analysis of chicken blood leukocytes (Fig. 8.3.2) demonstrates CD41/CD61⁺ CD45^{low} thrombocytes. Screening monoclonal antibodies specific for known protein structures against avian thrombocytes is also a method used to determine homologous thrombocyte-specific markers. This strategy was used to reveal that chicken thrombocytes exclusively express a CD51/CD61 integrin on their surface [17]. This group also showed that the CD51/CD61 positive population of cells expressed low levels of CD45 and were bound by K1, further indicating these cells to be thrombocytes.

In mammals, the development of megakaryocytes and the production of platelets is driven by thrombopoietin, a protein that interacts with a cell surface-expressed receptor, c-Mpl. Chicken thrombopoietin and c-Mpl have been identified, and it has been shown that c-Mpl is highly expressed on the surface of chicken thrombocytes, contributing to the proliferation and differentiation of thrombocytes and thrombocyte progenitors [5]. In an attempt to

TABLE 8.3.1 Surface protein expression and cross-reactive antibodies in avian thrombocytes.

Surface proteins	Description	References
B-G antigens	Part of chicken major histocompatibility complex	[15]
CD41/CD61	Chicken homolog of mammalian integrin	[16]
CD51/CD61	Chicken integrin	[17]
c-Mpl	Receptor for chicken thrombopoietin	[5]
CLEC-2	Innate pattern recognition receptor	[18]
MHC-II	Antigen presentation and formation of immune responses	[2]
CD40	Co-stimulatory molecule involved in immune response formation. Expressed at low level on chicken thrombocytes	[19]
SLAMF4	Immune response regulation	[20]
TREM-A1	Activator involved in immune response regulation	[21]
TREM-B1	Inhibitor involved in immune response regulation	[22]
TILR	Turkey immunoglobulin-like receptor. Fc receptor found on turkey thrombocytes	[23]
Antibodies	Method of production	References
2C12	Mice immunized with chicken Marek's disease virus-transformed cell line (MDCC-MSBI)	[24]
K1	Mice immunized with chicken spleen cells	[25]
11C3	Mice immunized with chicken peripheral blood leukocytes	[16]
HUKT	Mice immunized with thrombocyte-enriched preparations	[26]
GTr1 and GTr2 (guinea fowl)	Mice immunized with guinea fowl spleen cells	[27]
BA3 (duck)	Mice immunized with duck spleen cells	[28]

**FIGURE 8.3.2** Flow cytometry of chicken thrombocytes. Blood leukocytes were isolated by density gradient centrifugation and stained for flow cytometry using mAbs for chicken CD45 (clone 16-6) and CD41/CD61 (clone 11C3) and respective isotype specific secondary antibodies (anti-mouse-IgG2a-PE; anti-mouse-IgG1-APC); the sample was gated for viable, single cells. Note that thrombocytes (CD41/CD61 positive) show low CD45 expression. Dots in the FSC/SSC graph represent monocytes (red), thrombocytes (green), and lymphocytes blue.

find a homologous natural killer (NK) cell surface marker in chickens, researchers instead identified a c-type lectin receptor (CLEC-2) that was expressed on chicken thrombocytes [18]. Expression of CLEC-2 on thrombocytes was further supported by a lack of CD8 expression on CLEC-2 positive cells in addition to co-recognition by K1. Other proteins with immune system function have been found to be expressed on the surface of thrombocytes, including MHC-II [2], CD40 [19], signaling lymphocyte activation

molecule (SLAM)F4 [20], triggering receptor expressed on myeloid cells (TREM)-A1, which is an activating receptor involved in immunoregulation found on many cell types including a subset of thrombocytes [21], and TREM-B1, an inhibitory receptor found exclusively on thrombocytes [22]. Avian research in nonchicken species has made some additional findings, including the development of antibodies that exclusively recognize guinea fowl thrombocytes [27] and duck thrombocytes [28] in addition

to identification of a turkey homolog of an Fc receptor protein expressed specifically on thrombocytes [23].

8.3.3 Avian thrombocytes and immune responses

Many platelet-specific surface markers are involved in immune system function and regulation in other species. Therefore, it is of no surprise that research has demonstrated avian thrombocytes to possess a variety of functions related to the immune system. Thrombocytes have primarily been shown to contribute to innate responses in chickens; however, these functions are likely to also play a role in the formation of adaptive immune responses.

8.3.3.1 Innate responses

Innate responses in avian species provide initial protection against microorganisms and are constituted by physical barriers, the production of proteins and peptides, inflammatory responses, and a variety of specialized cells [32]. These cells include heterophils, macrophages, dendritic cells, NK cells, and gamma delta T cells, contributing to a variety of functions such as killing microorganisms via the release of granule contents and phagocytosis, production of inflammatory mediators, and antigen presentation. These cells also have the capacity to be stimulated by pathogen-associated molecular patterns (PAMP) through interaction with pattern recognition receptors (PRR), leading to the production of pro-inflammatory products, cytokines, and antimicrobial products.

In addition to their main hemostatic functions, avian thrombocytes also display some functions analogous to the functions of cells of the innate immune system. This is a current source of research, yet it is beginning to shape a better understanding of how thrombocytes contribute to innate responses in avian species. Specifically, research has demonstrated multiple findings on topics including thrombocyte responses to stimulation with PAMPs, phagocytosis, and production of antimicrobial components.

A recognized characteristic of innate responses is activation via stimulation of PRRs. Avian species express a variety of PRRs in many different cells and tissues, including the well-studied toll-like receptors (TLR) [33]. Chicken TLRs are expressed in endosomes (TLR3, TLR7, and TLR21) and on the surface of cells (TLR2, TLR4, TLR5, and TLR15) [34]. Chicken thrombocytes have been found to express different PRRs and have been shown to respond to stimulation with PRR-specific agonists. Levels of TLR mRNA transcripts in chicken thrombocytes have been assessed, demonstrating differences in expression levels [34]. Specifically, TLR2, TLR3, and TLR4 show the highest levels of expression, followed by TLR21 and finally TLR5 and TLR7, which were found to

be expressed at the lowest levels. It is noteworthy that other effector proteins with innate immune function were also identified at the mRNA level in thrombocytes, including inducible nitric oxide synthase and oligoadenylate synthetase. In addition to TLRs, thrombocytes have also been shown to express other PRRs on their surface, such as a C-type lectin receptor (CLR) which recognizes carbohydrates [18]. Furthermore, chicken thrombocytes express transcripts for nucleotide-binding oligomerization domain-like receptors, retinoic acid-inducible gene-I-like receptors, and inflammasome-related products [35]. Not only have PRRs been identified in chicken thrombocytes but outcomes of PRR stimulation have also been characterized. Lipopolysaccharide (LPS) and CpG ODN, ligands for TLR4 and TLR21, have been used to stimulate chicken thrombocytes, demonstrating upregulation of the expression of pro-inflammatory cytokines, including interleukin (IL)-6, IL-8, and IL-1 β , in addition to enhanced expression of IL-12 and the antiviral cytokine interferon (IFN)- α [34,36]. LPS stimulation of chicken thrombocytes results in the expression of inducible cyclooxygenase transcripts and the production of prostaglandins [37]. This provides evidence to support the notion that avian thrombocytes play a role in innate responses, as they are able to recognize pathogen-like structures and generate responses that can directly or indirectly influence immunity against pathogens. The signaling pathways in LPS-stimulated thrombocytes that lead to production of inflammatory responses have also been elucidated, demonstrating involvement of mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells [38]. RNA sequencing of LPS-stimulated chicken thrombocytes has further uncovered the innate functions of these cells, highlighting upregulation of transcripts associated with production of inflammatory cytokines and chemokines, apoptosis, activation of T and B cells, MAPK activation, IFN activation, and JAK/STAT signaling [7]. RNA transcript analysis of TLR2, TLR3, and TLR7 ligand treated thrombocytes has also been done, showing upregulation of similar pathways as TLR4 stimulation; however, TLR7 stimulation showed greater overall increases in transcriptional responses [35].

In addition to inflammatory responses, thrombocytes also seem to be able to exhibit direct pathogen-killing traits. This innate destruction of pathogens is mediated by the release of cytotoxic contents from large granules and through phagocytosis. Although initial reports dismissed chicken thrombocytes as phagocytes [39], newer studies demonstrated phagocytic activities of chicken thrombocytes in vitro against multiple species of bacteria, including *Escherichia coli*, *Pseudomonas*, *Staphylococcus aureus*, *Burkholderia cepacia*, and various *Salmonella* species [40]. After exposure to these bacteria in vitro, chicken thrombocytes may release reactive oxygen

species, suggesting they have cytotoxic potential for pathogen killing. Degranulation in chicken thrombocytes has also been shown to be mediated by stimulation, as CD107 expression was shown to appear on the surface of CLR stimulated cells [18]. Similarly, stimulation of chicken thrombocytes with TLR2, TLR3, TLR4, TLR7, and TLR21 ligands leads to an increase in production of nitrite [34,41] and phagocytic activity [34].

8.3.3.2 Adaptive immune responses

While innate responses in avian thrombocytes are starting to become better characterized, less is known about the function of these cells in adaptive immune responses. The ways in which thrombocytes contribute to adaptive responses are likely not through direct detection and killing of pathogens, rather, these cells promote immune responses indirectly, via the production of cytokines, presentation of antigens, and attraction of antigen-specific cells of the adaptive immune system.

A characteristic of thrombocytes that has been researched intensively is their response to microbial stimuli. A prominent finding in these studies is the increased expression or production of IL-6 in response to ligand stimulation [34,36–38,41]. IL-6 is an inflammatory cytokine that influences adaptive immune responses. The roles of IL-6 in adaptive immune system responses in humans have been reviewed and are known to include differentiation of naïve CD4 + T helper (Th) cells, enhancement of antibody production, and activation of CD8 + cytotoxic T cells [42]. This indicates that the production of IL-6 by thrombocytes in avian species likely enhances the formation of adaptive immune responses. Cytokine production mediated enhancement of adaptive responses is not limited to IL-6, for example, under stimulated conditions thrombocytes have also been shown to express IL-12 [34,36], which is understood to be involved in differentiation of naïve CD4 + Th cells into Th 1 cells, promoting cell-mediated immune responses. Therefore, similar to other cells of the innate immune system, thrombocytes in avian species may have the potential to direct adaptive immune responses based on the stimuli that are present during infection or vaccination.

Another function of thrombocytes that is becoming more understood is the ability to present antigens on MHC-II molecules. Early research demonstrated the phagocytic ability of thrombocytes suggesting they may be able to internalize pathogens and present peptides to immune system cells, although this was speculative [43]. However, more recent work has detected MHC-II transcripts in chicken thrombocytes [7,34], interestingly, these studies both showed increased expression following TLR stimulation. Beyond MHC-II expression, other transcripts relevant to antigen processing and presentation have also

been shown to be expressed in chicken thrombocytes, in addition to transcripts for genes involved in naïve T cell stimulation such as CD80 and CD40. Importantly, in addition to identification at the mRNA level, MHC-II, CD80, CD86, and CD40 proteins have also been detected on the surface of avian thrombocytes [2]. Given the high quantity of thrombocytes circulating in the blood of avian species, these cells may represent a major inducer of adaptive immune responses by functioning as antigen-presenting cells. Therefore, activating these cells may be a strategy for future vaccines directed against poultry pathogens.

Avian thrombocytes may also display migratory responses during infection. In chickens, it has been shown that thrombocytes migrate and adhere to virally infected cells. Additionally, thrombin-stimulated thrombocytes were demonstrated to release chemotactic factors that induce lymphocyte migration toward them [44]. These early observations raise the possibility that thrombocytes may play a role in immunity against infections in chickens by attracting effector cells to the site of infection. More recent research has made similar findings that thrombocytes can attract lymphocytes and heterophils; however, it was also revealed that supernatants from cells cultured for only 2 hours resulted in lymphocyte migration, suggesting that chemotactic factors are preformed in thrombocytes [45].

Other roles for thrombocyte participation in adaptive immune responses are implicated based on the expression of immune system receptors, including various members of the immunoglobulin superfamily. Fc Receptors (FcR) expressed on the cell surface bind to antibodies and facilitate a wide variety of functions related to killing pathogens, including degranulation, opsonization and phagocytosis, and antibody dependent cellular cytotoxicity. Additionally, FcRs can activate or inhibit cell activation, providing the ability for more precise regulation of immune response via the summation of signals. An activating FcR that can bind IgY was discovered in chickens and expression of gene transcripts for this receptor was found to be elevated in thrombocytes [46], suggesting thrombocytes may be able to respond to antibody-bound pathogens. A homolog of this receptor has also been identified on turkey thrombocytes [23]. Thrombocyte expression of SLAMF4 [20], TREM-A1 [21], and TREM-B1 [22] further promotes the notion that thrombocytes have some impacts on adaptive responses to infection as these receptors are all involved in immune system regulation. For example, TREM-B1 may have an inhibitory function, as stimulation of this receptor on chicken thrombocytes resulted in decreased degranulation as evidenced by a decrease in cell surface CD107 expression. Further research is needed to characterize the function of immune system regulation by these receptors on chicken thrombocytes.

8.3.4 Infection of thrombocytes

Avian thrombocytes may become infected with microorganisms. Due to their high numbers in systemic circulation, these cells are a potential target for infection and replication. Additionally, immune system related and unrelated functions of thrombocytes have been assessed during infection. Multiple viruses have been found to infect thrombocytes; however, some viral pathogens affect thrombocyte quantities without infecting thrombocytes exclusively. For example, chicken anemia virus infects erythroid progenitors in chickens ultimately leading to a reduction in thrombocyte as well as heterophils and erythrocytes [47]. In migration and chemotaxis experiments, Newcastle disease virus (NDV) was found to be internalized by thrombocytes *in vitro*; however, virus titers did not increase in culture over time [44]. Nevertheless, this work revealed that thrombocytes that had been infected with NDV exhibited decreased migration and phagocytosis capabilities. A recent interesting finding surrounding H5N1 highly pathogenic avian influenza virus infection demonstrated differences between chicken and duck thrombocyte infection levels [48]. Specifically, quantities of H5N1 virus in duck thrombocytes were much lower relative to chicken thrombocytes. Importantly, chickens demonstrated 100% mortality in these experiments, whereas mortality was 30% in ducks. This study did not determine whether thrombocytes phagocytosed the virus or if entry was infection-mediated. Furthermore, infection with a low pathogenic influenza virus did not result in high quantities in chicken thrombocytes [48]. Nevertheless, this study highlights a potential role that thrombocytes may play in the pathogenicity of highly pathogenic avian influenza virus in avian species. Beyond viruses, some parasites have been shown to infect thrombocytes of avian species, including *Leucocytozoon sabraezesi*, which was identified to infect thrombocytes exclusively in chickens [49]. Given the important immune system functions of avian thrombocytes, pathogenicity studies should be carried out to determine the effects that infection with relevant poultry pathogens may have on thrombocyte quantities and functionality.

8.3.5 Conclusion

Avian thrombocytes perform hemostatic functions similar to their mammalian counterpart, however, physical and functional differences exist. Beyond maintaining hemostasis, prominent roles of avian thrombocytes have been identified in the immune system. Most research concerning immune system functions thus far has focused on chicken thrombocytes, however, differences likely exist between different avian species. Identification and selection of thrombocytes for experimentation have been made

possible through the recognition of multiple proteins that are expressed on the cell surface in addition to the generation of antibodies that can exclusively bind thrombocytes. Research has begun to demonstrate that thrombocyte functions in the immune system include innate and adaptive roles. These cells are able to recognize pathogens or pathogen-derived structures and respond in multiple ways, including phagocytosis, oxidative burst, and the production of cytokines which could help to shape adaptive immune responses. They are a prominent cell type in the blood of avian species and, as a result, they can be a target for infection by pathogens. Despite numerous recent findings, further research is required to better understand the roles that thrombocytes play in innate and adaptive immune responses. Enhancing this collective knowledge will allow for the design of more effective vaccines or prophylactic treatments for chickens and other avian species.

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Natural killer cells

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8.4.1 Potential natural killer cell receptor families

In recent years three principal modes of target recognition by mammalian natural killer (NK) cells have been documented. The “missing-self recognition” postulates that NK cells are inhibited by MHC class I proteins expressed on normal cells which are downregulated after infection or transformation [1]. Alternatively, recognition of infected cells can also be mediated by pathogen-encoded molecules, and finally, pathogens or transformation can induce the upregulation of self proteins that ligate activating receptors on NK cells as stated in the “induced self-recognition” model. It has become evident that NK cells display many different inhibitory and activating receptors that mediate these functions. In most cases all of the three types of recognition are found in parallel and NK cell responses are fine-tuned by the integration of these various signals.

Receptors on NK cells can be grouped into two major classes that either belong to the Ig superfamily or are C-type lectins [2]. In both these families, two types of receptors can be distinguished, based on their cytoplasmic motifs and the nature of the transmembrane region. Inhibitory receptors are characterized by long cytoplasmic domains that harbor one or multiple immunoreceptor tyrosine-based inhibition motifs (ITIM). Upon phosphorylation, these motifs recruit tyrosine phosphatase which prevents further downstream signaling. Activation receptors, however, have a short cytoplasmic tail and a positively charged transmembrane residue which allows their association with adapter molecules that carry cytoplasmic immunoreceptor tyrosine-based activation motifs; this leads to activation. In many cases activation and inhibitory receptors share a high degree of homology in their extracellular regions and may even bind similar ligands.

In mammalian NK cells, there are two distinct multigene receptor families: the leukocyte receptor complex on human

chromosome 19 and the NK complex (NKC) on human chromosome 12. The LRC encodes several families of Ig-like receptors such as the killer-inhibitory receptors, the Ig-like transcripts, and the sialic acid-binding immunoglobulin-type lectins, whereas the NKC harbors multiple C-type lectin receptors, including NKG2D, CD94/NKG2A, and Ly49. Depending on the species, expansion or contraction with duplications or deletions of NK cell receptors are present. For instance, the human LRC contains multiple killer Ig-like receptors but the syntenic mouse regions lacks this multi-gene family, instead the Ly49 receptors have been expanded in mouse but are absent in man [3].

In the chicken, syntenic regions to both multigene families have been described. The chicken LRC is present on microchromosome 31, whereas the NKC is located on chromosome 1 [4–6].

There are several C-type lectin molecules that have been annotated within the chicken NKC and by sequence homology and structural criteria, they were designated as chicken CD69 homologue and chicken CD94/NKG2-like receptor, based on equal similarity to both mammalian CD94 and NKG2 [4]. We have generated a mAb specifically for this molecule and based on the expression on thrombocytes as well as some sequence features, we propose that this C-type lectin is a homologue of mammalian CLEC-2 [7]. The chicken NKC therefore may not harbor any NK cell-specific C-type lectins; however, the chicken CD69 and CLEC-2 genes are very far apart and there may be still other C-type lectins present between these two genes that have not been annotated. In fact, a recent search for molecules harboring a C-type lectin domain resulted in the discovery of novel C-type lectins located in the NKC [8]. These novel receptors seem to have an expression profile on myeloid cells; however, since a specific mAb is not available yet, it is possible that they are expressed on NK cells. Moreover, in the vicinity of the chicken MHC, there are two C-type lectins designated B-Lec and B-NK [9,10]. RT-PCR analyses of B-NK have indicated that it is expressed in chicken NK cells

expanded in vitro by IL-2. A B-NK-specific mab also revealed expression on NK cells as well as on T-cell subsets [11]. Functionally, B-NK represents an inhibitory molecule, based on the fact that it harbors ITIM and recruits intracellular tyrosine phosphatases [10]. The nature of its ligand is currently unknown; however, the ligand is expressed on activated T cells [11]. Interestingly, both B-NK and B-Lec are strikingly conserved in all genomes of birds analyzed so far. More details on B-NK and B-Lec are provided in Chapter 7.

The second multigene cluster potentially encoding NK cell receptors is found on microchromosome 31, the chicken LRC that encodes the chicken Ig-like receptors (CHIR) [5]. The CHIR represent a vastly expanded polygenic and polymorphic gene family encoding more than hundred receptors [12–14]. The CHIR can be subdivided into activating CHIR-A with short cytoplasmic domains coupling to the adapter protein Fc γ R, inhibitory CHIR-B with a long cytoplasmic domain containing ITIM, and bifunctional CHIR-AB combining features of CHIR-A and CHIR-B [6,15]. For most of the CHIR, it has not been proven whether they function as NK cell receptors and their ligands are currently unknown. The exception is CHIR-AB1, a chicken Fc receptor that binds to IgY with high affinity [16,17]. CHIR-AB1 is expressed on a variety of leukocytes, including granulocytes and monocytes, but it is also expressed on a lymphocyte subset that may resemble NK cells (see next). Using a method called reference strand-mediated conformational analysis, Meziane et al. showed that relatively simple patterns of CHIR-AB genes are found in experimental bird lines and laying chicken lines which is probably due to partial inbreeding, whereas commercial broiler lines and red junglefowl showed more complex patterns arguing for a larger extend in heterozygosity [18]. Some activating CHIR-A were identified as receptors for avian influenza suggesting that some of the various CHIR molecules could functionally resemble mammalian NKp46. [19–21]. In conclusion, the chicken may have an expansion of the LRC with multiple CHIR but seems to have lost most of the C-type lectins of the NKC, a situation similar to man. Interestingly, it has been argued that the chicken BF-1 MHC molecule functions as a restriction element for NK cells; however, it is not resolved which receptor can bind to the BF-1 molecule [22,23].

Apart from these two major multigene families, there are additional families or receptors scattered throughout the genome. The recent progress in various genome projects of birds has allowed the identification of several gene families with potential role for NK cells, including CD200R, CD300, SIRP, TREM, SLAM, and CD56 [24–28].

8.4.2 Phenotype of chicken natural killer cells

Just as for mammals, no unique NK cell marker has been identified for the chicken [29,30]. Consequently, the

phenotypic description of chicken NK cells was primarily based on the combination of the CD8 expression together with the lack of surface Ig or CD3. Chicken embryonic NK cells express the common leukocyte antigen CD45 and Fc receptor-like Ig-binding activity; they lack MHC class II antigens [31]. Like human fetal NK cells, chicken NK cells express cytoplasmic CD3 proteins, probably indicative of a common origin with T cells [31,32].

Using the phenotypic criteria outlined earlier, Göbel and coworkers have investigated the presence of putative NK cells in a variety of different tissues such as peripheral blood, spleen, cecal tonsils, bursa, thymus, and intestinal epithelial lymphocytes (IEL). Only about 0.5% to 3% of CD3⁺Ig⁺CD8 $\alpha\alpha$ ⁺ cells were detected in all tissues, except intestinal epithelium. The IEL population, however, contains a large population of putative NK cells. A monoclonal antibody (mab) designated 28-4 has been raised against this population. IEL expressing the 28-4 antigen showed spontaneous cytotoxicity against the standard NK target (LSCC-RP9) cells, whereas CD3⁺ intestinal T cells did not show this cytolytic activity [33]. We have identified the chicken CD25 as the antigen, recognized by the 28-4 mAb, therefore the IL-2 receptor α chain is differentially expressed on chicken intestinal lymphocytes. One member of the SLAM family, CD244 is also a useful marker for intestinal NK cells [25,34].

A mAb against the chicken Fc receptor CHIR-AB1 [16] detected a CHIR-AB1⁺ cell subset in peripheral blood mononuclear cells that expressed CD4 but not CD8. In addition, these cells were positive for CD57, CD244, the vitronectin receptor (α V β 3), and CD25. Moreover, this cell population degranulated upon stimulation as judged by CD107 expression and it produced interferon- γ [35]. We have also generated a panel of NK cell-specific mab and categorized them in several subgroups [36,37]. It remains to be seen which of these markers are useful for the identification of NK cells.

8.4.3 Natural killer cell function

Functional studies have detected NK cell-mediated cytotoxicity in spleen and blood cell populations. These functional analyses do not necessarily conflict with the phenotypical analyses described earlier, since a low frequency of NK cells could be responsible for the observed cytolytic activity. It has been demonstrated that $\gamma\delta$ T cells in spleen and intestinal epithelium also function as spontaneously cytotoxic cells [38]. These results suggest that NK cells and some $\gamma\delta$ T-cell subsets may functionally complement each other. Certain infections can cause a drastic, but transient, increase in the NK cell frequency. It has been reported, that *Mycoplasma gallisepticum* infection leads to an accumulation of TCR⁺ CD8⁺ cells in the tracheal mucosa during the first week of infection [39].

Obviously, the relevance of NK cells in various chicken viral infections models, such as avian influenza [40], infectious bronchitis virus [41], Marek's disease virus [42,43], Newcastle disease virus [44], and infectious bursal disease [45], has been thoroughly documented, especially in IEL.

Cytotoxicity assays are normally performed with effector cells isolated from either spleen or peripheral blood obtained from either normal or infected chickens. The LSCC-RP9 target cell line was derived from a lymphoid tumor induced by the retrovirus, Rous-associated virus 2 [46]. In contrast to standard mammalian target cell lines, RP9 is MHC class I positive and even at high effector to target ratios, only a fraction of the RP9 cells is susceptible to lysis. Most other chicken cell lines, including all MDV-derived cell lines so far tested, are resistant to NK cell lysis—the same applies to mammalian derived NK cell targets. However, it has been reported that chicken skin fibroblasts are highly sensitive for NK cell lysis [47]. The cloning of chicken perforin adds a new valuable tool for future studies of NK cells; however, an antibody is currently not available [48].

Culture experiments using embryonic NK cells have indicated the necessity of including growth factors to induce NK cell proliferation. Two major NK cell growth factors, IL-2 and IL-15, have been cloned [49,50]. Recombinant chicken IL-2 has been successfully employed to generate polyclonal NK cell in vitro cultures for several weeks. Although many chicken cytokines have been cloned and characterized recently, their effects on NK cells are not known. Oral administration of IFN- α decreased NK cell activity after MDV infection [51], whereas in vitro IFN- γ treatment increased NK cell activity under different circumstance [52]. In conclusion, studies on NK cell function are still lacking, mainly due to the dearth of appropriate phenotypic and molecular markers.

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Soluble components and acute-phase proteins

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8.5.1 Soluble components

8.5.1.1 Host defense peptides

Host defense peptides (HDP) are a diverse group of small, cationic peptides that are important components of the innate immune system. These peptides have been isolated from most living organisms and are considered an old defense mechanism capable of protecting the organism from infection. Although originally named antimicrobial peptides after their ability to kill bacteria *in vitro*, they are now also considered as immunomodulators due to their ability to steer the response of immune cells [1].

In aves, two major classes of HDP have been identified: cathelicidins and defensins. In addition to those, liver expressed antimicrobial peptide has also been identified [2]. Peptides from both HDP families are produced as longer precursor proteins, from which the active peptide is released through proteolytic cleavage. Cathelicidins derive their name from their conserved pro-sequence (which resembles cathelin) while sequence and structure of the active mature peptide can vary considerably. Defensins, on the other hand, are characterized by the conserved structural elements of the mature peptide, most notably the inclusion of three intramolecular disulfide bonds.

Currently, four cathelicidin-like peptides have been described, CATH-1-, 2, and -3 (sometimes also referred to as fowlicidins 1–3) and CATH-B1. The main gene expression site for CATH-1–3 is the bone marrow, while immunohistochemistry and mass spectrometry have shown that the peptides are mainly present in heterophilic granules [3,4]. In contrast, CATH-B1 is almost exclusively expressed in the Bursa of Fabricius by interfollicular secretory epithelial cells [5].

Fourteen different defensins (originally called gallinacins) have been reported in the chicken genome, clustered on chromosome 3 [6]. They are designated as avian β -defensins (AvBD)1–14, while α - and θ -defensins (other mammalian defensin classes) are absent. Gene expression studies indicate that several AvBDs are mainly expressed in the bone marrow and derived cell types, while some AvBDs such as AvBD11, 12, and 13 are more diversely expressed [7,8]. Interestingly many defensins are also detected in the testis, the ovary, and oviduct implicating an important role for β -defensins in protection of the reproductive system [9]. A new class of defensins, named ovo-defensins with specific expression in the oviduct was also described [10]. They structurally resemble β -defensins, are located near the AvBD cluster on chromosome 3, and are most likely derived from AvBD12 [11]. However, they contain a distinct intermolecular cysteine pairing [12]. Functionally, not much is known yet of these defensins except for weak antimicrobial activity, but their presence almost exclusively in the egg white predicts a protective function during embryonic development [13].

There are some discrepancies between studies describing the gene expression of HDPs. These are likely explained by the inducibility of HDP gene expression. Strong increases in HDP gene expression are described for bacterial infections or bacterial products, but also short-chain fatty acids and vitamin D3 have effect of HDP mRNA levels [14]. Finally, HDP expression is developmentally regulated with relatively higher levels just before hatch [15], implicating that many internal and external factors contribute to specific HDP expression profiles, and highlights the flexibility of immune responses by HDPs.

The classic function of HDPs is their antimicrobial activity against bacteria. A broad range of bacteria including avian pathogens and multiresistant strains, such as *Staphylococci*, *E. coli*, *Salmonellae*, *Klebsiellae*, and *Campylobacter* are susceptible to HDPs in vitro [16,17]. Generally, the peptides act by forming pores in-, or destabilizing the bacterial membrane, leading to cell death [18], although there are also some indications that peptides can have intracellular targets [19]. Besides bacteria, HDPs are also active against fungi [20], viruses [21,22] and based on homology with mammalian peptides possibly also parasites [23].

Besides direct killing of microorganisms, several immunomodulatory effects of HDPs have been described. Fueled by the observation that antimicrobial activity of many HDPs is hampered in more physiological conditions (high salt concentration, presence of serum proteins), the immunomodulatory activity of HDPs is gaining much interest. For example, HDPs can bind and neutralize lipopolysaccharide (LPS) or lipoteichoic acid (LTA), thereby lowering a possible unwanted excessive immune response toward neutralized nonviable bacterial cells [24,25]. In addition they are chemotactic for immune cells [24,26], affect cytokine and chemokine responses of immune cells,

and can stimulate uptake of bacterial DNA into macrophages leading to increased sensitivity toward this bacterial stimulus [27]. Further proof for the immunomodulatory effect of HDPs in vivo was observed in studies where chicken cathelicidins were either injected in ovo [28] or intraperitoneally [29] with subsequent protection against bacterial infection several days after. The low quantity of injected peptide in these experiments and the time gap between infection and prophylactic peptide treatment rules out that protection was due to direct killing of pathogens by the injected HDPs. An overview of described functions of HDPs is shown in Fig. 8.5.1.

An extensive phylogenetic study among 48 bird species indicated that both the defensin and cathelicidin family are conserved among birds [31,32]. With few exceptions, all defensins are present in all avian species tested, while several species in especially the family of passeriformes acquired novel AvBDs through gene duplication of AvBD1 or AvBD3. On the other hand, AvBD14 and AvBD7 developed in some species into a pseudogene. For cathelicidins similar conservation was observed although due to the current low assembly quality of the genomic regions, the cathelicidin gene cluster was missing in several bird species. However, it is likely that the functional roles

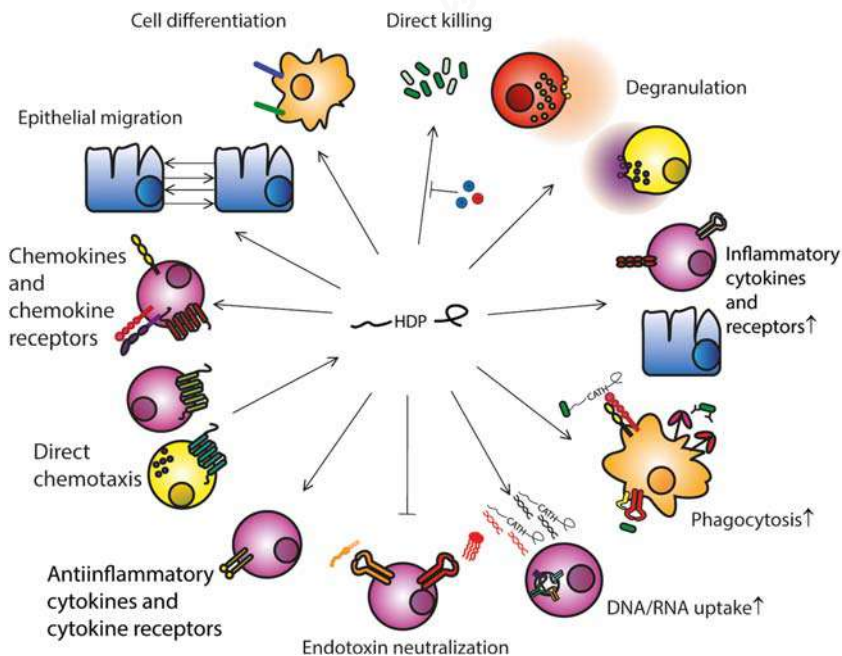


FIGURE 8.5.1 Antibacterial and immunomodulatory functions of HDPs. Overview of the described functions of HDPs, starting from the top going clockwise through the figure, HDPs have direct killing activity against both Gram-positive and Gram-negative bacteria; induce degranulation of neutrophils and mast cells; induce upregulation of inflammatory cytokines and cytokine receptors; enhance phagocytosis by opsonizing bacteria and upregulate bacterial recognition receptors; enhance DNA/RNA uptake, thereby boosting intracellular toll-like receptor (TLR) signaling; inhibit endotoxin-mediated activation of TLR2 and TLR4 by binding to lipoteichoic acid (LTA) and lipopolysaccharide (LPS), respectively; induce upregulation of anti-inflammatory molecules such as IL-10; be directly chemotactic for multiple immune cells; induce production of a variety of chemokines and chemokine receptors; induce migration of epithelial cells and thereby influence wound healing; and finally influence the differentiation of immune cells [30]. HDP, Host defense peptides. Adapted from van Harten RM, van Woudenberg E, van Dijk A, Haagsman HP. Cathelicidins: immunomodulatory antimicrobials. *Vaccines (Basel)* 2018;6(3), 63.

observed for chicken HDPs represent a template for their roles in the immune system in birds in general.

8.5.1.2 Collagenous lectins

The collagenous lectin family contains multimeric proteins containing both a carbohydrate-binding (lectin) domain and a collagen domain, such as collectins and ficolins. Nine different collectins have been identified in mammals so far, including mannose-binding lectin (MBL), surfactant protein A and D (SP-A and SP-D), Collectin 10 (collectin liver 1, CL-L1), Collectin 11 (Collectin kidney 1, CL-K1), Collectin 12 (collectin placenta 1, CL-P1), and three bovine-specific collectins: conglutinin, CL-43, and CL-46. The family of ficolins is smaller with only three members described in mammals: H-, M-, and L-Ficolin [33,34]. The avian collagenous lectin family differs from its mammalian counterpart. It obviously lacks the bovine-specific collectins, but also surfactant protein D is absent [35,36]. Finally, only one ficolin has been identified in chicken up to date [37]. Except for MBL, functionality of avian collectins is mainly based on homology with their mammalian counterparts.

The monomeric subunit of a collectin contains four regions: a cysteine-rich N-terminal domain, a collagen-like region, a coiled-coil neck domain, and finally a large globular lectin/carbohydrate recognition domain (CRD). The neck domain of approximately 30 amino acids initiates trimerization, which is further stabilized by the collagen. Higher oligomers of trimers are subsequently formed through the cysteines in the N-terminus resulting in protein structures where the carbohydrate-binding domains (CRD) are aligned to one side of the molecule, sometimes referred to as a “bouquet of tulips” for especially SP-A

and MBL (Fig. 8.5.2), or a more extended cross-like structure for SP-D, conglutinin, and CL-46. Although each CRD has relatively low affinity for a carbohydrate structure, the spatial aligning of CRDs and their subsequent multivalency optimizes binding to microbial surface polysaccharides or host macromolecules. Ficolins have some structural differences to collectins. They lack the neck region and trimerization is initiated and stabilized by the collagen domain. In addition, they use a fibrinogen (FB)-like domain to bind glycoconjugates instead of a classic C-type lectin domain of collectins. However, the resulting structure after oligomerization of ficolin trimers is very comparable to the structure of collectins.

The key functional aspect of the collagenous lectins is initiated through binding of polysaccharides to the CRD. Each family member has a slightly different preference for specific oligosaccharides but generally binds much stronger to bacterial—than mammalian carbohydrates. Binding can result in several functionalities (depending on the specific collectin member) such as activation of the complement cascade, aggregation and mucociliary clearance of microorganisms, or opsonization and increased phagocytosis of microorganisms. In addition, the CRDs can also bind DNA, which is thought to be involved in clearance of apoptotic cells.

8.5.1.3 Surfactant protein A and cLL

Pulmonary surfactant is a mixture of specific lipids and surfactant-specific proteins, in mammals secreted by alveolar type II cells in the lung, where its main function is to lower the surface tension at the air–water liquid interface. In aves, pulmonary surfactant is also present but less is

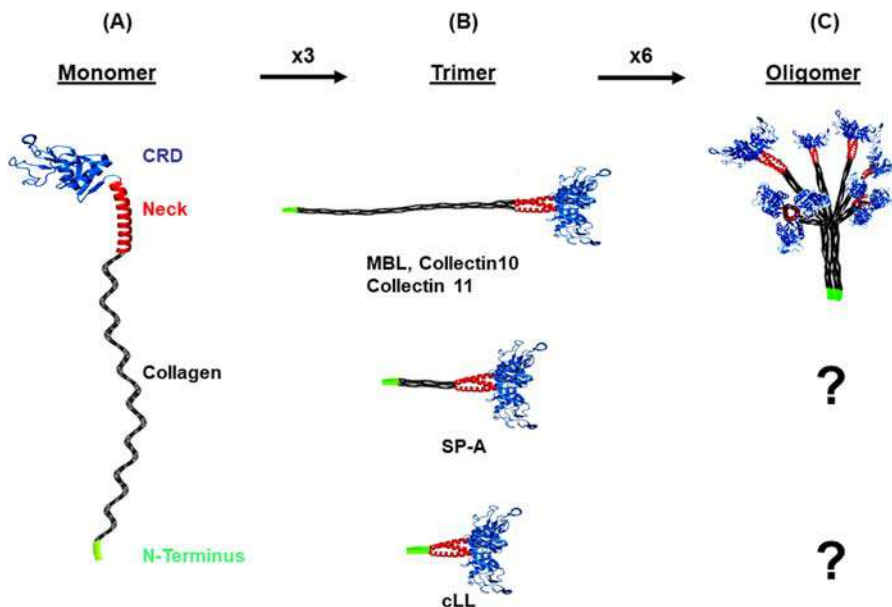


FIGURE 8.5.2 Structure of avian collectins. The four structural domains of a monomeric collectin molecule are depicted (A). Trimerization is initiated by the neck region and stabilized by the collagen domain, resulting in functionally active (carbohydrate binding) units for all avian collectins. Avian SP-A and cLL have a shorter or no collagen domain but are still able to form the functionally active trimer (B). Cross-linking of the N-termini leads to higher oligomeric structures for MBL, Collectin 10, and Collectin 11, while it is unknown whether SP-A and cLL can form higher oligomeric structures (C). No structural information is available for avian collectin 12 and has been left out of the figure, while SP-D is absent in Aves. MBL, Mannose-binding lectin.

known about specific production sites nor its necessity for lowering surface tension in the static birds lung. However, important additional roles in innate immunity have been assigned for the surfactant-specific collectins. SP-A can aggregate and thereby lower the infectious potential of bacteria and viruses, and thereby increase removal from the lung by mucociliary clearance. In addition, binding of SP-A to the surface of microorganisms leads to opsonization and activation of phagocytosis through interaction of SP-A with receptors on phagocytic cells. These interactions with immune cells also modulate the cellular inflammatory responses. Besides numerous in vitro studies on the functionality of SP-A, the importance of this collectin in vivo has been elegantly shown especially in genetically modified mice lacking SP-A. These mice were shown to be more susceptible to both viral and bacterial infections [38].

Interestingly chicken SP-A lacks a large part of the collagen domain. The structural and functional consequence of this adaptation for chicken is unknown but partial purification of cSP-A from lung lavage suggested that at least the existence of trimeric structures were present in vivo [39]. However, it is unclear if higher oligomers are formed and what form or shape these would have (Fig. 8.5.2). A second SP-A gene is present in chicken, named chicken lung lectin (cLL) because this SP-A completely lacks the collagen domain (Fig. 8.5.2). Gene expression of cLL and cSP-A largely overlap and is mainly expressed in the lung [35,40]. On a protein level, SP-A itself was localized to specific cells, likely resembling avian type II cells, in the atria and sometimes the infundibulum of the parabronchi. Functionally, cLL and SP-A are under-characterized but both are calcium dependently binding glycans [39,41] and both are transcriptionally regulated upon viral infections [42,43].

8.5.1.4 Mannose-binding lectin

Chicken mannose-binding lectin (MBL) was the first chicken collectin to be isolated in 1985 by Oka and colleagues from chicken liver [44]. Further characterization of the native and recombinant forms of MBL showed that it is very comparable to mammalian MBL in many aspects. Structurally, chicken MBL has a high sequence homology to MBL from other species and it was shown to form the collectin characteristic trimers and higher oligomeric forms (Fig. 8.5.2). The carbohydrate domain folds into a typical C-type lectin shape and it binds carbohydrates with a preference for high mannose glycans [45,46].

Chicken MBL is primarily produced in the liver and secreted into the blood, although low expression has also been demonstrated in the trachea-larynx, germinal centers of the cecal tonsils, abdominal air sac, infundibulum, and

thymus [35,47,48]. Serum levels remain relatively stable during development [49] but upon viral infection expression of MBL is increased in the liver and serum levels are approximately twofold to threefold higher [48]. Therefore MBL can be considered a mild acute-phase protein (APP). In infected tissues MBL can also be detected in other cell types than hepatocytes, including degenerating macrophages, infected tracheal cells, and precursor dendritic cells [48,50]. It is likely that these cases relate to increased local expression of MBL by these cells, but uptake or association of serum-derived MBL cannot be completely ruled out.

Functional studies on MBL have shown that it can bind and aggregate *E. coli* and *Salmonella* in vitro [45,51] and that this results (at least for *Salmonella*) in increased opsonization and phagocytosis. Although it was not measured in the study, phagocytosis of bacteria would generally lead to killing of bacteria, but also to modulation of cytokine expression, and drive the immune response. Recombinant MBL was shown to bind and aggregate infectious bronchitis virus and to prevent infection in vitro [52]. Binding of MBL to influenza A virus (H3N2 and H1N1) resulted in an inhibition of its hemagglutination activity. Finally, upon binding of mannan, MBL can activate the lectin pathway of complement activation, in vitro, as measured by C4 deposition on the MBL/MBL associated serine proteases (MASP) complex [49].

More studies on the innate immune role of MBL have been performed in vivo mainly using chicken strains selected for high- or low-serum concentration of MBL. These studies have consistently shown that a low amount of circulating MBL is associated with increased disease parameters after viral or bacterial infections. This correlates well to human studies where similar associations between susceptibility and MBL levels are described. For example, reduced growth rate was observed for low-MBL chicken after *E. coli* infection of chicken [53], while other detrimental effects of low-MBL concentration were observed in infection models for levels of *Pasteurella multocida* in the spleen [54] and *Salmonella* shedding [55]. From the in vivo studies it cannot be deduced whether it is complement activation or one of the other activities of MBL that forms the basis of the protection against pathogens, but the data clearly suggest that MBL plays a major role in disease severity in chickens.

8.5.1.5 Collectin 10, -11, and -12

Collectin 10 and Collectin 11 are two MBL-like collectins that were more recently discovered and have a broad and strongly overlapping gene expression profile [35]. Functional studies in chicken have not been performed but the strong homology with their mammalian

counterparts in sequence and location suggests a similar function. Based on mammalian data, both collectins are mainly complexed as heteromeric molecules with each collectin trimer containing two Collectin 11 and one Collectin 10 monomers [56]. Their level is unaffected by bacterial challenges, indicating that, unlike MBL, these collectins cannot be considered APP. In serum the heteromeric trimer complexes with MASPs and is able, upon binding of microbial polysaccharides, to activate the lectin pathway of complement activation, similar to MBL.

The final collectin, Collectin 12, is the only membrane-bound collectin. This protein also contains structural characteristics of a scavenger receptor [57], making it structurally different from the other collectins. It has a broad gene expression profile but seems mostly expressed by endothelial cells. In vitro Collectin 12 is effectively involved in endocytosis of glycans and microorganisms. Besides that, Collectin 12 interacts with C-reactive protein (CRP), thereby regulating subsequent complement activation through the classical pathway [58].

8.5.1.6 Complement

The serum complement system, a chief component of innate immunity, is an important part of the system of defense against invasion of pathogenic microorganisms [59]. Complement activation results in a fast neutralization of

bacteria by immune cells, and direct bacterial killing via membrane pore formation. Only a few minutes are required between initial detection of invading bacteria and subsequent permeabilization of their membrane by complement components, making complement one of the most rapid ways to kill invading bacteria [60]. The importance of the complement system in innate immunity is evident from the observations that complement deficiency (almost) always results in increased susceptibility [61] and the fact that pathogenic bacteria have evolved mechanisms to resist complement attack [62].

The complement system was initially recognized in mammals as the heat-sensitive factor in serum that “complemented” heat-stable antibody in lysis of bacteria and red blood cells. Nowadays, it refers to a series of about 30 serum proteins as well as 10 or more cell surface complement receptors and regulatory proteins that are present on a wide range of host cells [63]. The proteins circulate in an inactive form, but, in response to the recognition of molecular components of microorganisms, they become sequentially activated, working in a cascade in which the activation of one protein promotes the activation of the next protein in the cascade (Fig. 8.5.3). Besides direct killing of Gram-negative bacteria by the so-called membrane attack complex (MAC) formed from complement components, it also triggers many other innate processes. These include an enhancing effect on phagocytosis

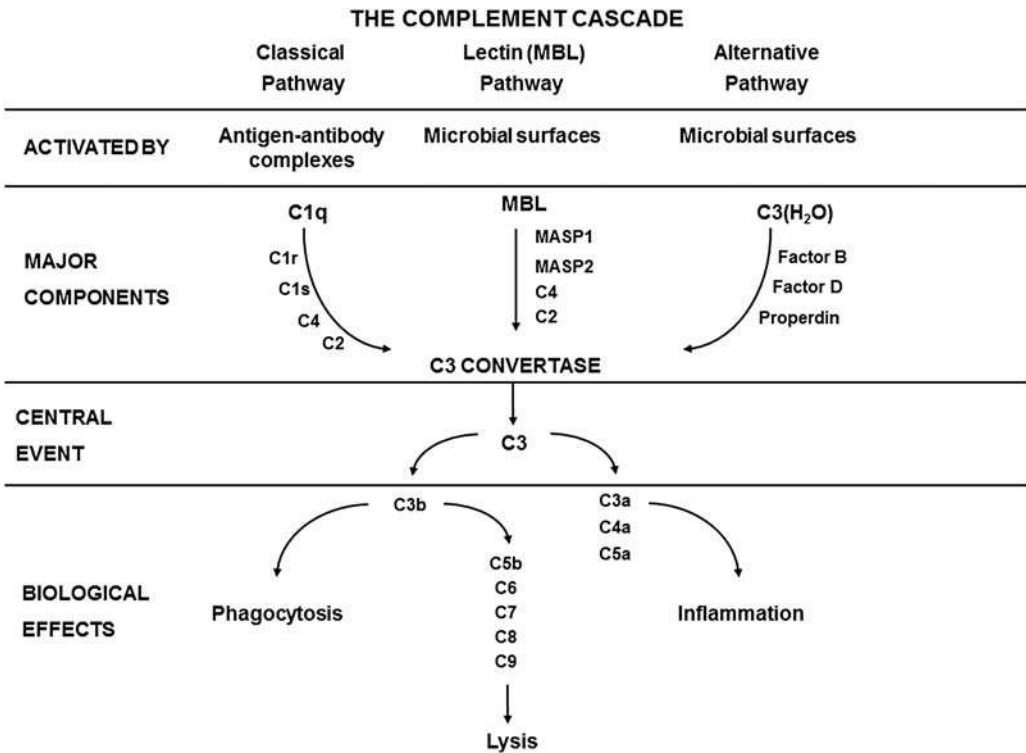


FIGURE 8.5.3 Overview of the main components and effector actions of the complement cascade.

(opsonin activity), the ability to induce an inflammatory response, and enhancing B and T cell responses.

Although the full mechanism of action of complement is quite complex, three clearly distinguishable phases can be described, at least for mammals. The first phase is recognition of microorganisms, or more general foreign elements, leading to initiation of the complement cascade. Three different pathways leading to activation are described: (1) the classical pathway is mainly activated by antigen–antibody complexes; (2) the lectin pathway is mainly activated by the interaction of microbial carbohydrates with, for example, MBL in serum and tissue fluids; and (3) the alternative pathway is less clearly defined but is mainly initiated by a slow spontaneous hydrolysis of C3 in plasma to form C3(H₂O) [63]. All three pathways ultimately produce the key enzyme, C3 convertase. The second phase of the complement cascade is the formation and activity of the complement C3- and C5 convertases, which are able to cleave and produce multiple active complement components that can interact with host (immune) cells and pathogens. Finally, the third phase is formation of the membrane active complex, a pore-forming structure that kills bacteria.

Avian orthologues of most of the complement factors have been identified in the available genome sequences, making it likely that the basic principles will be similar to the mammalian complement system. However, a very limited amount of functional data of the avian complement system is available, mainly showing general cytolytic activity of the complement system [64–66].

Therefore, for an in-depth understanding of the complement cascade and its functionalities, the reader is referred to some of the excellent reviews that are available on this subject [60,63], while the paragraphs below will describe the characterized avian components of complement or the apparent absence of them.

8.5.1.7 Components of the classical pathway

The chicken C1q molecule has been identified and functionally characterized. C1q is part of the classical pathway of complement activation where it binds to the Fc part of the antigen-bound immunoglobulins after which two serine proteases, C1r and C1s, attach in a calcium-dependent way to form the C1 complex. Chicken C1q, like its human counterpart, has a characteristic “bouquet of tulips” structure (comparable to collectins) with six globular subunits. It consists of a hexamer of heteromeric trimers of three C1q proteins, A, B, and C. The globular subunits are ideally spaced to interact with multimerized Fc units of IgM explaining why activation of complement does not occur in serum when all components are in circulation. Chicken C1q has the ability to generate full C1 hemolytic activity when mixed with the human

complement subcomponents C1r and C1s [67]. Gene expression of the three chicken C1q chains has been identified (XM 417654.6, XM_004947521.3, XM 417653.5) with the genes located at chromosome 21. In addition, the nucleotide sequence for chicken C1s and C1r has been determined (NM_001030777.1, XM 416518) and mapped to chromosome 1.

The chicken C4 component has not been isolated but its predicted amino acid sequences were identified (NM_001077233.1). In addition, C4 cleavage, resulting in the formation of C3, one of the key proteins (convertase) in the complement cascade has been detected in chicken serum using human and guinea pig reagents, indicating the presence of a functional C4 component in the chicken. Also, a chicken serum protein has been found to cross-react with antihuman C4 serum [68]. It, therefore, seems reasonable to assume that chickens possess a functional C4 orthologue and likely a normal classical complement pathway.

8.5.1.8 Components of the lectin pathway

As described above, the initiators of the lectin pathway, MBL, Ficolin, and Collectin 10/11 have been identified in chicken, of which MBL has been functionally characterized. In addition the cloning of chicken MASP-2, MASP-3, and MAP-19 (NM 213587, AY 567828, AY 567830) has been described [37]. Interestingly, chicken MASP-1 is not functionally present because of the loss of an exon in the gene. Despite this apparent lack of MASP-1, chicken MBL has the ability to activate the complement system when tested in a heterologous system by deposition of human C4 on the chicken MASP complex. This correlates with the observation that also in mammals MASP-1 does not seem to be essential for complement activation [69]. Complement activation was directly associated with the concentration of MBL in serum indicating the importance of MBL for the total complement response to infection [49].

8.5.1.9 Components of the alternative pathway

Activation of the alternative complement pathway is less defined than the classical- and lectin pathways because it lacks clear ligands. It is speculated that spontaneous low-level hydrolysis of C3 in plasma occurs to form C3(H₂O) can bind to Factor B to initiate the further complement cascade (see Fig. 8.5.3). Alternatively, a variety of “natural” activators of the alternative pathway have been postulated such as microbial polysaccharides (LPS, zymosan, inulin), fungi, viruses, and more [70]. Finally, a third hypothesis is that the alternative pathway could function mainly as an amplifying mechanism, which serves to increase the density of C3b molecules on the bacterial

surface, initially started by the classical or lectin pathways [60].

Of the alternative pathway components, chicken Factor B has been described. Chicken Factor B is a glycoprotein exhibiting a genetic polymorphism with three common phenotypes [71,72]. When activated in a magnesium-dependent way, but not in a calcium-dependent way, the chicken factor B is cleaved into a Ba fragment and a Bb fragment, leading to creation of an active C3 convertase [71,73]. Depletion of factor B abolished the lytic activity of complement, indicating that factor B participates in the alternative pathway of complement activation in chicken [73].

8.5.1.10 Downstream components of complement

Chicken C3 as a key element of complement where all three activation pathways merge has been isolated, mapped, and characterized [74,75]. It has a double-chain structure with an α -chain and a β -chain; both have been cloned (NM 205405). Normally, chicken C3 is present in serum, but recently it was also found in egg yolk [76]. Upon cleavage of chicken C3 by C3 convertase, two fragments are generated: the small anaphylatoxin C3a and the major fragment C3b [75].

Components of the MAC have not been isolated or characterized. However, chicken serum is able to lyse red blood cells indicative of the presence of an active MAC [77,78]. In addition, predicted amino acid sequences have been determined for chicken C5–C9, (XM 415405, XM 429140, XM 424775, XM 415563, AI 980217), and C6 and C8 were also identified in a proteomics approach of egg yolk components [79]. These findings are all supporting a MAC process similar to that in mammalian species.

8.5.2 The acute-phase response

Inflammation in vertebrates is accompanied by a large number of systemic and metabolic changes, collectively referred to as the acute-phase response (APR). Stimuli that commonly give rise to an APR include bacterial infection, surgical or other trauma, bone fractures, neoplasms, burn injuries, tissue infarctions, and various immunologically mediated inflammatory states. Among the changes in homeostatic settings described during an APR are fever, somnolence, anorexia, increased synthesis of a number of cytokines and endocrine hormones, decreased erythropoiesis, thrombocytopenia, alteration in plasma cation concentration, inhibition of bone formation, negative nitrogen balance with consequent gluconeogenesis, alterations in lipid metabolism, and, finally, major alterations in the concentration of some plasma proteins called acute phase proteins (APP).

APP have empirically been defined as those proteins whose plasma concentrations change by 25% or more during an APR. Proteins that increase during the response are designated positive APP, while those that decline are termed negative APP. Furthermore, the positive APP can be divided into three groups: those increasing by onefold (group I), those increasing by twofold to fivefold (group II), and those increasing up to 1000-fold (group III) [80].

Some APP increase in concentration as early as 4 hours after the inflammatory stimulus, attain a maximum level at 24–72 hours, but decline very quickly. Other APP begin to increase 24–48 hours after stimulus, reach a maximum in ~ 7 –10 days and return to normal after ~ 2 weeks. Most positive APP are glycoproteins synthesized in the liver upon stimulation with pro-inflammatory cytokines and glucocorticoids; they are subsequently released into the bloodstream. In mammals, the main pro-inflammatory cytokines involved are IL-1, IL-6, and tumor necrosis factor- α . These cytokines are produced by tissue macrophages and peripheral blood monocytes, which are likely cells become activated, for example, upon encounter with foreign organisms. Over 12–24 hours, the liver responds to the cytokine exposure and starts to produce the positive APP and decrease the production of the negative APP. In addition, extrahepatic production of APP has been reported in most mammalian species studied. Finally, psychological stress has been shown to elevate the level of IL-6 and APP. It is not known how stress induces APR, but activation of the hypothalamic–pituitary–adrenal axis may trigger systemic or local cytokine production by stress signals, thereby stimulating APP production [81,82].

Chicken acute-phase proteins are produced mainly in the liver but APP mRNA has been detected in a variety of other tissues from healthy chickens. In 55 days old broilers, extrahepatic Serum amyloid A (SAA) mRNA was shown in amounts comparable to liver in lung (70%) and cecal tonsils (72%) and to a lesser extent in spleen (26%). Also, extrahepatic expression of Ovotransferrin (OVT) was shown in lung (20% of liver expression). CRP, PIT54 and alpha-1-acid glycoprotein (AGP) mRNA was detectable in several tissues but in very low amounts compared to liver [83]. The monitoring of chicken APP has so far mainly relied on determination of individual proteins by clinical biochemistry and enzyme-linked immunoassays, often using partially cross-reactive murine or human antibodies. Recently, more integrated proteomic approaches have been developed [84,85].

8.5.2.1 C-reactive protein

Human CRP is a cyclic oligomer, which binds to phosphocholine in a Ca^{2+} dependent manner and plays an important role in protection against infection, clearance of

damaged tissue, prevention of autoimmunity, as well as in regulation of the inflammatory response [86]. CRP is an evolutionary well-conserved protein also found in primitive arthropods, where it is expressed constitutively and not as part of an APR [87]. In mammals, CRP is identified as a major acute-phase protein and is widely used for diagnostic purposes in both human and veterinary medicine [88]. The value of using CRP as an acute-phase response marker in chickens is not well established.

Constitutive hepatic expression of CRP is reported and examples exist of slightly increased mRNA levels in chickens fed with aflatoxin [89] or in transport stressed turkeys [83]. Also, slightly increased CRP mRNA expression was reported in spleen of chickens infected with *Ascaridia galli* [90].

A commercial CRP ELISA with potential use for chicken samples is available. Sohail et al. report that they found that 50% of the serum samples from heat-stressed broilers were CRP positive compared to 20% of the samples from a thermoneutral control group [91]. Also, small increases (onefold to twofold) in CRP serum concentrations were reported in broilers after LPS inoculation in the crop [92] or infection with IBV 4/91 [93]. In summary, no evidence exists for CRP being a major acute-phase protein in the chicken.

8.5.2.2 Serum amyloid A

SAA is an apolipoprotein associated with high-density lipoproteins in plasma. In mammals, SAA is a major acute-phase protein produced in the liver for release into blood circulation as a response to, for example, infection, trauma, and stress. Modulation of lipoprotein transport and metabolism is an important SAA function as well as pathogen opsonization, immune cell recruitment and prevention of oxidative tissue damage [94,95].

Multiple SAA genes have been described in mammals [94,96], but in chickens, only one SAA gene has been identified [97]. The chicken SAA gene is mainly expressed in liver and hepatic SAA mRNA expression was shown to increase in response to infection with *S. gallinarum* (33-fold) [98]. In addition, increased expression of SAA mRNA was reported in the spleen of chickens infected with *S. enteritidis* (55-fold) [99] or H5N1 influenza (40-fold) [100].

Increased plasma levels of chicken SAA during an APR have been reported for infection with IBV (fivefold to sixfold) [101]; IBDV (1.5-fold to twofold) [102]; NDV (1.5-fold) [103]; *S. typhimurium* (fourfold) [104] poultry red mite (fourfold) [85] and after LPS challenge (sixfold) [84].

In mammals, SAA is the precursor of amyloid A, the tissue deposit causing secondary amyloidosis [105]. SAA plasma concentrations were shown to be elevated in broilers with experimentally induced amyloid arthropathy [106] and

fibroblast-like synoviocytes were identified as a potential source of articular SAA during inflammation [107].

8.5.2.3 α 1-acid glycoprotein

AGP is a highly glycosylated plasma protein with branched carbohydrate side chains composed of monosaccharides such as mannose, galactose, and glucose. AGP is involved in binding and transport of various drugs including heparin, serotonin, steroids, and histamine. Numerous immunomodulatory functions of AGP are described and ability to bind to LPS implicates the importance of AGP in antiinflammatory responses. The immunomodulatory, as well as the binding activities of AGP, is largely dependent on carbohydrate composition [108]. As in mammals, avian disease responses may change the glycan composition, which Inoue et al. [109] showed by ConA interaction studies using AGP from chickens infected with IBDV [109].

Moderate increases of AGP levels in plasma have been reported for chickens infected with IBV or ILTV (twofold to threefold) [110], IBDV (threefold to sixfold) [109], LPPI (threefold) [111], and *C. perfringens* (twofold) [112]. Increases in AGP plasma levels were in some cases shown to directly correlate with disease severity or virulence of the pathogenic challenge strain used [111]. Also, inoculation with LPS has been shown to increase plasma levels of AGP (threefold to sixfold) [84,113,114].

Apart from infectious diseases, plasma levels of AGP are influenced by external factors including nutrition [115] stress [116], housing [117], as well as gut barrier function/failure [118].

8.5.2.4 (Ovo)transferrin

Two different soluble glycoproteins of the transferrin family are present in mammals; transferrin in plasma committed to iron binding/transport and lactoferrin in extracellular fluids committed to antibacterial and immunomodulatory activities [119]. In chickens, OVT is the only soluble glycoprotein of the transferrin family and the protein is present both in plasma and eggs although differing in glycosylation [120]. Hepatocyte production of OVT for secretion into the blood is influenced by pro-inflammatory cytokines, whereas OVT produced in the oviduct for incorporation into the egg is influenced by estrogen [121].

In mammals, transferrin is a negative APP and expression is downregulated under an APR [122]. In chickens, mRNA expression of OVT in the oviduct was reported but at the same time, OVT serum levels, as well as liver mRNA expression, was increased [121]. Infection studies confirmed that OVT is a positive APP in chickens and plasma levels were shown to increase 7 days after

challenge with ILTV (threefold), *E. coli* (sevenfold), FPV (twofold), ARV (eightfold), IBDV (threefold), IBV (threefold) [123]. Infection with LPAI was also shown to increase plasma OVT (twofold) especially in unvaccinated chickens [111]. Furthermore, OVT levels were reported to increase in response to *E. maxima* and *E. tenella* infection (twofold to fourfold) [124] as well as *Gallibacterium anatis* and *Streptococcus zooepidemicus* infection (1.5-fold to twofold) [125]. Even though OVT is considered a positive APP in chickens a single study reports a small but significant decrease in plasma OVT 2–3 days after *S. gallinarum* infection [126].

Recently OVT content in feces has been identified as a potentially valuable tool to measure gut barrier failure caused by enteric pathogens. In experimental infection trials, it was shown that fecal OVT levels were significantly correlated with the severity of gut barrier failure caused by either coccidiosis or necrotic enteritis [127].

8.5.2.5 PIT54

In mammals, haptoglobin (Hp) binds free hemoglobin (Hb) from lysed erythrocytes and inhibits its toxic, pro-inflammatory and oxidative activity. The Hp-Hb complex is recognized by CD163 and scavenged by phagocytes [82,128]. No Hp gene has been identified in the chicken genome but a functional homolog in terms of Hb binding was identified as being PIT54, a soluble member of the family of scavenger receptor cysteine-rich proteins which also contain CD163 [129]. The *PIT54* gene was previously characterized and the antioxidative properties of the protein were established [130]. PIT54 is a single-chain polypeptide bound to a carbohydrate component, unlike the glycosylated tetramer structure of mammalian Hp. Also Hb binding capacity differs between PIT54 and Hp, that is, PIT54 binds with lower affinity to chicken Hb than mammalian Hp binds to mammalian HB [131].

Nazifi et al. found no significant increase in plasma PIT54 levels upon IBDV infection, whereas IBV infection induced a small increase (1.5-fold to twofold) [101,102]. Increased plasma PIT54 levels were also shown after challenge with LPS from *S. typhimurium* (1.5-fold) [132] or *E. coli* infection (1.5-fold) [133]. Only a single study report increases in plasma PIT54 levels as high as sixfold to eightfold upon infection with *S. gallinarum* [126].

8.5.2.6 Hemopexin

Hemopexin (Hx) is involved in removal of free heme from circulation by binding and transporting it to the liver, where it is taken up by parenchymal cells in part via the CD91 receptor for further catabolism and excretion [134]. Free heme released from damaged erythrocytes provide a source of iron for invading bacteria but is

also highly toxic via its ability to induce free radicals. Hx thus protects against heme toxicity, sequesters heme from potential pathogens, and supports iron homeostasis and antioxidant production [135]. Hx was shown to be a positive chicken APP in early studies where plasma levels increased in chickens injected with turpentine (fivefold) [136]. Others have shown that Hx increase (threefold) after injection with LPS [137,138] and interestingly in one study Hx levels remained higher than in control animals up to 14 days after the LPS challenge [139]. Furthermore, Hx was increased in chickens infected with *S. typhimurium* (1.5-fold) [126],

8.5.2.7 Ceruloplasmin

Ceruloplasmin (Cp) belongs to the multicopper oxidase family of plasma proteins and contains the majority of copper found in mammalian blood [140]. Avian blood has a very low copper content and as such the Cp concentration is around 10% of the Cp concentration in, for example, human blood [141]. Cp is involved in both storing, transporting, and donating copper via Cp receptors expressed on various cell types in addition to having a principal role in the prevention of iron-induced oxidative stress [140]. Early studies established Cp as a positive APP in chickens as Cp content in plasma measured by p-phenylenediamine oxidase activity was shown to increase twofold to fivefold after LPS challenge [142]. Plasma Cp content also increased after *E. coli* infection coinciding with the clinical onset of the infection (twofold to fivefold) [133,143] as well as after *E. tenella* infection (twofold) [133,144]. Interestingly, others identified decreased Cp plasma levels in chickens infected with IBDV [85,102].

8.5.2.8 Fibrinogen

FB is involved in hemostasis, providing a substrate for fibrin formation, and in tissue repair, providing a matrix for the migration of inflammatory-related cells. Pro-inflammatory functions of FB are associated with its ability to bind to a variety of immune cells and hepatic production is associated with APRs in mammals [82,145]. During a turpentine-induced APR in chickens, FB plasma levels were shown to increase (twofold to 2.5-fold) [136,146]. Infection with *E. coli* or *E. tenella* also induced an increase in plasma Fb levels (twofold to fourfold) [133].

8.5.2.9 Other potential chicken APPs

Fibronectin (FN) is a glycoprotein, which exists in circulation and in tissue. The liver synthesizes most of the soluble FN found in the plasma, whereas fibroblasts, endothelial

cells, and other cell types synthesize the insoluble tissue FN. FN influences a wide range of cellular properties such as growth, differentiation, wound healing, migration, and apoptosis [147]. After treating chickens with turpentine, the plasma FN concentration was found to increase (2.5-fold) [146]. In vitro studies have confirmed that chicken hepatocytes secrete increased levels of FN after treatment with glucocorticoid or chicken IL-6 [148].

Pentraxin 3 (PTX3) is a member of the Pentraxin protein family, which all contain a C-terminal eight amino acid pentraxin motif. The short pentraxins include classical mammalian APPs such as CRP and SAP, which comprise solely the pentraxin domain. PTX3 belongs to the long pentraxins, which possess a second N terminal domain. Mammalian PTX3 is a soluble pattern recognition receptor with multiple roles not only in defense against infectious agents but also in tissue repair and cancer-related inflammation (reviewed in Ref. [149]). Chicken PTX3 mRNA was shown to be constitutively expressed in various tissues but interestingly in spleen only after LPS stimulation or IFN- α administration [150,151]. RNA-seq data further supported that gene expression in Bursa of Fabricius and spleen were increased upon experimental infection with bacterial as well as viral pathogens. Finally, challenge dose-dependent increases were shown in spleens of chickens infected with avian pathogenic *E. coli* [150].

Albumin is a small globular but highly abundant plasma protein, which contributes to the colloid osmotic pressure, serves as a carrier protein for a number of compounds, and contributes to extracellular antioxidant defenses [152]. An APR triggers downregulation of albumin production in the liver in combination with loss by renal and gastrointestinal systems leading to a decrease in plasma concentration. Albumin is a negative APP in all mammals studied [81,82]. LPS or turpentine administration was shown to lower albumin plasma levels in chickens [136,139].

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Pattern recognition receptors

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8.6.1 Introduction

The term “pattern recognition receptor” (PRR) was first coined by Janeway and colleagues [1–3] to describe the families of receptors that interact with conserved pathogen-associated molecular patterns (PAMPs). Many of the molecular patterns that stimulate PRRs are also found on non-pathogenic microbes and for that reason some authors prefer to use the term “microbe-associated molecular pattern” (MAMP). The general nature of a PAMP (or MAMP) is a conserved component or product of a pathogen (or microbe) that is not easily modified. In the late 1990s or early 2000s there was an intense discussion on the importance of pathogen (microbe) recognition versus the sensing of damage (or danger). These two camps were brought together when it was recognized that the PRRs were activated not only by both PAMPs and MAMPs but also by damage-associated molecular patterns (DAMPs). In many ways, many of the DAMPs are molecularly similar to the P(M)AMPs, for example, mitochondrial DNA-derived unmethylated CpG motifs [4]. Other DAMPs are less obviously related to microbial products but are recognized by the same classes of PRRs that recognize microbes [5]. Whatever the stimulus, the consequence of PRR activation is to initiate downstream immune cascades that typically lead to initiation of antimicrobial pathways, inflammation, and/or activation of immune cells. The concept of PRR holds true across the entire spectrum of life with even the simplest organisms having sensory mechanisms to detect and respond to the presence of potential pathogens. Within multicellular organisms, many of the PRR molecules are highly conserved and orthologues of many of those that we will consider in the rest of this chapter can be readily identified in other vertebrates and invertebrates. Indeed, most of the work on avian PRRs is heavily informed by biomedical studies in humans and rodents.

The families of PRRs include soluble components [lipopolysaccharide (LPS)-binding protein, collectins,

pentraxins, and alternative pathway complement components] and cell-associated components [C-type lectins, scavenger receptors (SRs), formyl-peptide receptors, Toll-like receptors (TLR)], as well as various cytoplasmic receptors [e.g., nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) and retinoic acid-inducible gene I (RIG-I)-related receptors]. Collectively, PRR families form the basis for the activation of innate immunity and underpin the ability of the host to recognize and react to invading pathogens or cellular damage/stress. In multicellular organisms, pathogens are typically located in three different types of compartment: in tissue fluids, on mucosal surfaces, or on/in cells. Unsurprisingly, this distribution is matched by the location of PRRs; some are soluble, while others are transmembrane (surveying the extracellular environment close to cells) and others are cytoplasmic. The positioning of PRR in each of these locations covers those niches occupied by all major pathogen groups. Redundancy in recognition of particular PAMP structures and coordination of different PRR to sense any single invading class of microorganism are important features of the PRR network. In essence, the outcome of PRR-mediated responses is to initiate downstream antimicrobial cascades and cellular activities that represent the earliest events in a developing immune response. Indeed, without the activity of PRR, under most circumstances, the body and the immune system ignore the presence of nonself-antigens. Moreover, the “adjuvant effect,” which is central to successful vaccination, is largely mediated by PRR stimulation with microbial PAMPs or their synthetic mimics.

Within the field of avian immunology, the PRRs are receiving considerable attention although there is still a strong bias toward certain groups of PRRs, most notably the TLRs and some of the intracytoplasmic PRR, particularly those responsible for recognition of viral agonists. The chapter will first briefly consider the work on soluble PRR before focusing on those that are cell associated. For

cell-associated PRRs, it is important to note that some cells express wider ranges of PRRs than other cell types, for example, those cell types with a wide range include classical immune cell types such as heterophils, macrophages, and dendritic cells [6–12]. The completion of a full genome sequence for the chicken [13] hugely simplified the identification of PRR and related pathways. The recent availability of a wide range of avian genomes [14] will open the door to a wide array of comparative studies and most PRRs can be readily identified across a range of avian taxa. In most cases functional studies are limited to the chicken, and sometimes ducks with relatively little in other species. This chapter will focus mostly on areas which include functional studies and will therefore be biased toward work on chickens. The following sections will subdivide the PRRs according to those that are found in tissue fluids or secreted and those that are associated with cells (either on the cell surface or intracellular).

8.6.2 Tissue fluid and secreted pattern recognition receptors

There are a range of soluble PRRs including those found in the blood and tissue fluids as well as some that are secreted at the mucosal surfaces. In terms of tissue fluid-associated PRRs, these often initiate extracellular cascades, for example, the complement cascade. Some are constitutively found in tissue fluids (e.g., the mannose-binding protein), while others are dramatically upregulated during inflammatory responses and can form part of a group of changes known as the acute-phase response [e.g., C-reactive protein (CRP)]. This section deals with soluble PRR and the reader is also directed to Chapter 8.5 for a discussion of the soluble pathways as a whole.

8.6.2.1 C-reactive protein

CRP is a ring-shaped protein and member of the pentraxin family often upregulated as part of the acute-phase response. It is a PRR and recognizes phosphocholine on microorganisms and lysophosphatidylcholine that is upregulated on the surface of dead and dying cells. CRP is implicated in protection against infection, clearance of damaged tissue, prevention of autoimmunity, and regulation of the inflammatory response. It has both proinflammatory and antiinflammatory effects *in vitro* and *in vivo*. Blood CRP levels typically increase in response to a variety of inflammatory triggers and is often linked to activation of other cell-associated PRR (see below) triggered by PAMPs or DAMPs. CRP is also upregulated in response to increasing levels of inflammatory cytokines (e.g., IL-6). It also acts as an opsonin, binding polysaccharide residues on bacteria, fungi, and parasites to activate the complement and phagocytosis [15]. In chickens, infections with a wide array of pathogens induce

increased levels of CRP [15–17]. Infection of Japanese quail with *Aspergillus* has also been reported to lead to rapid upregulation of CRP [18].

8.6.2.2 Collectins

The collectin family of molecules is calcium-dependent multimeric proteins with carbohydrate-recognition domains (CRD) aligned in a manner that facilitates binding to microbial surface polysaccharides or host macromolecules. Nine collectins have been identified in mammals, including mannanose (or mannan)-binding lectin (MBL), surfactant protein A (SP-A), surfactant protein D (SP-D), collectin liver 1 (CL-L1), collectin kidney 1 (CL-K1, collectin 11), collectin placenta 1 (CL-P1), conglutinin (CG), collectin of 43 kDa (CL-43), and collectin of 46 kDa (CL-46). The basic subunit of the collectins is a trimer with three identical monomers each containing four regions: a cysteine-rich N-terminal domain, a collagen-like region, a coiled-coil neck domain, and finally a large globular CRD.

The three polypeptide chains forming the trimer also assemble into larger oligomers via cysteine residues in the N-terminal domain except for CL-43 and CL-L1, for which the trimer is the native form [19]. MBL, SP-A, and FCN may consist of up to six subunits forming a “bouquet of tulips,” whereas the collectins CG, SP-D, and CL-46 are assembled by four subunits in a cruciform structure [19–21]. CL-K1 seems to form dimers or trimers of the trimeric subunit only [22]. The multidomain nature of these molecules is important to increase binding capacity [23]. The functions of the collectins, depending on the specific lectin, include aggregation and neutralization of the microorganism, opsonization and activation of phagocytosis, complement activation, modulation of the inflammatory responses, and modulation of the immune system. A wide range of collectins have been identified in the chicken [24] which vary in expression levels according to tissue [25] and these levels are modulated by infection [26].

8.6.2.3 Mannose-binding lectin

MBL is one of the most studied of the collectins; it is a soluble PRR found in body fluids and plays a key role in activating the complement cascade via a pathway known as the lectin pathway (also see Chapter 8.5). As the name suggests MBL binds mannose and acts as sensor for the density of mannose or *N*-acetyl glucosamine residues which are at high density on the surface of many microbes.

Chicken MBL (chMBL) is primarily produced in the liver and secreted into the blood, although low expression has also been demonstrated in the trachea–larynx, abdominal air sac, infundibulum, and thymus [27,28]. chMBL, like mammalian MBL, is a weak acute-phase

reactant with infection often resulting in less than twofold increases in serum concentration [29]. In contrast to most mammals, only one form of MBL has been found in chickens [30,31]. Electron microscope studies revealed that the chMBL is an oligomer of subunits, each composed of three 30–40 kDa polypeptides joined in a collagen-like triple helix with three globular CRD [30]. To activate the complement, cascade MBL complexes with a set of serine proteases known as MASPs (MBL-associated serine proteases).

Studies of the ontogeny of MBL levels in the chickens revealed that serum chMBL levels increase from 11 days before hatching until 1 year of age, although levels varied between different types of birds [27–29]. chMBL is detectable in yolk but not the egg white and it is likely that maternal chMBL is transferred from the yolk sac to the embryo [27].

Nielsen et al. [28] reported the distribution and function of chMBL in different tissues after infection with infectious laryngotracheitis virus (ILV) and infectious bursal disease virus (IBDV). In noninfected chickens, chMBL was found in the cytoplasm of a few liver cells, but the level increased in virus-infected birds. chMBL-positive cells were also detected in germinal centers within cecal tonsils, on the surface and within ILV-infected tracheal cells, and in the cytoplasm of splenic macrophage-like cells from IBDV-infected birds. Complement activation was shown to be directly associated with the concentration of MBL in serum [29] and it is noteworthy that chMBL can also directly bind the spike glycoprotein of infectious bronchitis virus viral [32].

Comparing the MBL sequence across different chicken breeds/lines has revealed considerable variation particularly in the promoter region of the MBL gene [24]. Studies in chicken lines selected for high- or low-serum concentration of MBL revealed that a low amount of circulating MBL is associated with increased disease severity after IBDV infection [33] and with reduced growth rate after *Escherichia coli* infection [34]. Low MBL concentration was also associated with increased numbers of *Pasteurella multocida* in the spleen [35]. These results confirm that MBL, as proven in mammals, plays a pivotal role in the chicken immune system.

8.6.2.4 Ficolins

Members of the ficolin (FCN) family bind carbohydrates (typically *N*-acetyl glucosamine and mannose structures [36]) in a calcium-independent manner; three ficolins have been identified in humans: H-FCN, L-FCN, and M-FCN [19]. Ficolin has a globular C-terminal fibrinogen-like domain as the functional carbohydrate-binding domain. FCNs were originally discovered as transforming

growth factor- β -binding proteins in the porcine uterus [37] but have since been identified in a wide range of vertebrates and invertebrates [38]. Human L- and H-FCN are serum proteins mainly produced in the liver. H-FCN is also produced in the lung. M-FCN is a nonserum FCN that is mainly synthesized by monocytes (reviewed in Ref. [38]). The PRR function of FCN, like MBL, is to activate the complement and act as an opsonin for phagocytosis of pathogens or apoptotic cells [39]. Ficolins activate complement through a pathway shared with MBL by being dependent on MASPs. In chickens, a single FCN gene has been identified, which appears to represent an ancestor of L-FCN and M-FCN [40], but no functional studies have been reported.

8.6.2.5 Surfactants: surfactant protein A and surfactant protein D

The lung collectins SP-A and SP-D are mainly produced by nonciliated bronchial epithelial cells and secreted to the lung mucosa, but both forms have also been detected in nonpulmonary tissues [41]. Both lectins are PRR involved in agglutination, enhancement of phagocytosis, growth inhibition of pathogens, clearance of apoptotic cells, and modulation of the immune response (reviewed in Ref. [42]). In chickens, a gene for SP-A and a lung lectin named chicken lung lectin (cLL) that lack the collagen domain have been sequenced and located on chromosome 6 (AF411083, DQ129667) [25]. There is no evidence of an SP-D gene in the chicken. Reemers et al. [26] found that SP-A and cLL mRNA expression was downregulated in the lung of avian influenza virus (AIV)-infected chickens but upregulated in the trachea.

8.6.2.6 Other collectins

Three other collectins have been identified in mammals: CL-L1, CL-P1, and CL-K1. CL-L1 and CL-K1 are soluble serum collectins, whereas CL-P1 is membrane-bound. Interestingly, CL-K1, which binds L-fucose and D-mannose, is able to activate the complement cascade via MASPs as reported for MBL and FCN [43–45].

Three collectins have been identified in chickens and designated: chCL-1, chCL-2, and chCL-3, which resemble the mammalian lectins CL-L1, CL-K1, and CL-P1, respectively [24]. These collectins are expressed in a wide range of tissues throughout the digestive, reproductive, and lymphatic systems, which indicates that these proteins may have an important role in avian host defense [25]. After an infection with avian influenza A, the mRNA expression of CL-K1 was downregulated in the lung, whereas the CL-L1 and CL-K1 were upregulated in the trachea [26].

8.6.2.7 Chicken mannose (or mannan)-binding lectin-associated serine protease proteins, linking soluble pattern recognition receptor to complement activation

MBL, FCN, or CL-K1 binds to carbohydrate groups on the surface of pathogens and associates with the two serine proteases: MASP-1 and MASP-2 [40,46]. A third MASP, MASP-3, and a nonenzymatic MBL-associated protein (MAP)-1 are alternative splice variants from the MASP-1 gene [47]. A second MAP protein MAP-19 is an alternative spliced variant from the MASP-2 gene [47]. The lectin–MASP complex then cleaves C4 and C2 to form the C3 convertase (C4bC2b) [19,40]. The nonenzymatic protein MAP-1, lacking the CRD domain of the MASP-1 gene, competes with all three MASPs for ligand binding and mediates a dose-dependent inhibitory effect on the lectin complement pathway [48]. MBL, FCN, and a CL-K1-like genes have been identified in chickens (see earlier). Chicken MASP-2, MASP-3, and MAP-19 have been identified and characterized. Despite the lack of a functional MASP-1 in chickens (due to the loss of specific exons in the MASP-1/3 gene), chicken MBL has the ability to activate the complement pathway [40]. The complement pathway is described in more detail in Chapter 8.5.

8.6.3 Cell-associated pattern recognition receptors

The cell-associated PRR can be divided into those that sense PAMPs at the cell surface or have been taken into intracellular membrane-bound vesicles, and those that act as cytoplasmic pathogen detection systems. The cell surface group includes some of the membrane-anchored C-type lectins (e.g., Dectin 1, Dectin 2, and mannose receptor), SRs, formyl-peptide receptors, and the TLRs. Of these the TLRs have received by far the greatest attention within the field of avian immunology. Many of the other cell surface PRRs can be identified in the chicken genome but functional analyses are often lacking. The cytoplasmic PRRs detect products of pathogens that replicate or are translocated into the cell cytoplasm (typically derived from viruses and some bacteria) or cytoplasmic DAMPs (e.g., mitochondrial products). Cytoplasmic PRRs include NLRs and RIG-I-like receptors. The ever increasing availability of genomic resources has fueled the identification of cell-associated PRR in a multitude of species (including birds) although in many cases functional analyses have not yet been completed.

8.6.3.1 Avian Toll-like receptors

Background

The Toll protein was originally described as a regulatory protein involved in dorsoventral axis formation of

Drosophila melanogaster [49] but was later identified as a receptor initiating upregulation of the antifungal peptide drosomycin [50]. The discovery of human TLR [2,51,52] was soon followed by the characterization of 13 mammalian TLR, and since then members of the family have been described in fish, amphibians, reptiles, and birds [53]. A substantial body of evidence has accumulated to identify the adapter molecules, signaling pathways, and downstream events. A wide range of PAMPs are reported to stimulate TLR; these are often referred to as TLR agonists. The nature of TLR–agonist interaction is under intense scrutiny, and for many vertebrate TLR, the residues responsible for ligand interactions have been identified (e.g., Ref. [54]), but it is important to remember that for some interactions, these may involve other molecules (e.g., MD2 with TLR4-based recognition of LPS). Analysis of the increasing array of genome sequences has revealed that most bird genomes encode a similar set of TLRs (typically 10–12), although there have been some deletion and duplication events in some lineages [53] including birds (e.g., Refs. [55–58]).

The general structure of TLR comprises an extracellular N-terminal leucine-rich repeat (LRR) domain with one or two cysteine-rich regions, a transmembrane domain, and a C-terminal domain referred to as Toll/IL-1 receptor (TIR), reflecting a similarity with the IL-1R intracellular domain. The number and distribution of LRRs within the ectodomain is a defining feature of specific TLR and affects agonist recognition. The TIR domain is relatively conserved between different TLR and contains three highly conserved regions that are important in recruitment of adapter proteins and in signaling [59].

The avian Toll-like receptor repertoire

The TLR molecules can be subdivided into phylogenetically related families, and most vertebrate genomes have representatives of at least one member from each family [53,55–57]. Based upon phylogenetic analyses, the main groups of vertebrate TLR can be considered ancient and are predicted to have diverged during, or before, the Cambrian period (505–590 million years ago [53]). These families are as follows: the TLR1/2 group (with TLR1, 2, 6, 10, and *Fugu* TLR14); the TLR3 group; the TLR4 group; the TLR5 group; and the TLR7 group (including TLR7, -8, and -9). Other TLRs have also been described in mouse (TLR11, -12, and -13), human (a TLR11 pseudogene), and fish (e.g., the related TLR21 and -22 molecules). The chicken TLR (chTLR) repertoire includes representatives of the major groups and relevant components of conserved TLR signaling pathways. The first chTLRs to be identified and characterized were two genes with high homology to mammalian TLR2 [60,61], followed by chTLR4 [62], chTLR5 [63], and chTLR7

[64]. Expressed sequence tags (ESTs) were identified with homology to human TLR1, -6 or -10, TLR2, TLR3, TLR4, TLR5, and TLR7 [65,66]. Subsequently, identification of chTLR from the chicken genome sequence (with EST annotation) has been reported [67]. Unfortunately, in this report there are some errors in the annotation of some chTLR sequences. Two further chTLRs have been identified that are not orthologues of any mammalian TLR, and these have been termed chTLR15 and chTLR21 [53,66,68]. Despite extensive analysis, no orthologues of mammalian TLR9, TLR11, TLR12, or TLR13 can be identified in any of the available avian genomic resources. Moreover, it is clear that remnants of a chTLR8 molecule can be detected in the chicken genomic sequence (validated by independent sequencing of this region), but this is disrupted in galliformes and many other avian groups with multiple frame shifts and a ~6-kB insertion containing the CR1 element [64,69]. In summary, the chTLR repertoire to date includes two TLR1/6/10-like molecules; two TLR2, TLR3, TLR4, TLR5, and TLR7 orthologues; and two TLRs that are found only in birds. Those chTLRs with mammalian orthologues can be differentiated relatively easily according to amino acid identity, predicted patterns of LRR, and gene position in areas of conserved synteny with mammalian genomes. A range of conserved components of the known TLR signaling pathways (reviewed in Ref. [59]) have been identified in EST and genomic resources [64–66]. The components that can be identified in the sequence resources include coreceptors (e.g., CD14, dectin-1, and MD2); adapter proteins (e.g., MyD88, Tirap, and TRIF); signaling intermediates (e.g., TRAF-6, IRAK, and MAPK); and associated transcription factor components (e.g., NF- κ B and IRF7). To date, there is little confirmation of the precise pathways initiated by chTLR, although it is predictable that the gross features of chTLR signaling will be conserved with other avian species. This statement is supported by the pharmacological inhibition of oxidative burst in heterophils [70,71] and nitric oxide production with the macrophage-like cell line HD11 [72] and by a comprehensive study of TLR regulation in HD11 cells [11].

8.6.3.2 TLR1/6/10-related molecules

In humans, TLR1, -6, and -10 represent a closely related group of TLR receptors. Functionally, TLR1 and TLR6 have been demonstrated to form heterodimers with the related molecule TLR2, influencing the agonist-driven response providing a degree of specificity as seen with recognition of triacylated peptides (TLR1) or diacylated peptides (TLR6) [73,74]. In the chicken, it is clear that two TLR1/6/10-like molecules exist (with 44%–46% amino acid identity with all three human molecules),

which are broadly expressed in tissues and different cell types [10,67]. The two TLR1/6/10-like molecules have very high sequence identity with evidence of gene conversion—the longer version has been attributed as TLR1 type 1 and a second shorter gene (with a truncated LRR domain) is identified as TLR1 type 2 [75]. It is clear that avian macrophages and heterophils recognize both diacylated and triacylated peptide agonists [11,76] although it is less clear whether this relates to differential usage of the two TLR1/6/10-like molecules since both agonists can be recognized by TLR1 type 1 [75,77] (note: the TLR16 in Ref. [75] is accepted as TLR1 type 1).

8.6.3.3 TLR2

In mammals, the TLR2 molecule has been demonstrated to respond to a wide variety of PAMPs, including bacterial lipoproteins, arabinomannan, peptidoglycan, fungal zymosan, and GPI anchors from protozoan parasites [78–84]. As indicated in the preceding section, the spectrum of mammalian TLR2 agonists is influenced by the capacity to function as homodimers or as heterodimers with TLR1 or TLR6. The two chTLR2-like molecules have 88.5% nucleotide identity with each other and ~50% amino acid identity to human TLR2. chTLR2 genes are tandemly arranged on chromosome 4, in a region with conserved synteny with the mammalian TLR2-containing genomic regions [60,61]. The two forms of chTLR2, termed type 1 and type 2, are differentially distributed in tissues and cell populations [10,61]. ChTLR2 type 1 has more restricted distribution, being highly expressed only in spleen, cecal tonsils, and liver tissues, and most intensively expressed on heterophils, the DT40 B-cell line, and the macrophage-like HD11 cell line (but not on blood monocyte-derived macrophages). In contrast, chTLR2 type 2 is more broadly distributed [10,61].

The oxidative burst activity of heterophils in response to lipoteichoic acid (from *Staphylococcus aureus*) is inhibited (25%–30%) by application of a goat antihuman TLR2 or antihuman CD14 antisera [85]. Evidence for conservation of the signaling pathways with TLR2-mediated responses in avian cells is evident from studies on the pharmacological inhibition of the heterophil oxidative burst and the transcriptional profile of the HD11 chicken macrophage-like cell line [11,71].

8.6.3.4 TLR3

The TLR3 agonist, double-stranded (ds) RNA, was widely known as an immunostimulatory compound in both mammals and birds, long before the discovery of TLR molecules [86]. Human and chicken TLR3 share 58% overall amino acid identity and are expressed in a wide range of

tissues and cell populations [10]. The chTLR3 gene is located on chromosome 4, again in a region of conserved synteny with mammalian TLR3 genes. In mammals, the response to TLR3 agonists is typified by upregulation of type-I IFNs, and exposure of chicken cells to the synthetic dsRNA polyinositol cytosine (polyI:C) stimulates upregulation of IFN α and IFN β mRNA [64,87]. The cellular response to polyI:C also includes induction of an oxidative burst and degranulation response in isolated chicken heterophils [88] as well as production of nitric oxide in the macrophage cell line HD11 [72]. In mammals, TLR3 is the only TLR with a signaling dependence on TRIF and this component is present in avian genomes (see below and Refs. [56,89]). Pharmacological inhibition studies and analysis of phosphorylation status of various signaling components support the general finding that the TLR pathways are conserved between chickens and mammals [11,72,88]. The specificity of chTLR3 for polyI:C was also confirmed using a heterologous expression system [90]. TLR3 has also been characterized in ducks and geese [91–93].

8.6.3.5 TLR4

The response of TLR4-intact cells to LPS exposure is typified by high levels of nitric oxide (NO) production and expression of the proinflammatory cytokines, including TNF α in mammals and IL-1 β . Different mammalian species react more, or less, intensely to the effects of LPS, although with most mammals, B-cell proliferation is induced by exposure to LPS. This effect is not seen with chickens, except at very high doses of LPS [94]. Chickens are relatively resistant to systemic administration of LPS [95] and, although the nature of this resistance is unclear, the lack of B-cell proliferation may be a contributory factor.

Studies examining the effect of LPS on chicken cells [85,96–102] predate the formal identification of chTLR4 [62,103,104] or other avian TLR4 genes [105,106]. Early efforts to identify a TLR4-mediated response relied upon the cross-reactivity of antibodies raised against human TLR4, which interfered with LPS-mediated induction of NO synthesis, inducible nitric oxide synthase (iNOS) mRNA, and the cytokines IL-1 β and IL-6 mRNA [85,100,101]. The chTLR4 gene encodes a protein with 44% amino acid identity with human TLR4 and is located on the microchromosome 17 [62]. Sequence polymorphisms in chTLR4 were detected in several inbred lines of White Leghorn chicken, and inheritance of the TLR4 locus was associated with survival after exposure of chicks to systemic infection with *Salmonella enterica* serovar Typhimurium [62]. As with mammalian TLR4 [107], chTLR4 mRNA is expressed in a wide range of tissues, immune cell populations, and stromal cells; it is particularly highly expressed on macrophages and heterophils [10,62,76]. The expression level of antihuman

TLR4 reactivity on chicken blood monocyte-derived macrophages also differs between chicken lines. These levels were correlated with the ability of the cells to produce NO in response to LPS [100]. Hence, chTLR4-mediated LPS responsiveness may be affected by both sequence polymorphism and amounts of protein expressed on the surface of target cells. Interestingly, while TLR4 engagement stimulates strong IL-1 β , IL-6, and NO responses, very little IFN α or IFN β response is reported which may be a consequence of a lack of the TRAM sorting adapter that usually recruits TRIF to TLR4 in mammals [103].

8.6.3.6 TLR5

TLR5 responds to flagellin, the most abundant building block of bacterial flagella, and TLR5 orthologues have been reported in mammals, fish, and birds. The chicken TLR5 gene is located on chromosome 3 in the chicken genome and shares 50% amino acid identity with human TLR5 [63]. The tissue distribution of chTLR5 mRNA is as extensive as mammalian TLR5 and is found in both immune and stromal cell types [10,63,107]. Exposure of chTLR5⁺ cells to purified flagellin led to an upregulation in IL-1 β mRNA levels, and the magnitude of the response correlated with the relative levels of chTLR5 mRNA detected by RT-PCR. In these experiments, no change was seen with IL6 mRNA in any of the cell types tested, [63] contrasting with the IL-6 detected after stimulation of mammalian TLR5 under similar conditions [108]. The specificity of chicken, duck, and goose TLR5 for flagellin has been confirmed using transfection-based experiments [109–112] although there are some interesting differences in the structural requirements for flagellin-based activation of chTLR5 compared with mouse and human TLR5 [113].

The higher invasiveness of aflagellate *Salmonella* serovars (e.g., Gallinarum and Pullorum) compared with flagellated serovars (Typhimurium and Enteritidis) suggested a potential role for TLR5 in limiting the level of invasion from the gut. This hypothesis was supported by the increased invasion rates obtained after oral infection of chickens with an aflagellate mutant *S. enterica* serovar Typhimurium [63].

8.6.3.7 TLR7 and TLR8

Analysis of the TLR7/8 locus revealed that chTLR7 is present in the chicken genome (with 62% amino acid identity with huTLR7), but chTLR8 is fragmented and disrupted by a large CR1-containing insertion [64]. The TLR8-CR1 disruption can be identified in the genomic DNA of a wide range of birds [64,69]. The implications for this finding are difficult to ascertain, but in mammals both TLR7 and TLR8 respond to a series of “synthetic modified nucleotide” or virus-associated single-stranded

RNA agonists. In humans, TLR7 and TLR8 exhibit differential agonist specificity and are expressed on different cell types, suggesting distinct function [107,114,115]. All of the known agonists for TLR7/8 in mammals appear to be recognized by chTLR7.

The tissue and cellular distribution of chTLR7 mRNA is relatively focused and similar to that described for mammals [10,64,107,116], with strong expression in B cells. The mammalian plasmacytoid DC is also reported to respond to TLR7/8 agonists, and it is likely that at least some chicken DC subsets have a similar capacity; however, the paucity of reagents to differentiate chicken DC subsets has precluded this type of analysis. Nonetheless, chTLR7 mRNA is present at lower levels in other cell subsets, including blood-derived heterophils, which also respond to exposure with TLR7 agonists [88]. Similar observations have been reported with murine eosinophils [117]. Although very little TLR7 mRNA can be detected in fresh blood monocyte-derived macrophages, the macrophage-like cell line HD11 is strongly positive (using RT-PCR) and responds efficiently to application of the R848 agonist [11,64]. The specificity of chTLR7 for the mammalian TLR7 agonists is supported by transfection studies [66].

Exposure of chicken splenocytes or HD11 cells to a wide range of TLR7/8 agonists induced upregulation of a range of cytokine mRNA in a dose-dependent fashion. These include IL-1 β , IL6, and IL8, but not IFN γ or IFN β [64], as well as increased levels of NOS-2 (iNOS) mRNA. In contrast, with purified heterophils, loxoribine exposure downregulated the levels of IL-1 β , IL6, and IL8 (CXCL8) mRNA [88], although respiratory burst activity and degranulation were increased by this treatment. It is important to note that in another study loxoribine-treated chicken splenocytes did result in upregulation of type-I IFNs [118]. The lack of type-I IFN response (or at least its inconsistency) was somewhat surprising, since mammalian peripheral blood lymphocytes respond to TLR7/8 agonists by upregulating these antiviral molecules [119]. Moreover, exposure of chickens or chicken splenocytes to the imidazoquinolinamine S-28828 led to an increase in type-I IFN-like activity as measured by bioassay [120]. The apparent discrepancy in type-I IFN production may be resolved by the differential activity of different TLR7 agonists, differences in experimental systems (e.g., in vivo or *ex vivo* application will interact with different cell subsets), the response of different cell types, or differences in the chicken lines tested. Application of TLR7 agonist did result in increased levels of type-I IFNs in goose splenocytes [121,122]. The implications of reduced (or variable) type-I IFN responses after TLR7 activation are potentially very important in protection against a wide range of viral pathogens. For this reason, they deserve further investigation.

8.6.3.8 The absence of TLR9

Although it is clear that chickens respond to the classical TLR9 agonist based upon a short unmethylated CpG motif [88,123–132], there is no evidence for an orthologue of mammalian TLR9 in birds. The responses of chicken heterophils, monocytes, or peripheral blood leukocytes to application of CpG DNA include production of nitric oxide and upregulation of IL-1 β and IFN- γ [88,125–127,129] consistent with the activity of a PRR. Functional data with CpG motifs indicate that birds respond to the appropriate TLR9 agonist. It is now clear that chTLR21 is activated by DNA containing unmethylated CpG motifs (see below) [133,134].

8.6.3.9 Avian Toll-like receptor without mammalian orthologues: chTLR15 and chTLR21

Two TLR genes can be located in the chicken genome: chTLR15 (chromosome 3) and chTLR21 (chromosome 11 [42,68]).

Initial characterization of chTLR15 indicates that this molecule is not orthologous to any known TLR molecule in mammals or fish [53,68], but it loosely groups with the TLR1 and TLR2 molecules in phylogenetic analysis. More detailed phylogenetic analysis revealed that the TIR domain sequence of TLR15 clearly groups within the TLR1/2 family; however, when the extracellular domain sequence was considered, TLR15 is clearly distinct from TLR1 or -2 family members [135]. TLR15 genes can be readily identified in other avian genomes (turkey, duck, and zebra finch), and a partial gene was also identified in a reptilian genome (*Anolis carolinensis*) [135,136]. This suggested that this receptor arose ~320 million years ago after the divergence of the reptilian/avian lineage from the mammalian lineage [135]. However, a recent report indicating the presence of TLR15 in the Australian ghostshark (but not any other fishes or amphibians examined) suggests that this receptor may have more ancient origins [137]. The origin of TLR15 is very interesting and deserves further attention.

The expression of chTLR15 mRNA was highest in bone marrow, bursa, and spleen, and expression in the cecum was increased by infection with *S. enterica* serovar Typhimurium, as was expression of chTLR2 mRNA, suggesting that these TLR may be found on cells infiltrating into the gut (perhaps even on the same cell subset) [68,135]. Moreover, exposure of chicken primary embryonic fibroblast cultures to heat-killed *Salmonella* led to upregulation of chTLR15 mRNA [68], which may be the result of activation of other chTLR constitutively present on the cultured cells. ChTLR15 is activated by a heat-labile yeast-derived agonist and this activity is sensitive to inhibition with phenylmethylsulfonyl fluoride (PMSF),

suggesting a requirement for enzymatic function [135,138]. Direct cleavage of TLR15 (with protease K or microbial-derived proteases) has been proposed as a mechanism of activation for TLR15 [138]. It is also appropriate to note that siRNA targeting TLR15 reduced nitric oxide production of HD11 cells in response to *Mycoplasma*-derived diacylated lipopeptide [139].

A further chicken TLR gene was identified and termed chTLR21 because of amino acid sequence homology with *Fugu* TLR21 [53]. However, the level of an identity with *Fugu* TLR21 and TLR22 is very similar at 44% and 38%, which is reminiscent of the level of identity seen between different members of a TLR subfamily. For example, whereas the chTLR7 amino acid sequence is 62% identical to huTLR7, it is also 40% and 35% identical to huTLR8 and huTLR9, which are also members of this TLR subfamily. However, since papers have begun to appear using the TLR21 identifier, we will retain the use of chTLR21 in this chapter to avoid confusion. The expression of chTLR21 mRNA can be detected in a wide range of tissues from adult White Leghorn chickens, including the spleen, bursa, lung, and gut [133], and in the macrophage cell line HD11 [11]. Strikingly, chTLR21 has been shown to respond to DNA containing unmethylated CpG motifs that require endosomal maturation for activation, indicating that chTLR21 performs a recognition function similar to that of TLR9 in mammals [133,134]. Functional work with duck and goose TLR21 indicates that these also recognize unmethylated CpG motifs and can mediate antiviral effects [140,141].

8.6.3.10 Toll-like receptor signaling pathways in chickens

The consequences of TLR activation are diverse; they include activation of cytokine, IFN, and chemokine production; cell maturation; induction of degranulation; and the direct production of antimicrobial peptides. The literature covering the effects of TLR activation in mammalian systems is vast, whereas much more limited information is available on avian systems. For detailed coverage of signaling cascades associated with TLR activation, the reader is referred to Refs. [2,59,142] as only general aspects will be covered here.

The optimal activation of at least some TLRs involves the coordinated action of a variety of molecules that have been most intensely studied with TLR4–LPS interactions and shown to include LBP, CD14, and MD2. Genes encoding orthologues of each of these molecules can be identified in the chicken genome resources based upon sequence identity, gene structure, and conserved synteny. However, there is some evidence that in chickens CD14 may not be GPI-anchored as it is in mammals [143].

Activation of the TLR leads to recruitment of adapter proteins to the TIR domain, and for most TLR, these

include the MyD88 adapter. With TLR3, however, the only known adapter-mediating signal transduction is TRIF. With TLR4, it is clear that a wide range of adapters are involved with signal transduction, including MyD88, TIRAP, TRIF, TRAM, and SARM, and it is likely that each configuration of adapter molecules invokes different downstream modifications to the signaling cascade. For example, with TLR4, recruitment of TRAM to the TIR domain leads to interaction with TRIF, which results in induction of type-I IFN responses [144]. Notably in chickens TLR4 activation does not efficiently induce type-I IFNs and TRAM does not appear to be present in the chicken genome sequence resources [103]. The downstream components of TLR signaling cascades in mammals include IRAK-1, IRAK-4, TAK-1, TRAF-6, MAPK pathway elements, ESCIT, BTK, I κ B kinases, NF- κ B, and IRF family members. Very little work has been completed in chickens, although many of the elements of these pathways are clearly conserved and can be identified using EST and genomic resources [64,65,89]. Further evidence for the conservation of TLR signaling requirements in chickens and mammals comes from the conserved effects of a range of pharmacological inhibitors of heterophils and the HD11 macrophage-like cell line [11,70–72]. Nonetheless, there also appear to be some species-specific differences in the consequences of TLR activation, as judged by the analysis of cytokine and IFN production with chTLR4, chTLR5, and chTLR7 (see earlier).

8.6.3.11 Genetic diversity and evidence of selection in avian Toll-like receptors

Over recent years those interested in genetic diversity and immune selection in wild and domesticated birds have begun to examine avian TLRs finding that these genes frequently exhibit signatures of positive selection [58,145–150]. Although this topic will be considered in more detail in the context of wild bird immunology elsewhere in this book (Chapter 20), it is worthy of brief comment here. One key question is why are PRRs suitable targets for these types of analyses? First, these molecules are on the front line of the evolutionary battle between pathogens and their hosts; hence, changes in pathogen biology or pathogen profiles can exert strong selective forces on these receptors. Second, the PRR molecules are detecting the presence of molecular patterns from pathogens (called PAMPs) as well as damaged self (DAMPs), therefore the outcomes of selective forces are often evident within the coding sequence. Third, while, by definition, PAMPs are conserved, there are variations in the structure of PAMPs, for example, with bacterial flagellin or LPS. These direct influences and

selection on coding sequences are somewhat different to many other immune genes where selection for variance is more often related to expression (rather than ligand binding) and therefore found in promoter or regulatory regions. Of course, the other main genes often considered in immune selection studies are those encoding the MHC (see [Chapter 7](#)), which are highly polymorphic in regions focused on the binding of peptides from pathogens. Hence, with both PRRs and MHC, changes in parts of the coding sequence have direct effects on the recognition of elements derived from pathogens.

The main PRRs being studied in the context of birds are the TLRs and there are a few points that are relevant to this chapter. There is considerable genetic diversity in many of the avian TLRs either in wild birds (e.g., Refs. [\[58,145,147,149\]](#)) or in domesticated chickens [\[148\]](#). Evidence of positive selection can be detected with avian TLRs either within species or more broadly across species (e.g., Refs. [\[58,145,147–150\]](#)). As might be expected for genes under selection by a changing pathogen profile, there is evidence for periodic positive selection across the birds [\[147\]](#). Most positively selected residues tend to be observed in regions of the TLR involved in recognition of PAMPs, that is, in the LRR domains in the extracellular/intraendosomal part of the molecules [\[58,145–149\]](#). In contrast, the transmembrane domain and signaling domain (i.e., TIR domain) are under stronger purifying selective pressures, as would be expected since these parts of the molecule are highly constrained (by needing to be in the membrane or by having to interact with conserved signaling molecules). There are some circumstances where positively selected sites are located in these regions and these are likely to have strong effects on TLR signaling capacity as demonstrated by functional studies with TLR5 polymorphisms in Gentoo penguins [\[149\]](#). In the context of domestic chickens, the high standing diversity in TLR genes [\[148\]](#) may reflect the complex origins (from geographically distinct populations of red jungle fowl), interactions with other native breeds, and the diversity of selection for production and other traits over history [\[151–158\]](#).

Interestingly, not all TLRs exhibit the same degree of positive selection and generally those found at the cell surface (e.g., TLR4 and 5) exhibit higher levels of positive selection than those found solely in the endosomal location (e.g., TLR3 and 7) (e.g., Refs. [\[58,145–149\]](#)). These differential patterns with TLR4 and 5 versus TLR3 and 7 most likely relate to the level of structural diversity in their respective agonists. The TLR1 and 2 molecules are diverse and found at the cell surface but selection analyses are complicated by the effects of gene conversion. TLR15 is also diverse but often exhibits little evidence of positive selection (e.g., Refs. [\[58,148\]](#)) which may relate to the activation of this TLR by enzymatic processes [\[138\]](#) or highly conserved, invariant agonists.

The patterns of selection seen in TLRs have been highly informative in terms of both the evolutionary history and function of these genes. It is predictable that similar analyses of other avian PRRs are likely to identify interesting patterns of selection that will help us to understand the varying evolutionary effects of pathogens on domesticated and wild populations of birds.

8.6.3.12 Other transmembrane pattern recognition receptor

There are a wide range of non-TLR PRRs that have been extensively studied in mammals, including the C-type lectins, SRs, and formyl-peptide receptors. In the context of avian immune systems, this large group of molecules has received very little attention. Many of these molecules combine PRR-like activities with other functions, including promotion of phagocytosis and other physiological roles; these receptors can be considered more than PRR. In birds, among the C-type lectins, a lectin 1-like gene has been identified as have various mannose receptors. In the latter case, these include the target of one of the most widely used “macrophage” detecting monoclonal antibodies KULO1 which has recently been demonstrated to recognize a homologue of the macrophage mannose receptor MRC1 termed MRC1LB [\[159\]](#). Indeed, the MRC1 family of PRR comprises five genes in chicken, turkey, and zebra finch (MRC1LA to MRC1LE) compared with the single gene found in mammals and represent a clear product of gene duplication [\[159\]](#). The tissue distribution of this family was determined by RT-PCR, but no functional work has been reported. Another C-type lectin of the mannose receptor group is DEC205, it is found on dendritic cells and associated with a variety of functions, including enhancing phagocytosis and recognition of dying cells [\[160–162\]](#). An orthologue of this receptor has been identified in chickens [\[163\]](#) and the antibodies raised against this molecule as well as CD83 [\[163,164\]](#) will help greatly with the work on avian DC (see [Chapter 8.1](#)).

A diverse set of SRs are found in mammals with numerous subclasses that differ in their structure, expression patterns, and recognition preferences [\[165,166\]](#). A range of putative avian SRs have been identified in the chicken genome [\[167\]](#). These include three SRA family members: macrophage receptor with collagenous structure, SR class A member 5, and SR with C-type lectin molecules. Three members of the SRB family (SCARB1 to SCARB3 with SCARB3 also known as CD36), two members of the SRF family (SCARF1 and SCARF2), and one SRH-like gene (FELE1) were identified. No homologues of mammalian CD68 (SR-D), LOX-1 (SR-E), and R-PSOX (SR-G) were identified in the chicken genome

resources [167] SRs are mostly found on immune cells such as macrophages, heterophils, and DCs. Engagement of SRs leads to diverse outcomes that include targeted phagocytosis of pathogens or apoptotic cells as well as induction of inflammatory responses. The targets for SRs are also diverse and include acetylated or oxidized low-density lipoproteins, heat shock proteins, polyG, polyI, sulfated polysaccharides (e.g., fucoidin), and phosphatidylserine (found on the surface of apoptotic cells) as well as various surface components of pathogens. Exposure of HD11 cells, heterophils of chicken monocytes to a range of different SR agonists led to differential upregulation of a variety of cytokine transcripts or nitric oxide production according to the agonist employed and the cell type [167].

8.6.4 Cytosolic pattern recognition receptor

While TLRs detect PAMPs either on the cell surface or in the lumen of intracellular vesicles, it has been shown in mammals that a multitude of additional PRRs exist for the detection of intracellular PAMPs in the cytosol. These can be classified into RLRs (RIG-I-like receptors), NLRs, and several cytosolic nucleic acid sensors such as members of the AIM2 (absent in melanoma-2) family (reviewed in Ref. [168]).

8.6.4.1 Nucleotide-binding oligomerization domain-like receptors

NLRs are found in plants and animals and are built up of three domains: an N-terminal protein interaction domain, a central nucleotide-binding domain, and a C-terminal LRR. To date, at least 23 human and 34 murine NLR genes have been identified, but for many of them the physiological function is poorly understood [169]. NOD-1 and NOD-2 (also known as NLRC1 and NLRC2) are well-characterized NLRs that bind peptidoglycan (PGN) derivatives and are important for the host response against bacteria. NOD-1 recognizes γ -glutamyl diaminopimelic acid (iE-DAP), a PGN-breakdown product of all Gram-negative as well as several Gram-positive bacteria [170]. NOD-2 recognizes muramyl-dipeptide, a PGN motif common to most bacteria [171]. Signaling through NOD-1 and NOD-2 activates NF- κ B and results in the induction of inflammatory cytokines and other antimicrobial genes, for example, α - and β -defensins in the Paneth cells of the gut [172,173]. A chicken homologue of NOD-1 can be identified in the genome, but NOD-2 seems to be absent in birds, reptiles, and amphibians [174]. The agonist iE-DAP stimulates chicken and duck NOD-1, resulting in cytokine expression via NF- κ B and MAPK pathways [175–177]. The levels of NOD-1 mRNA were also

reported to be increased during infection with *S. enterica* serovar Pullorum [177].

A subgroup of NLRs is activated by many PAMPs and senses various forms of cellular damage and stress, such as uric acid crystals, amyloid- β , or UV irradiation [178,179]. These NLRs assemble with an adapter protein and caspase-1 to form a complex named the inflammasome. Based on the involved NLRs, inflammasomes are grouped into three main types: NLRP3 (NALP3), NLRP1 (NALP1), and NLRC4 (IPAF). By catalyzing the cleavage from pro-IL-1 β and pro-IL-18 to mature IL-1 β and IL-18, the inflammasome is responsible for the maturation and release of these proinflammatory cytokines [180,181]. The immature cytokines are often induced by a variety of PRRs which are then processed by inflammasome-induced caspase-1 to a functional form [182]. Interestingly, the NLRP3 inflammasome is also a crucial element in the adjuvant effect of alum [183]. The chicken NLRP3 sequence is annotated in the genome, and functional studies have been performed with duck NLRP3 in relation to avian pathogenic *E. coli* [184].

The more recently identified NLRC5, functions as a positive and negative regulator of antiviral immune responses and is implicated in the regulation of MHC class I transcription (reviewed in Ref. [185]). Lamont and coworkers have shown that chicken NLRC5 mRNA is upregulated in HD11 cells after LPS stimulation [186]. chNLRC5 inhibition using siRNA affected the expression of type-I interferons, suggesting, that the chicken molecule, like mammalian NLRC5 is involved in the regulation of intracellular inflammatory immune responses [187]. ChNLRC5 is upregulated during infection of chickens with *S. enterica* serovars Enteritidis and Pullorum [188,189] and polymorphisms in the promoter region are reported to affect the splenic immune response.

8.6.4.2 Retinoic acid-inducible gene-like receptors

The RLR family is widely expressed and has three members: RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). RLRs are RNA helicases and are essentially involved in the recognition of viral RNA in the cytoplasm [190]. RIG-I and MDA5 have a tandem N-terminal CARD domain, the central DExD/H box helicase domain, and a C-terminal regulatory or repressor domain (CTD), which binds RNA. LGP2 contains only the helicase domain and the CTD [190]. RNA binding of the RIG-I CTD leads to the interaction of the CARDs with the mitochondrial adapter molecule CARDIF, which then initiates a downstream signaling cascade,; activates NF- κ B,

MAPK, and IRF3; and finally induces secretion of proinflammatory cytokines and type-I IFNs [191]. Since LGP2 lacks the interacting CARD domain, the molecule has no signaling capacity [192], but by binding to RIG-I or MDA5, it acts as a regulator of RLR-specific antiviral responses [193].

RIG-I recognizes only short, blunt 5'-triphosphate (ppp) double-stranded (ds) RNA, which can be found in the panhandle structures of negative-strand RNA viruses [194,195]. MDA5 binds long dsRNA and is also activated by the synthetic dsRNA analogue polyI:C [196]. Hence, MDA5 senses especially positive-strand RNA viruses such as *Picornaviridae* and *Flaviviridae* [197] but can also recognize some negative-strand RNA viruses such as AIV [198].

In general, the RLR family and its signaling cascade are conserved among vertebrates, but the RIG-I gene could not be found in the chicken genome [199,200]. Because ducks possess functional RIG-I, Barber and coworkers suggested that the lack of RIG-I-mediated IFN induction in chickens could be responsible for the increased susceptibility to AIV of chickens as compared to ducks [199]. However, infection studies in chickens have demonstrated that infection with H5N1 AIV rapidly induces high amounts of IFN, but this does not mediate protection against highly pathogenic AIV [201]. Chicken MDA5 was recently identified and shown to mediate the production of type-I IFN in cells after stimulation with polyI:C [202]. Ruggli and coworkers identified chicken LGP2 and demonstrated that chMDA5 is the key PRR for the recognition of AIV in chicken cells and senses AIV in a 5'-ppp-independent manner. Like mammalian LGP2, chLGP2 acts as a positive regulator of MDA5 signaling and is essential to mediate RLR-specific antiviral immune responses [203]. These data clearly show that chicken cells possess functional sensors to detect AIV infection and induce IFN production. For this reason, the lack of RIG-I seems not to be the reason for the high AIV susceptibility of chickens. Yet such species-specific differences in the PRR repertoire could have an effect on virus evolution, which is supported by the fact that the influenza A NS1 protein is able to inhibit MDA5-mediated IFN production [203]. The subtleties of RLR function in different bird species may turn out to be very important in terms of sensitivity to a range of viral infections, in particular with avian influenza.

8.6.5 Closing comments: general considerations in pattern recognition

PRRs can be subdivided, according to the locality of PRR–PAMP interactions, into those that operate at body surfaces, those in body fluids, and those that are cell associated; the latter can be further subdivided in terms of monitoring the external milieu (e.g., TLR) or cell

cytoplasm (e.g., NODs, MDA5, and RLRs). Moreover, many of the PRRs that monitor different areas in the host recognize related groups of PAMPs such as are seen with LPS, peptidoglycan (and derivatives), and double-stranded RNA. Thus the host is provided with efficient pathogen detection in multiple compartments. For example, the capacity to detect intracellular pathogens within the cytoplasm of the first infected cell, in neighboring cells, and systemically, during pathogen translocation between cells provides a multilayered recognition/defense system.

The cellular responses to PRR stimulation differ considerably, depending upon the cell type and the context of PAMP exposure. In chickens, this can be seen with the differential responses to TLR5 and TLR7 agonists in heterophils [88] and other cell types [63,64]. Moreover, any individual pathogen type usually presents a variety of PAMP structures to the host, and the form taken by the developing immune response is, at least partly, driven by integration of a range of PRR signals. The importance of PRRs cannot be overstated, they are the interface with the microbial world and the sensors of damage, without PRRs the immune system would simply not function. That said they are also very dangerous receptors and can trigger many immunopathologic cascades as evidenced by the role for TLR4 in endotoxic shock.

The PRRs come in many guises and are key receptors for initiating and directing immune responses in all organisms. In terms of birds, there are many features conserved with other vertebrates and this makes the differences all the more interesting. From a practical perspective, the avian PRRs are the receptors through which the “adjuvant effect” of vaccines is mediated. The avian PRRs continue to be a fertile field of research and one where we can expect significant advances in the coming years. The impact of these studies will be felt in many areas ranging from the fundamental biology of host–microbe interactions, evolution, and conservation as well as the applied aspects of developing better vaccines tailored to the avian immune system.

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This chapter aims to provide an overview of avian PRR biology and it was not possible to comment on all publications in this field. Our apologies to any authors whose work that was not specifically mentioned in this chapter.

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Avian cytokines and their receptors

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9.1 Introduction

The immune system is designed to defend against infection and disease, and while pathogens invade the host via a large spectrum of mechanisms, the immune response has evolved to provide a specific, complex, and efficient response to control and eliminate these problems. However, in a large multicellular organism synchronising this intricate response throughout the host relies on coordination and suitable communication between cells to generate an appropriate and balanced protective response [1]. The term “cytokine” was first coined by Cohen et al. (1974) [2], to describe regulatory peptides with molecular weights typically less than 30,000 Daltons that act as extracellular signals between cells during the course of immune responses. Cytokines both elicit and regulate immune responses and can be produced by many cell types, having pleiotropic effects on cells of the immune system, as well as modulating inflammatory responses. Cytokines exist as families of proteins and play major roles in cell-to-cell communication during both innate and adaptive immune responses [3]. Cytokine families include the interleukins (IL), the interferons (IFN), the transforming growth factor- β (TGF- β) family, the tumor necrosis factor (TNF) superfamily (TNFSF), the colony-stimulating factors (CSF) and the chemokines. Cytokines are secreted by a variety of cell types in response to a number of stimuli, and bind to specific cell surface receptors that trigger signal-transduction pathways in target cells. Their receptors exhibit very high affinity and specificity for the cytokine ligand, and because of this high affinity, local picomolar amounts of cytokine can mediate biological effects typically in local microenvironments.

Broadly speaking the cytokine families play distinctive roles within the immune system—the ILs have functional activities involving lymphocytes; the IFNs have antiviral effects; the TGF- β family has a crucial role in regulating

inflammatory reactions; CSFs play a role in immune cell generation and differentiation, and the TNFSF family has a wide range of activities related to any antitumor activity. The immunological effect depends on the cytokine and the target cells involved. These molecules can affect cell activation, division, apoptosis, or movement and they act as autocrine, paracrine, or endocrine messengers. Moreover, their role in helping define and control an appropriate immune response is critical. The innate and adaptive immune responses are often described as two separate arms of the host response, however, both systems work together and frequently this team work is directed by cytokines. Innate immunity provides an immediate first line of recognition of and defense against invading organisms by the innate immune system which leads to the release of cytokines resulting in initiation of downstream pathways leading to protection. Adaptive immunity involves the recognition and interaction of lymphocytes containing highly diverse antigen receptors specific for foreign antigen. Once again, cytokines are vital in determining the type and the extent of these responses as well as playing an important role in the host–pathogen interaction and the successful resolution of disease [4].

As cytokines are regulatory proteins that play a crucial role in controlling the immune response, they are increasingly being assessed as candidates for therapeutic use. Furthermore, the recent identification of a large number of chicken cytokines has provided the possibility to determine their effectiveness in enhancing the immune response during infection or vaccination. As such, the use of recombinant cytokines as immune modulators and vaccine adjuvants has attracted considerable attention. Additionally, the worldwide trend towards a reduced reliance on in-feed antibiotics for livestock has put pressure on the development of new alternative strategies to maintain poultry health and production. With this in mind,

there is a greater emphasis to harness natural host immune products, like cytokines, and develop these for health benefits [5]. Therefore it is important to develop a strong understanding of the role of cytokines in the avian immune response.

9.2 Avian cytokine and chemokine families

There are many structural and functional differences between the immune system of birds and mammals, for example, the bursa of Fabricius and different immunoglobulin (Ig) types as well as the lack of lymph nodes in birds. These types of remarkable differences highlight the need to understand the immune system in birds and, therefore, the genes involved in regulation of immune responses to understand the difference in the evolution of immune system between birds and mammals. Nevertheless, our knowledge of the repertoire of cytokines and chemokines in the chicken has always lagged behind mammals. For example, although IFN was first described in the 1950s (paradoxically in the chicken), it was more than three decades ago that the first IFN gene was identified and cloned. However, the release of the chicken genome sequence in 2004 provided the opportunity to start identifying the immune regulating genes in chickens, particularly the orthologues of those known in mammals. Even so, in comparison to mammals, progress in the identification and characterization of chicken cytokines has been somewhat slow due to the evolutionary distance between birds and mammals and relatively low amino acid sequence identities. In the past, traditional cross-hybridization or degenerative polymerase chain reaction approaches have been difficult to employ as in evolutionarily distinct organisms, many cytokine orthologues could not be identified by simple sequence comparison. Initially, only genes of highly conserved cytokines were cloned on the basis of sequence similarity. Therefore progress in isolating chicken orthologues of mammalian cytokines was slowed by the generally low level of sequence similarity, typically in the range of 20%–50%, and as a consequence, their identification by orthologous probes, or amplification of genes using primers based on mammalian sequences, has been difficult and somewhat unproductive. However, the availability of the chicken genome sequence has improved our ability to understand the repertoire and thereafter the biology of avian cytokines and chemokines. Additionally, next-generation sequencing techniques which reveal the transcriptome are also assisting in identifying gene expression. Nonetheless, while many avian orthologues of mammalian cytokine genes continue to be identified, many of these are yet to be functionally characterized.

9.3 The interleukins

9.3.1 The interleukin-1 family

The human IL-1 family consists of a large group of cytokine ligands and their receptors which stimulate both pro and antiinflammatory activities. This family contains 11 ligand genes encoded across 3 separate loci. Of these genes, 9 are present at a locus on chromosome 2, with IL-18 and IL-33 differentially located on chromosomes 11 and 9, respectively (Table 9.1; Fig. 9.1). These cytokine ligands were originally given an IL-1 family (IL-1F) designation [6], however, this nomenclature was subsequently revised as functions were identified and individual IL designations assigned [7]. The IL-1 family members share similar functions with the Toll-like receptor families in that both families have a cytoplasmic Toll-IL-1-Receptor domain which is important in signaling. Of the IL-1 family members, 7 ligands have pro-inflammatory activity (IL-1 α , IL-1 β , IL-18, IL-33, IL-36 α , IL-36 β , IL-36 γ) while other members can have antagonistic antiinflammatory activities (IL-1 β , IL-1 receptor antagonist (IL-1RN), IL-36RN, IL-37, IL-38) (Fig. 9.2).

As is often the case, fewer members of the IL-1 family have been identified so far in the chicken compared to mammals. There are now four IL-1 family members that have been cloned and characterized in the chicken. ChIL-1 β [8] and ChIL-18 [9] were identified some time ago, and both have roles in inflammatory reactions. A cDNA expression library from a stimulated chicken macrophage cell line was used to identify and clone the ChIL-1 β gene [8]. IL-1 is produced as an inactive precursor molecule, proIL-1 which is converted into an active form by calpain. IL-1 β requires caspase-1 (IL-1 β Converting Enzyme or ICE) processing to become active [10]. The secretion of biologically active, mature forms of IL-1 β and IL-18 is dependent on the proteolytic activity of ICE. ICE itself requires activation via the cytosolic protein scaffold, known as the inflammasome. ChIL-1 β activity can be measured by its ability to stimulate a CXC chemokine (K60) in the target cell line CEC-32 [11]. IL-1 β in mammals has a diverse range of effects including induction of fever, elevation of corticosterone levels, general activation of the cytokine network and induction of B cell maturation and antibody production. In light of this, ChIL-1 β has been examined for its immunoadjuvant activities and when administered as recombinant protein was found to increase antibody responses compared to when administering the antigen alone [12].

In mammals, IL-18 was first described in 1989 as an IFN- γ inducing factor [13] and subsequently cloned in 1995 [14]. Mammalian IL-18 is produced by many cell types, both hematopoietic cells and nonhematopoietic cells, have the potential to produce IL-18 and it is constitutively expressed in nonhematopoietic cells, such as

TABLE 9.1 Comparison of IL-1 and IL-1R family members in human and chicken with chromosomal locations.

Human IL-1 family	Ligand also known as	chr	Ligand in chicken genome	chr
IL-1F1	IL-1 α	2	IL-1 β IL-36RN	22 Un
IL-1F2	IL-1 β	2		
IL-1F5	IL-36RN	2		
IL-1F6	IL-36 α	2		
IL-1F7	IL-37	2		
IL-1F8	IL-36 β	2		
IL-1F9	IL-36 γ	2		
IL-1F10	IL-38	2		
IL-1F3	IL-1RN	2		
IL-1F4	IL-18	11	IL-1RN IL-18	Un 24
IL-1F11	IL-33	9		
Human IL-1 receptor family	Receptor also known as	chr	Receptor in chicken genome	chr
IL-1R1	IL-1RI	2	IL-1R1	1
IL-1R2	IL-1RII	2	IL-1R2	1
IL-1R3	IL-1RAcP	3	IL-1RAcP	9
IL-1R4	ST2	2	ST2	1
IL-1R5	IL-18Ra	2	IL-18Ra	1
IL-1R6	IL-36R	2	IL-36R	1
IL-1R7	IL-18Rb	2	IL-18Rb	1
IL-1R8	SIGIRR	11	SIGIRR	5
IL-1R9	TIGIRR-1	X	TIGIRR-1	4
IL-1R10	TIGIRR-2	X	TIGIRR-2	1

chr, Chromosome; Un, unannotated.

intestinal epithelial cells, keratinocytes, endothelial cells, activated macrophages, Kupffer cells, and dendritic cells to act as an inducer for Th1 cells [15]. IL-18 is synthesized as an inactive precursor molecule, proIL-18, which requires caspase-1 directed cleavage, to become a mature, active IL-18 protein [16]. IL-18 signals through its cognate cell surface molecule IL-18 receptor (IL-18R), a heterodimeric receptor composed of two subunits IL-18R α and IL-18R β [17] (Fig. 9.2). ChIL-18 was first identified by Schneider et al., (2000) [9] and similar to mammalian IL-18, was found to induce high levels of ChIFN- γ secretion and cellular proliferation when added to splenocytes [18]. Additionally, ChIL-18 binding protein (ChIL-18BP) was identified and shown to effectively inhibit ChIL-18 stimulate ChIFN- γ production [19]. It has been suggested that, due to its role in chicken T cell proliferation and antigen upregulation, ChIL-18 plays a major role in chicken Th1 inflammatory responses [20].

Three novel ChIL-1 family sequences were recently identified from expressed sequence tag libraries. Two were shown to be the secretory and intracellular structural variants of ChIL-1RN [21] and further splice variants were isolated. Both full-length ChIL-1RN structural variants exhibited biological activity similar to their mammalian orthologues, in that they inhibited the pro-inflammatory effects of ChIL-1 β on macrophages, whereas the splice variants were not biologically

active. The third sequence was confirmed as ChIL-36RN, although its biological activity remains to be fully determined [22]. Surprisingly, ChIL-1RN and ChIL-36RN are not encoded at the same locus as ChIL-1 β (Fig. 9.1). This observation underlies the difficulty of determining the full repertoire of ChIL-1 family members. However, it remains likely that more members of the ChIL-1 family are still to be identified, as the chicken genome encodes the full repertoire of IL-1 family receptors seen in mammals (Table 9.1).

9.3.2 T-cell proliferative interleukins

Outside of mammals, arguably the best characterized immune system is that of the chicken, including knowledge of cytokines in other nonmammalian species. The advancement of research into the chicken immune response has been driven by the increasing incidence of infectious viral diseases in poultry which raises significant animal welfare and food production issues as well as zoonotic disease potential. Moreover, the increase of viral prevalence and the decrease in vaccine efficacy has spurred interest into avian cytokine research and their potential use as therapeutics and the notion of immune response regulation through a greater understanding of the T cell responses.

The existence of a T cell growth factor with IL-2-like properties in chickens was known for many years [23],

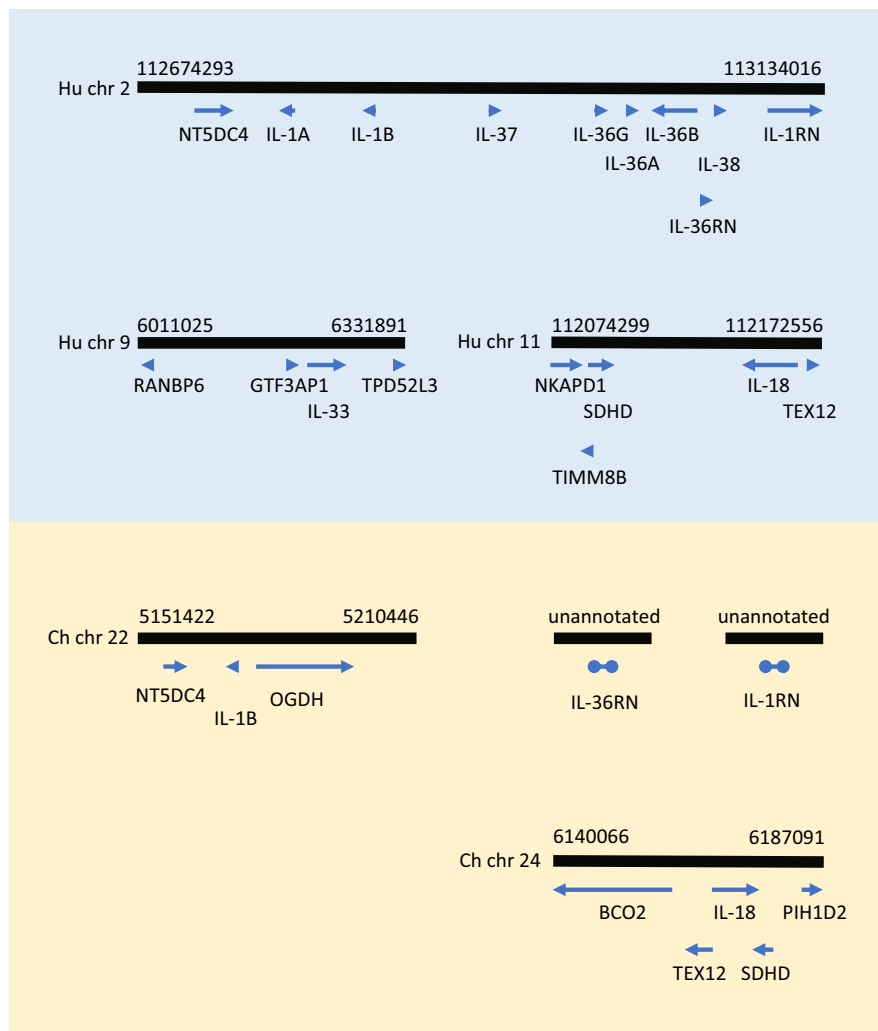


FIGURE 9.1 Representation of the genomic organization of the IL-1 family gene cluster. The information for human (Hu) and chicken (Ch) is from the assembly databases GR38.p13 and GRCg6a, respectively. Positions on the chromosomes (chr) are indicated and arrows show transcriptional orientation. Hu IL-1 gene family members mapped to chromosome (chr) 2, 9 and 11 and chicken family members mapped to chromosome 22, 1 or are currently unannotated.

however, it was some time before the gene was cloned [24]. Due to the phylogenetic distance between chickens and mammals, screening of chicken cDNA libraries for ChIL-2 by hybridization with mammalian IL-2 probes was unsuccessful [25]. Efforts to purify ChIL-2 from Concanavalin A activated spleen cell supernatants also failed to yield protein of sufficient purity for sequencing. A chicken lymphokine gene was cloned from an activated chicken spleen cDNA library, and the expressed product showed T cell proliferative activity [24]. This gene had a 429 base pair open reading frame that codes for a protein of approximately 14,500 Daltons and shares 24% and 25% amino acid identity with bovine IL-2 and IL-15, respectively [24]. The cloned chicken cytokine shared similar properties with mammalian IL-2 by being produced by activated T cells, having a 20-nucleotide 5' untranslated region and containing a short signal peptide. However, unlike mammalian IL-2 genes which contain three conserved cysteine residues, it contained four cysteine residues which is a characteristic of mammalian

IL-15. Gene structure analysis subsequently confirmed the gene to be the chicken orthologue of mammalian IL-2 [26]. The gene structure of ChIL2 more closely resembled that of IL-2 rather than IL-15 since it comprises four exons and three introns, similar to the genes for mouse and human IL-2. The promoter was also highly orthologous to the promoter of mammalian IL-2, with conservation of most of the transcriptional factor binding sites both in nucleotide sequence and order. In the chicken the genes encoding the T cell proliferative ILs, ChIL-2, ChIL-15 and ChIL-21 [27], are all located on chicken chromosome 4, although they are not syntenic. The biological activities of all three cytokines have been characterized [28–30].

9.3.3 T-helper interleukins

T cells can function as regulators and effectors of immune responses and therefore have a critical role in many inflammatory processes [31]. The CD4 lineage of T cells

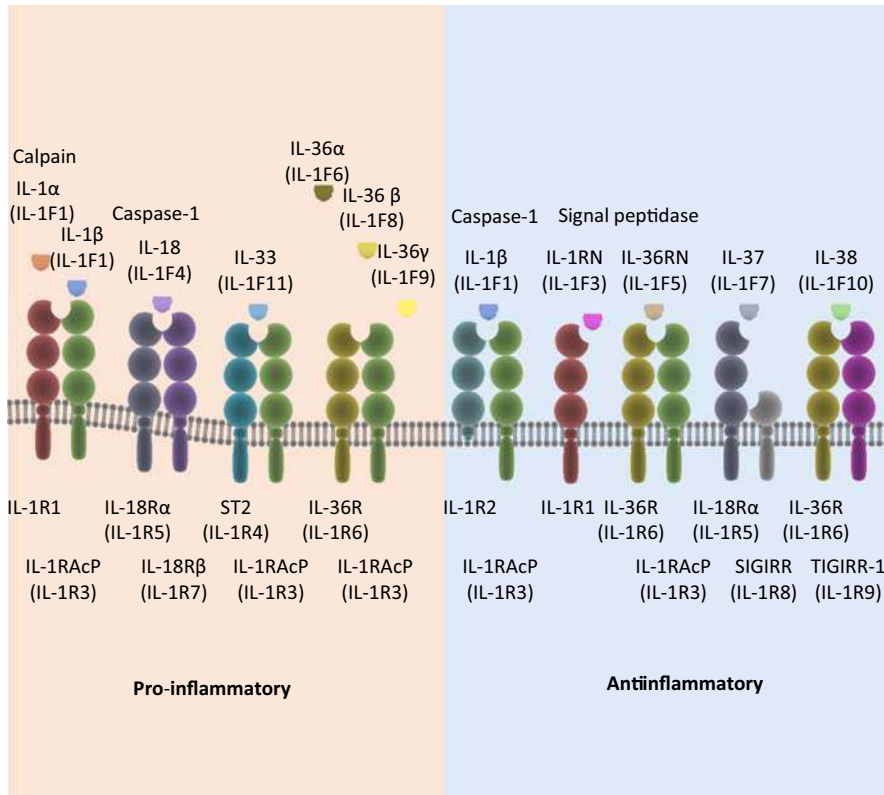


FIGURE 9.2 IL-1 family ligands and receptors. A representation of ligands and receptor chains in the IL-1 family constructed from mammalian information. Ligand and receptor engagement in some family members leads to a pro-inflammatory response (red shaded area) and in other results in an anti-inflammatory response (blue shaded area). Additionally, some ligands are expressed as a pro-protein and are cleaved by enzymes to produce an active form.

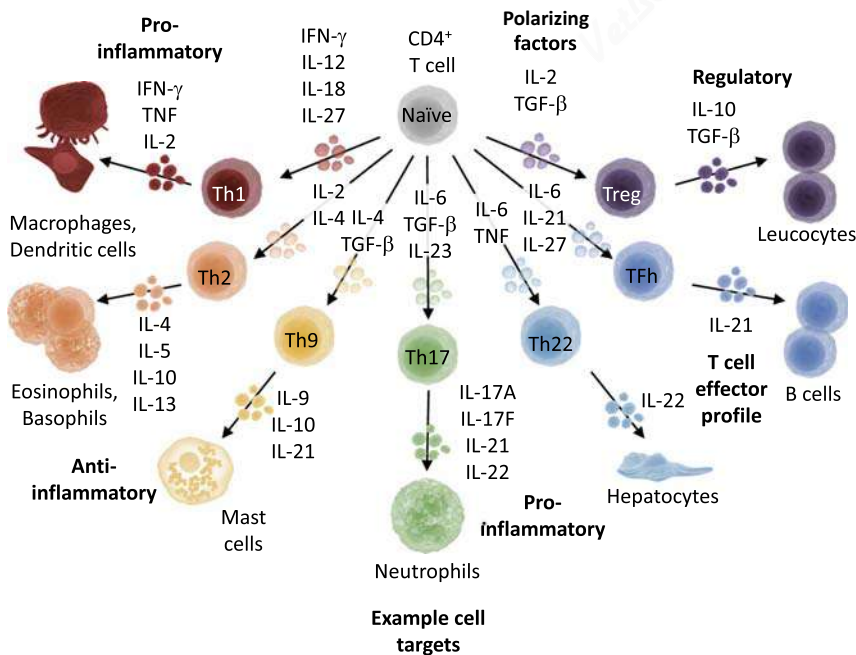


FIGURE 9.3 Cytokines in $CD4^+$ T cell differentiation. Naïve $CD4^+$ T cells differentiate into T helper cell subsets influenced by polarizing cytokines based on human and mouse data. The subsequent subset is defined by their pattern of cytokine expression. These expressed cytokine arrays modify the response of other cell types, which in turn impacts host response.

is of particular interest in the immune inflammatory response and in mammals has been shown to comprise of multiple distinct T-helper (Th) subsets [32]. In mammals, these subsets are characterized by the type of cytokines they release and are designated as Th1, Th2, Th9, Th17,

Th22, Follicular Th (TFh), and Regulatory T (Treg) subsets (Fig. 9.3). These Th cell subsets arise as a consequence of naïve T-cell differentiation in an appropriate stimulating microenvironment. The Th1–Th2 paradigm has been central to our understanding of adaptive immune

responses. Largely, mammals generate an immune response that is functionally polarized into either type 1 or type 2 immune pathways that resolve infections with intracellular or extracellular pathogens, respectively. The divergence of the response is largely regulated by antigen-specific Th cells. Th1 cells drive cell-mediated, inflammatory responses, whilst Th2 cells facilitate responses against larger extracellular pathogens such as helminths. Therefore to understand Th development it is critical to understand CD4 T-cell responses.

9.3.4 Th1 interleukins

Th1 responses are directed at increasing cell mediated immunity, driven to protect against intracellular pathogens and protozoa. Th1 responses are activated by the main polarizing factors IL-12 and IL-18, which stimulate a T cell effector profile dominated by the IFN- γ and IL-2 [15] (Fig. 9.3). Generally, Th1 cytokines drive the activation of various cell types, in particular antigen presenting cells, like macrophages and dendritic cells. The cytokines secreted by Th1 cells can activate macrophages and increase their expression of major histocompatibility complex on their cell surface and promote phagocytotic activity. Th1 cytokines can enhance the production of nitrogen free radicals which compliments increased enzymic activity and helps eliminate intracellular pathogens.

Studies defining Th1 cytokines in poultry have demonstrated close functional capabilities of avian cytokines compared with mammalian Th1 cytokines. Over the past few decades, Th1 cytokine research has progressed tremendously with the identification of many chicken cytokine and chemokine genes and the sequencing and cloning of Th1 cytokines. Basic Th1-like responses in chicken have been identified, however, key Th1 checkpoints have not yet been extensively investigated. In the absence of IL-12, the Th1 response is heavily impaired due to insufficient production of IFN- γ [33]. Prior to the release of the chicken genome in 2004, the β -chain of ChIL-12 was successfully cloned. However, following the release of the chicken genome, single chain, full length functional ChIL-12 was cloned and expressed. Therefore ChIL-12 has two components, ChIL-12 α (p35) and ChIL-12 β (p40) [34,35]. The ChIL-12p70 molecule was generated by transient coexpression of monomeric ChIL-12p35 and ChIL-12p40 and as a fusion protein, in a fusion linker construct. This fusion protein synergistically induce ChIFN- γ together with ChIL-18 to promote Th1 cell proliferation [36]. Additionally, in human and mouse, it has recently become apparent that there are at least two other members of the IL-12 family, IL-23 and IL-27, which also regulate Th cell responses. IL-27 is also a heterodimer, in this case made up of EBI3, an IL-12 p40-

related protein, and p28, an IL-12 p35-related protein. It has both pro-inflammatory and antiinflammatory properties. So far there is no indication of IL-27 in chicken, nevertheless, continuing analysis is important for understanding immune responses to pathogens in the chicken and to help with the development of control mechanisms for dysregulated cytokine production.

9.3.5 Th2 interleukins

In mammals Th2 cells assist in triggering a humoral immune response typically against metazoan parasites. Th2 cytokines are generated by the polarizing cytokines IL-4 and IL-2, and induce the effector cytokines IL-4, IL-5, IL-10, IL-13, and IL-25. The main target effector cells are eosinophils and basophils as well as B cells (Fig. 9.3). IL-4 acts as an autocrine cytokine for Th2 cells stimulating their differentiation. As a part of the humoral response to parasites, Th2 driven IL-4 production activates B cells to produce IgE and other antiparasitic factors. Additionally, the Th2 cytokines activate eosinophils to further enhance the antiparasitic immune response.

The presence of a Th2 cytokine gene group in chickens, the first cluster identified in a nonmammalian species, contains the orthologues of cytokines IL-4, IL-5, and IL-13 [35]. This chicken Th2 cytokine gene assembly possesses an extra family member, KK34 [37]. The KK34 gene has only recently been identified in mammals, such as cattle and pigs [38]. Similar to chickens, KK34 is located with IL-3 and CSF2. ChIL-4, ChIL-5, and ChIL-13 are found on chromosome 13 [35] as well as CSF2, ChIL-3, and KK34 (Fig. 9.4). Studies have shown evidence of a typical Th1 or Th2 based cytokine response in the chicken involving experimental viral and helminth infections, respectively, adding further evidence to the theory that the Th1/Th2 type immunity extends beyond the mammalian species. ChIL-4 activity has been less well studied compared to some other avian cytokines, nevertheless, it was shown to reduced nitric oxide production increasing reactive oxygen species production in macrophages when exposed to bacteria, implying a potential antiinflammatory role for ChIL-4 similar to that observed in mammals [39]. Likewise, Chaudhari et al., (2018) [40] showed that recombinant ChIL-4 inhibited nitric oxide production in lipopolysaccharide (LPS)-stimulated HD11 cells and monocyte/macrophages as well as upregulating arginase activity. In addition, ChIL-13 reduced LPS-stimulated nitric oxide production and inducible nitric oxide synthase expression in monocytes and macrophage cells [41]. ChIL-5 and KK34 has been expressed as recombinant proteins, however, little is known about their biological activity [42].

In mammals Th2 responses play a critical role in IgE production and in eosinophil activation. It is intriguing to

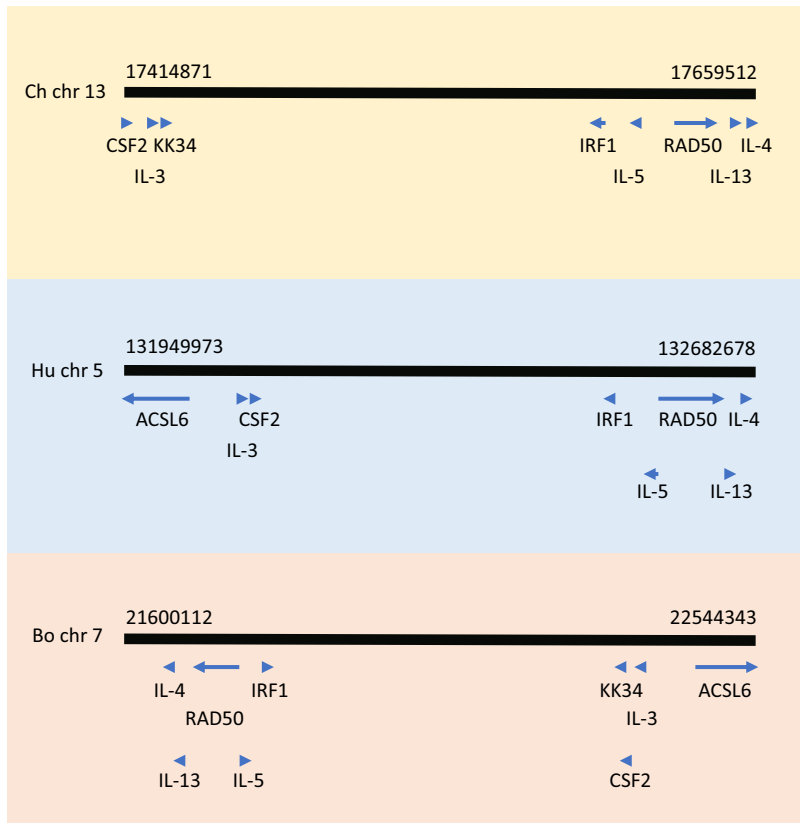


FIGURE 9.4 Schematic illustration of the syntenic maps of the Th2 cytokine gene cluster. The information for chicken (Ch), human (Hu) and bovine (Bo) is from the assembly databases GRCg6a, GR38.p13 and ARS-UCD1.2, respectively. Positions on the chromosomes (chr) are indicated and arrows show transcriptional orientation.

understand the role of Th2 cytokines in the chicken given their lack of IgE. It has been suggested that compared to mammals, chickens have fewer mast cells, basophils, and eosinophils as well as an absence of the eotaxins and their receptors. Additionally, there is also no IgG subclass switching in the chicken, indeed there is only a single chicken IgG equivalent, known as IgY. ChIL-5 mRNA expression is switched off during Th2 responses [43] and Th2-associated allergies have not been described for birds. As such, it is fascinating that chickens have the full complement of Th2 cytokines but so far appear to lack some of the effector functions vital in a mammalian Th2 response.

9.3.6 Th1–Th2 paradigm

Until recently, the existence of the Th1–Th2 paradigm outside mammalian species was not known. However, the paradigm was initially couched in terms of the cytokine profiles that dominate after infection with either intracellular or extracellular pathogens. The cytokine response to an intracellular pathogen is dominated by IFN- γ and to an extracellular pathogen by IL-4. In the chicken an upregulation in ChIFN- γ expression has been observed in response to ChIL-12 and ChIL-18 stimulation [20] and both ChIL-4 and ChIL-13 were the predominant cytokines

produced following *Ascaridia galli* infection [44] supporting the polarization of Th cell responses in the chicken and extending the paradigm beyond mammals. It remains to be determined whether this paradigm holds at the cellular and molecular levels and whether avian Th cells can become terminally polarized to a Th1 or Th2 phenotype.

9.3.7 Other Th subsets

Recently, other Th cell lineages have been described in mammals [45] (Fig. 9.3).

Th9 cells

In mammals, Th9 cells are stimulated to produce IL-9 and IL-10 and require TGF- β and IL-4 for their induction. IL-4 plays a role in STAT6 driven IL-9 production and TGF- β drives SPI1 driven IL-9 activity. Th9 cells express Foxp3 and IL-4, nevertheless in contrast to other similar Th subsets, such as regulatory T (Treg) cells and Th2 cells, they express these molecules at lower levels. Although little is known about the Th9 subset in chickens, many of the important components of the Th9 subset have been identified. ChIL-4 and ChIL-10 have been identified [35,46] while SPI1 [47] is situated on chromosome 5 and STAT6 on chromosome 33. While it is

unclear if FOXP3 exists in the chicken, other forkhead box proteins, like FOXP1 [48] have been identified. Importantly, ChIL-9 [49] has been identified and is positioned on chromosome 13.

Th17 cells

Th17 differentiation of naïve T cells has been found in mammals to be dependent on TGF- β and IL-6 [50]. Furthermore, the orphan nuclear receptor ROR γ t has an association as a transcription factor that directs this differentiation [51]. Maintenance and further differentiation of Th17 cells relies on the pro-inflammatory cytokine IL-23 which restimulates IL-17 production through the IL-23 receptor (IL-23R) bound on Th17 cells. Furthermore, IL-23 is the other predominant cytokine released by Th17 cells and has also been linked to the inflammatory response [52]. In addition, IL-23R is comprised of two subunits, the IL-12p40 subunit that is shared with IL-12, and the p19 subunit, which is unique to IL-23R. IL-12 and IL-23 are both heterodimers which share a common subunit, p40, and bind to a common receptor subunit, IL-12R β 1. However, their receptors also have a ligand-specific subunit resulting in distinct functions of each cytokine [53].

There has been progress in the area of the Th17 cytokines in the chicken. There is now evidence that ChIL-23 is situated on chromosome 33 with the cloning of ChIL-23p19 (ChIL-23 α) [54]. Furthermore, Truong et al., (2018) showed the ChIL-23 α has pro-inflammatory properties and activates the JAK/STAT signaling pathway presumably through the ChIL-23R, gene on chromosome 8 [27] and the predicted IL12R β 1.

Unlike the other cytokine multigene families, the IL-17 family in mammals is not syntenic but, in general, is dispersed throughout the genome. Five of the six IL-17 family members described for human (IL-17A-F) have been identified in the chicken genome (Table 9.2) [27]. The ChIL-17 cytokines that have been cloned and characterized include ChIL-17A [55], ChIL-17B [56], ChIL-17D [57], and ChIL-17F with these cytokines sharing some synonymous functions [58].

TABLE 9.2 IL-17 family members in human and chicken with chromosomal locations.

Human	Chromosome	Chicken	Chromosome
IL-17A	6	IL-17A	3
IL-17B	5	IL-17B	13
IL-17C	16	IL-17C	11
IL-17D	13	IL-17D	1
IL-17E	14		
IL-17F	6	IL-17F	3

Th22 cells

The Th22 subset is a more recently defined CD4⁺ T cell lineage subset. Naïve T cells differentiate into Th22 cells in the presence of IL-6 and TNF- α and are characterized by their production of IL-22. Although IL-22 maybe produced by other cell types, such as NK cells and Th17 cells, Th22 cells are restricted in their expression of IL-22 [59]. ChIL-22 [60] is located on Chromosome 1 in close proximity to ChIFN- γ (Fig. 9.5) and is a member of the ChIL-10 family.

Follicular T-helper cells

As for all of the Th subsets, the cytokine polarizing factors are critical in the development and differentiation of the subpopulation. The development of follicular T-helper (TFh) cells in mice has identified that IL-6, IL-21, and IL-27 encourage Bcl-6 expression and subsequent differentiation to TFh cells [61]. In humans, TGF- β in cooperation with IL-12 and IL-23 may add to the induction of human TFh cells [62]. TFh cells, by way of their location, can contact B cell follicles and impact germinal center responses [63]. As such, TFh cells express large amounts of IL-21 in the B cell follicles [64]. The chicken orthologue of IL-21 was cloned and is expressed in most lymphoid tissues, predominantly by CD4⁺ TCR $\alpha\beta$ ⁺ T cells [30]. As in mammals, ChIL-21 synergistically enhances T cell proliferation and inhibits maturation of dendritic cells [30]. In the chicken it has been suggested that TGF- β ⁺ CD4⁺ T cells in the gut associated lymphoid tissues may act as TFh cells [65].

Regulatory T cells

Regulatory T (Treg) cells play a role in regulating the immune response assisting in preventing aberrant responses. Natural Treg (nTreg) cells differentiate in the thymus and migrate to peripheral tissues whereas inducible Foxp3⁺ Treg (iTreg) cells differentiate in secondary lymphoid organs and tissues [66]. Treg cells are characterized by their expression of both CD4 and CD25 as well as FoxP3. Additionally, they produce IL-10 and TGF- β and modulate inflammatory responses. Chicken Treg cells have similarly been defined by their expression of both CD4 and CD25. As previously discussed, FOXP3 has not been unequivocally identified in the chicken but a FOXP3-like gene appears to be apparent in other birds [67]. It was hypothesized that chicken CD4⁺CD25⁺ cells exhibit suppressive and functional properties similar to that of mammalian CD4⁺CD25⁺Foxp3⁺ Tregs. Shanmugasundaram and Selvaraj (2011) [68] showed that chicken CD4⁺CD25⁺ cells proliferated in response to ChIL-2, suppressed T cell proliferation and produced ChIL-10 mRNA suggesting that chicken CD4⁺CD25⁺ cells are somewhat comparable to mammalian Tregs.

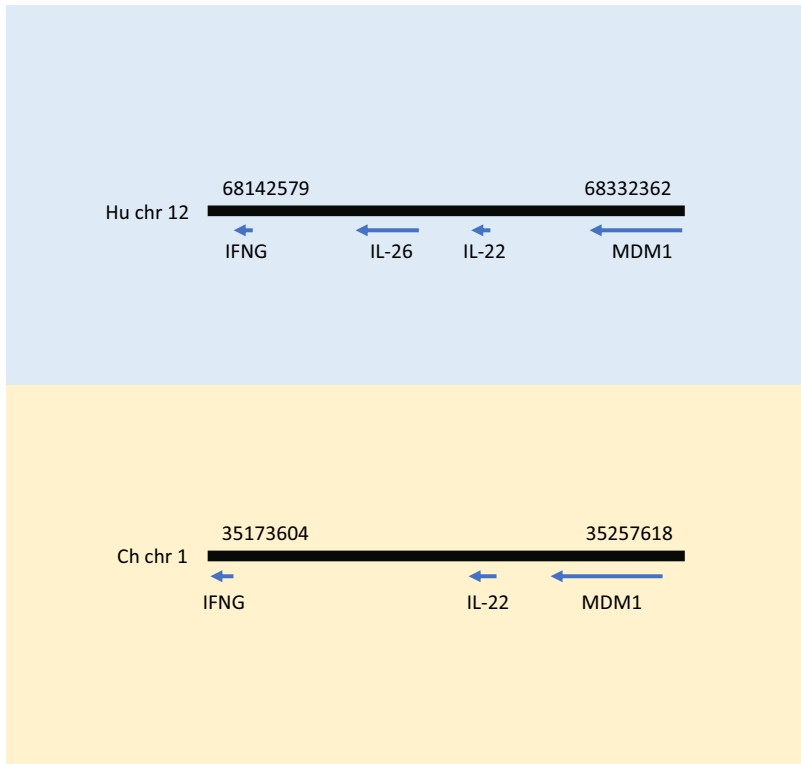


FIGURE 9.5 Schematic illustration of the genomic organization of IL-22 and IFN- γ . The information for human (Hu) and chicken (Ch) is from the assembly databases GR38.p13 and GRCg6a, respectively. Positions on the chromosomes (chr) are indicated and arrows show transcriptional orientation.

ChTGF- β producing Treg cells have been identified in various lymphoid tissues, with the highest levels found in the gut-associated lymphoid tissue [65]. There is evidence that these gut Treg cells may be influenced by the microbiota [69].

Although new chicken cytokines and immune associated factors are constantly being identified, there is still some way to go in generating the data to definitively support the existence of chicken orthologues of the many Th subsets that have been defined in mammals. Nevertheless, given that a great number of the associated polarizing factors and effector cell profile cytokines appear to exist in the chicken genome, it may not be long before these questions can be answered.

9.4 Other interleukins

In humans, the list of ILs currently stretches from 1 to 41 [70]. So far not all of the orthologues for these have been identified in the chicken, however, there are many important ILs that have been identified.

9.4.1 The interleukin-10 family

The human IL-10 family comprises six members (Fig. 9.6), encoded in two clusters on different chromosomes. IL-10, IL-19, IL-20, and IL-24 are syntenic on chromosome 1, while IL-22 and IL-26 are syntenic on chromosome 12. By

comparison to human IL-10 family members, the mouse has five members, lacking IL-26. So far, the chicken appears to have only four members of this family including ChIL-10 and ChIL-20 on chromosome 26 and ChIL-22 and ChIL-26 have been reported to be located on chromosome 1 [27]. In mammals IL-10 is produced by Tregs, B cells, and monocytes and can act to decrease cytokine production impacting cell activation. The function of IL-10 is conserved in the chicken, in that it acts as an antiinflammatory cytokine, downregulating the effects of ChIFN- γ [46]. In mammals, IL-19 has been described to have both pro- and antiinflammatory roles depending on the circumstances [71]. The very similar cytokine, IL-20 is produced by monocytes and keratinocytes and has a role in regulating proliferation and differentiation of keratinocytes during inflammation. Recombinant ChIL-20, (originally annotated as ChIL-19), stimulated chicken splenocytes to express ChIL-4, ChIL-13, and ChIL-10, and stimulated chicken monocytes to express ChIL-1 β , ChIL-6, and ChIL-19 [72]. In mammals, IL-22 and IL-26 are involved in inflammatory responses. IL-22 upregulates the expression of β -defensins in keratinocytes [73] and induces IL-10 expression in epithelial cells [74]. ChIL-22 induces ChIL-10 expression, as well as the expression of pro-inflammatory cytokines, chemokines and antimicrobial peptides in chicken kidney cells. In mammals IL-26 was initially identified as an orthologue of IL-10 [75], nevertheless, it induces distinct activities contrasting IL-10, as mediated

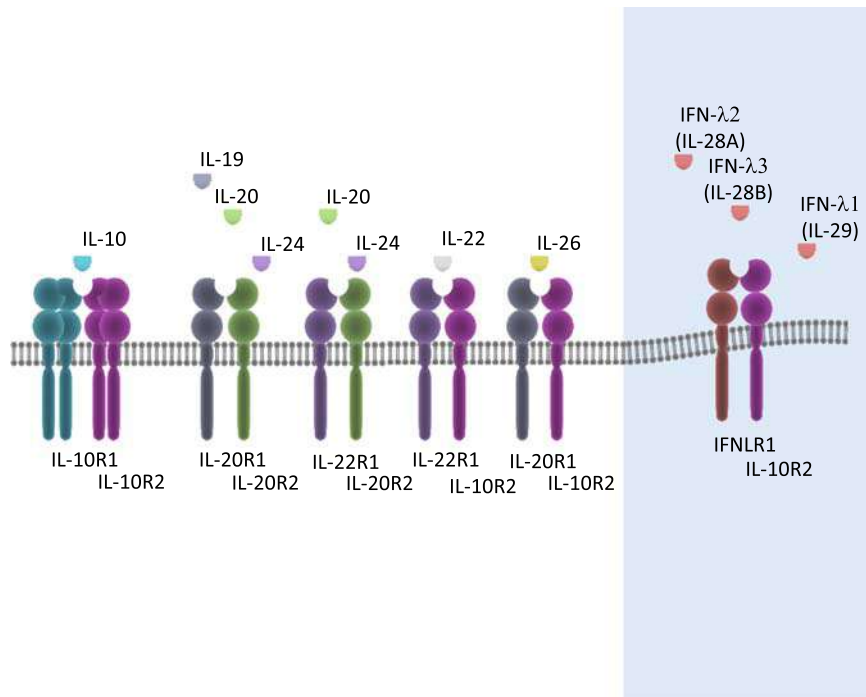


FIGURE 9.6 Schematic illustration of the mammalian IL-10 family receptor complex. The IL-10 family receptors signal through heterodimeric receptor subunits. IL-10 signals through a complex of two IL-10R1 and two IL-10R2 subunits. IL-22 signals through an IL-22R1 subunit in combination with an IL-10R2 subunit. IL-19 signals through the type 1 IL-20R consisting of IL-20R1 and IL-20R2 subunits. IL-20 and IL-24 can signal via either type 1 IL-20R or the type 2 IL-20R consisting of IL-22R1 and IL-20R2 subunits. The blue shaded area identifies the IFN- λ signaling complex which also uses the IL-10R2 chain.

through the restricted expression of IL-26R compared to IL-10R [76], and also shows some sequence homology with IFN- γ and shares a common enhancer sequence [77]. IL-26 is induced in T cells, NK cells following stimulation with specific antigen or mitogens. ChIL-26 has been cloned and expressed and is produced by T cells and stimulates the production of various cytokines and chemokines [78]. (Fig. 9.7)

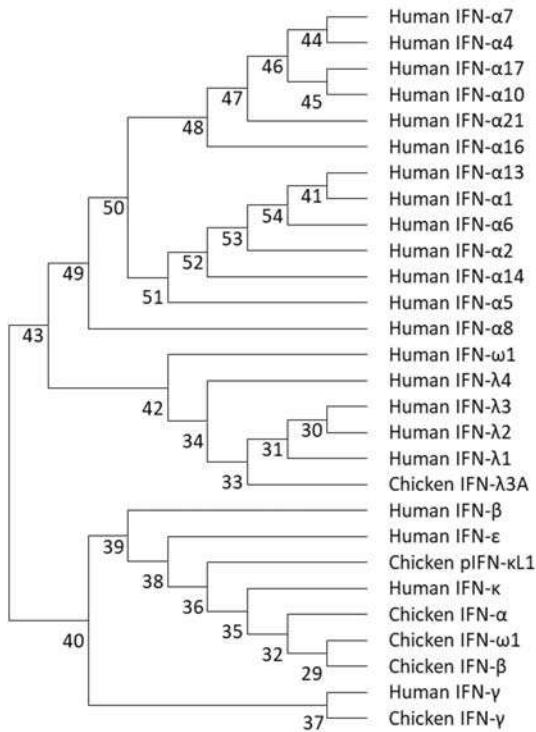
9.4.2 The interleukin-6 family

In mammals, the IL-6 family of cytokines is an assemblage that comprises IL-6, IL-11, IL-27 and the factors ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin 1 (CFT1), and cardiotrophin-like cytokine (CLCF1). These molecules are clustered together in a family based on receptor complex usage, with each incorporating the signaling receptor subunit gp130. The IL-6 family members show broad ranging activities and because of their use of a common signaling receptor subunit, these functions can be somewhat similar as well as having a specific role [79].

IL-6 is known to have important roles in the immune response, inflammation, hematopoiesis, and has effects in the endocrine and nervous systems. Moreover, IL-6 has various functions including induction of differentiation of B cells to antibody producing plasma cells; T cell growth and differentiation; the differentiation of leukemic cell lines into macrophages; megakaryocyte maturation; and acute phase protein synthesis in hepatocytes. Upon

expression, IL-6 triggers downstream events by activating effector functions in different organs as part of the host defense response. In mammals, IL-6 was initially given a variety of names based on biological function including IFN- β 2, B cell differentiation factor, T cell replacing factor, hepatocyte stimulating factor, hybridoma plasmacytoma growth factor and B cell stimulatory factor 2 based on its functions. The ChIL-6 gene was cloned from spleens of chickens treated with Imidazoquinoline S-28463, an immune modulating agent displaying antiviral and antitumor activity [80]. The cysteine residues of ChIL-6 in the central part of the molecule are also conserved amongst different mammalian IL-6 indicating a similar tertiary structure and high degree of conservation [80]. Full length ChIL-6 cDNA encodes a protein with only 35% amino acid identity to human IL-6, yet recombinant ChIL-6 induced proliferation in the IL-6 dependent murine hybridoma cell line 7TD1 [81]. Since the early discovery of IL-6 in chickens, a number of studies have shown that avian cytokines exhibit conservation of genetic location, gene structure, and regulatory sequences with their mammalian orthologues, with ChIL-6 being located on chromosome 2 [82].

Human IL-11 has been shown to be involved in the regulation of many functions, such as platelet maturation and osteoclastogenesis as well as adipogenesis and neurogenesis [83]. The human IL-11 gene is located on chromosome 19 and encodes a 23,000 Dalton protein [84]. IL-11 specificity is conditional on IL-11R α subunit engagement. This receptor appears to be expressed on



Gene	Accession number
Human IFN- α 1	NM 024013.3
Human IFN- α 2	NM 000605.4
Human IFN- α 4	NM 021068.3
Human IFN- α 5	NM 002169.3
Human IFN- α 16	NM 002173.3
Human IFN- α 7	NM 021057.2
Human IFN- α 8	NM 002170.4
Human IFN- α 10	NM 002171.2
Human IFN- α 13	NM 006900.4
Human IFN- α 14	NM 002172.3
Human IFN- α 16	NM 002173.3
Human IFN- α 17	NM 021268.2
Human IFN- α 21	NM 002175.2
Human IFN- β	NM 002176.4
Human IFN- ϵ	NM 176891.5
Human IFN- κ	NM 020124.3
Human IFN- ω 1	NM 002177.3
Human IFN- γ	NM 000619.3
Human IFN- λ 1	NM 172140.2
Human IFN- λ 2	NM 172138.2
Human IFN- λ 3	NM 001346937.1
Human IFN- λ 4	NM 001276254.2
Chicken IFN- α	U07868.1
Chicken IFN- β	GU119897.1
Chicken IFN- ω 1	NM 001024836.1
Chicken pIFN- κ L1	XM 015280184.1
Chicken IFN- γ	NM 205149.1
Chicken IFN- λ 3A	NM 001128496.1

FIGURE 9.7 Phylogenetic relationship between the IFN family members from humans and chickens. The evolutionary history was inferred by using MEGA7 software. The phylogenetic trees were constructed based on protein sequence alignment using the maximum likelihood method with a bootstrap test. Accession numbers of the genes used in the analysis are shown.

fibroblasts and stromal cells but less so on immune cells. This is in contrast to IL6 receptors that can be expressed on immune cells and potentially lower levels on stromal cells [85]. IL-11 has recently been identified in the chicken and is positioned on chromosome 22. Practical analysis of ChIL-11 showed an interaction with the appropriate receptors and activation of signaling pathways with its activation causing a variation of Th profile [86].

Of the other IL-6 family members there is variable information in the chicken. ChCNTF is positioned on chromosome 5. A purified protein product was identified and amino acid sequencing suggested it had close orthology with mammalian CNTF [87]. Correspondingly, ChLIF has been identified on chromosome 15 and has been cloned [88]. ChLIF has approximately 40% amino acid sequence identity to mouse LIF and has been shown to maintain chicken embryonic stem cell cultures in the undifferentiated state [88]. Less is known about the remaining family members. As yet OSM has not been identified in the chicken, nevertheless, the OSM receptor

can be identified on the Z chromosome. Likewise, IL-27 and CFT1 remain to be identified in the chicken, however, ChCLCF1 can be found on chromosome 5.

9.4.3 Other interleukins

There are other ILs which are not part of multigene families but nevertheless play vital roles in the chicken's immune response. ChIL-7 has been cloned and expressed and has been shown to induce lymphocyte proliferation as well as stimulate ChIFN- γ production and downregulate ChTGF- β expression [89]. Additionally, ChIL-16, located on chromosome 10, has been cloned and expressed and has been shown to functionally act as a chemoattractant for splenocytes [90].

9.5 The interferons

The difficult task of managing viral infections in poultry necessitates a perpetual investigation for new and

alternative antiviral strategies. Furthermore, there has recently been a great deal of concern with regard to poultry viruses with the observed outbreaks of avian influenza (AI) which can spread rapidly and cause high morbidity and mortality in both poultry and human populations. Clearly, improved control of poultry viruses would benefit the poultry industries (in terms of health, welfare, and productivity) as well as help to reduce the risk of transmission of zoonotic viruses to humans. The first IFN to be discovered was actually in the chicken in the 1950s by Isaacs and Lindenmann while studying influenza virus. They described IFN as a substance that was able to interfere with the ability of the virus to infect cells and thus coined the term “interferon” [91]. It is now recognized that there are in fact several different IFN types that are collectively crucial for defense against pathogens, contributing to the induction and regulation of both innate and adaptive antiviral mechanisms [92]. Three distinct IFN families have been identified in vertebrate species, type I, II, and III, and are defined by their use of specific receptor complexes. These are distinguished by their distinctive signaling pathways and patterns of gene induction, although there is considerable overlap in these features and some shared biological function between families [93]. All three IFN families are collectively important for an effective immune response [94]. They play critical roles in sensing and containing pathogens, providing the host with the means to survive otherwise lethal infections by delaying spread of the pathogen and enabling the adaptive immune system to mount an efficient response.

9.5.1 Type I interferon

Type I avian IFNs were originally designated as IFN1 and IFN2 until Lowenthal et al., (2001) [95] proposed the standard type I nomenclature of the mammalian IFNs be applied, thus becoming ChIFN- α and ChIFN- β , respectively [95]. Consistent with mammals, ChIFN- α exists as a multigene family whereas there is a single copy of ChIFN- β , all of which are intronless and encoded on the short arm of the Z chromosome [96]. Although ChIFN- β displays high amino acid sequence identity (57%) with ChIFN- α , they are clearly different cytokines since anti-ChIFN- α antibodies do not neutralize the antiviral activity of ChIFN- β [96]. These chicken type I IFNs are strongly induced in response to a number of viral infections, such as AI virus and Newcastle Disease Virus (NDV) [80]. Examination of the promoter regions of chicken type I IFNs has revealed putative binding sites for interferon regulatory factors in both and a NF κ B site only in the ChIFN- β promoter, similar to that observed in mammals [97]. Recombinant ChIFN- α and ChIFN- β expressed in bacteria or CV-1 in origin and SV40 (COS) cells display

appreciable antiviral activity that is comparable to their mammalian orthologues [96]. Recombinant forms of ChIFN- α suppressed the growth of many viruses in vitro, such as Marek's disease virus (MDV) [98]. Additional type I IFN have been identified in the chicken genome. ChIFN- κ has been found located on the Z chromosome and has type I IFN activity, triggering interferon stimulated gene production [99].

9.5.2 Type II interferon

The type II IFN, ChIFN- γ [100], was identified on chicken chromosome 1 as a single-copy gene, similar to its mammalian counterpart, with which it shared highest nucleotide sequence identity. Furthermore, the biological activity of recombinant ChIFN- γ was found to be similar to mammalian IFN- γ , whereby stimulation of macrophages with recombinant ChIFN- γ induced nitric oxide production, an important cellular inhibitory mechanism against virus. ChIFN- γ also displayed antiviral activities against Vesicular Stomatitis Virus in vitro, as well as inducing the upregulation of MHC class II antigen [101].

9.5.3 Type III interferon

The discovery of type III IFNs in humans by computational techniques facilitated the identification of ChIFN- λ on chromosome 7, however, whereas there are three IFN- λ genes in mammals, only one ChIFN- λ gene exists [27,102]. The ChIFN- λ gene has a higher amino acid identity to human IFN- λ 2 compared to IFN- λ 1 and IFN- λ 3, and a lower identity to the chicken type I or type II IFNs [102]. The genomic structure of both mammalian and ChIFN- λ genes is similar, each organized into 5 exonic regions. Recombinant ChIFN- λ has been produced and assayed in a number of biological systems to compare its activity to type I chicken IFNs. While ChIFN- γ is a potent stimulator of nitric oxide, ChIFN- β and ChIFN- λ enhanced nitric oxide to a low extent [102]. ChIFN- λ displayed viral inhibitory activity against Semliki Forest Virus and influenza virus, in a similar manner to that observed for the type I ChIFNs [102,103].

9.6 Other factors

9.6.1 The transforming growth factor- β family

The TGF- β family, in chickens and mammals, plays an important role in immunoregulation is an essential cytokine in the cell differentiation process. Three isoforms are known to exist in mammalian cells (TGF- β 1–3), however, TGF- β 1 was found to be the crucial isoform involved in Th17 induction [104]. Despite there being four ChTGF- β genes identified in the literature and

databases [105], most avian immunologists now accept that there are only three in the chicken, as in mammals. In the chicken genome the gene *TGFB1* (with the protein also known as ChTGF- β 4) is situated on chromosome 32, *ChTGFB2* gene is on chromosome 3 and *ChTGFB3* gene is positioned on chromosome 5. As such, the chicken orthologue of mammalian TGF- β 1 is ChTGF- β 4 [106]. ChTGF- β 2 was recognized as a growth factor gene shown to be expressed in chicken embryonic ovaries and testes during embryonic development [107]. Additionally, ChTGF- β 3 has been identified and it has been shown that human TGF- β 3 genes have areas of nucleotide sequence identity [108].

9.6.2 The tumor necrosis factor superfamily

Members of the TNFSF and TNF receptor (TNFR) SF have crucial roles as key players in providing signals essential for the innate and adaptive response, including inflammation, apoptosis, cell proliferation and stimulation of the immune system. In humans there are currently 19 TNFSF ligands and 29 TNFRSF receptors [109]. Many of the TNFSF molecules are type II transmembrane proteins found as membrane-bound form which can then be cleaved to yield a soluble ligand. With this in mind, many of the TNFSF members may somewhat be considered as costimulatory molecules rather than cytokines. Additionally, often the membrane-bound form may signal more efficiently than the soluble ligand. Nevertheless, TNF- α , lymphotoxin (LT)- α , LT- β and B cell-activating factor (BAFF) are members which can be considered cytokines [109].

Analysis of the chicken genome has identified several ChTNFSF ligands, their receptors and intracellular binding partners (Table 9.3). Usefully, for some members both the ligand and the receptor have been identified, nevertheless, in some instances, the ligand has been identified and the receptor is yet to be determined, and in others the receptor has been identified and the ligand is thus far not found. Nevertheless, there are some members where there is an absence of both ChTNFSF member and its cognate receptor, this possibly provides evidence for the nonexistence of that specific TNFSF member in the chicken.

In mammals, TNF- α , LT- α and LT- β bind to the death receptor TNFRSF subfamily cytokines [109]. Recently, the ChTNF orthologue was identified and shown to have a highly GC-rich gene, the protein product has 45% sequence identity to mammalian TNF [110]. Similar to mammals, LPS stimulation of monocytes upregulated TNF expression in the chicken. In mammals, the LT genes are crucial to the development of secondary lymphoid organs, including lymph nodes [111]. LT and the LT specific receptor has yet to be identified in the chicken. It is intriguing to postulate that the lack of lymph nodes in the chicken might be due to the lack of LT

genes. Other death receptor TNFRSF binding molecules have also been reported, for example, Fas ligand [112] and a putative Fas receptor have been identified as well as ectodysplasin and its receptor EDAR [113].

Additionally, ChTNFSF molecules which interact with receptors having a TRAF-interacting motif have been found. For example, ChBAFF has been cloned [114,115] and, as in mammals, mediates B cell survival. Also, CD30 [116] and CD30L [117], which are involved in T cell activation, have been characterized in chickens. Likewise, the cloning and expression of the ChTNF receptor-ligand pair OX40-OX40L has also been reported [118]. Similar to CD30-CD30L, OX40 and OX40L embody significant costimulatory molecules for T cells activation [118]. Glucocorticoid-induced TNFR (GITR) and its ligand, GITRL, have been identified in the chicken and shown to be upregulated by both $\alpha\beta$ and $\gamma\delta$ T cells on activation [119].

Several TNFRSF decoy receptors have been found in the chicken. Chicken osteoprotegerin (OPG) and decoy receptor 3 (DcR3) have been identified [120] as well as some of the ligands that bind them such as TRAIL [117] and RANKL [121], with OPG and FS-7 associated surface antigen (Fas) ligand [112], and VEGI [122] with DcR3.

9.6.3 Colony-stimulating factors

The CSF encompass a family of homodimeric cytokines essential in mediating the development of myeloid cells from pluripotent hematopoietic stem cells. Family members include macrophage-CSF (M-CSF), granulocyte-CSF (G-CSF), granulocyte/macrophage-CSF (GM-CSF), and can also include IL-3 and IL-34. ChM-CSF (or ChCSF1) promotes macrophage growth from chicken bone marrow cells in culture [123]. ChGM-CSF (or ChCSF2) lies in the Th2 gene cluster on chromosome 13 (Fig. 9.4). It is expressed in the bone marrow and other tissues, and can stimulate the proliferation of chicken bone marrow cells [35] and in conjunction with ChIL-4 it allows culturing of dendritic cells from bone marrow [124]. ChG-CSF (or ChCSF3) drives differentiation of myelomonocytic cells [125]. ChIL-34 is a recently revealed cytokine that performs as a second ligand to ChCSF1R. Comparable to ChM-CSF, ChIL-34 also stimulates bone marrow-derived monocyte survival and differentiation into macrophages. ChIL-34 functions by binding to ChCSF-1R and activating signaling pathways which regulate subsequent cytokine expression supporting the role of ChIL-34 in innate and adaptive immunity [123,126].

9.6.4 Cytokines and factors in other birds

Although the focus of this chapter has been on cytokines and factors in the chicken, continual progress is being

TABLE 9.3 TNF, TNFSF and TNFR superfamily members in chicken.

TNFRSF member	Receptor also known as	Receptor in chicken	Intracellular binding partner	Binding partner in chicken	TNFSF member	Ligand also known as	Ligand present in chicken
1A	TNFR1	Yes	TRADD, FAAD, RIPK1	Yes, Yes, Yes	2	TNF- α , LT- α , LT- β	Yes, No, No
1B	TNFR2	Yes	TRAF1–3	Yes	2	TNF- α	Yes
3	LTR	No	TRAF2, TRAF3, TRAF5	Yes, Yes, Yes	3	LT- β	No
4	OX40	Yes	TRAF1–3, TRAF5–6	Yes, Yes	4	OX40L	Yes
5	CD40	Yes	TRAF1–3, TRAF5–6	Yes	5	CD40L	Yes
6	FasR	Yes	FADD	Yes	6	FasL	Yes
6B	DcR3	Yes			6, 14, 15	FasL, LIGHT, VEGI	Yes, No, Yes
7	CD27	No	TRAF2–3, TRAF5	Yes, Yes	7	CD26L	No
8	CD30	Yes	TRAF1–3, TRAF5	Yes, Yes	8	CD30L	Yes
9	4–1BB	Yes	TRAF1–3	Yes	9	4–1BBL	No
10A	Dr4	No	TRADD, FAAD, RIPK1	Yes, Yes, Yes	10	TRAIL	Yes
10B	Dr5	Yes	TRADD, FAAD, RIPK1	Yes, Yes, Yes	10	TRAIL	Yes
10C	DcR1	No			10	TRAIL	Yes
10D	DcR2	No			10	TRAIL	Yes
11A	RANK	Yes	TRAF1–3, TRAF5–6	Yes, Yes	11	RANKL	Yes
11B	OPG	Yes			11	RANKL	Yes
12A	TWEAKR	No	TRAF2, TRAF6	Yes, Yes	12	TWEAK	No
13B	TACI	Yes	TRAF2–3, TRAF6	Yes, Yes	13, 13B	APRIL, BAFF, CAMLG	No, Yes, Yes
13C	BAFFR	Yes	TRAF2–3, TRAF6	Yes, Yes	13B	BAFF	Yes
14	HEM	Yes	TRAF1–3, TRAF5	Yes, Yes	14	LIGHT	No
17	BMA	No	TRAF1–3, TRAF5–6	Yes, Yes	13B	APRIL, BAFF	Yes
18	GITR	Yes	TRAF1–5	Yes	18	GITRL	Yes
19	TROY	Yes	TRAF2, TRAF5, TRAF6	Yes, Yes, Yes		unknown	
19L	RELT	Yes	TRAF1	Yes		unknown	
21	Dr6	Yes	TRADD, RIPK1	Yes, Yes	NA	N-APP	No
25	Dr3	No	TRADD, FADD	Yes, Yes	15	VEGI	Yes
27	EDAR2	Yes	TRAF3, TRAF6	Yes, Yes		EDA-2	No
	EDAR	Yes	EDARADD	Yes		EDA	Yes

made in identifying similar molecules in other birds, especially other poultry (see chapter 19).

9.7 Chemokines

Chemokines, or chemotactic cytokines, are a family of messenger molecules produced by a wide range of immune cells that control the trafficking of various immune cells and, additionally, have roles in other functions, such as embryonic development [127]. Importantly, the chemokine system works to regulate the circulation and the recruitment of lymphoid cells to various sites of infection [128]. Although chemokines were originally considered just as inducers of focused chemotactic movement, they are also able to stimulate an assortment of purpose driven and undirected migratory actions as well as other activities such as bringing about cell arrest or influencing cell adhesion [129].

In mammals, the chemokine repertoire is thought to be derived from multiple gene duplications followed by sequence divergence. This expansion is more prevalent in the inflammation associated genes (induced during inflammation) with the generation of three major clusters in both humans and mice, whilst the constitutively expressed homeostatic genes are found as individuals or in small clusters. There are several subfamilies of chemokines, for example the C, CC, CXC, and CX3C clusters,

where each is demarcated by the spacing between the first two conserved cysteines at the amino termini of these proteins, with the exception being XC where the first cysteine is absent [130]. Chemokine ligands are designated with an L (for example, CCL) and receptors with an R (such as, CCR).

In the chicken there are many orthologues of mammalian chemokines with more than 20 being so far identified [27] (Table 9.4). Still, examination of the chicken genome has revealed that the chicken has a diverse, but a reduced, collection of chemokines and receptors similar to those of mammals and other species. Chicken chemokines share little sequence identity with mammals and, as such, it can be problematic to allocate chicken chemokines to a precise mammalian counterpart built on sequence data alone. This complexity has led to difficulties in identifying orthologues and necessitated adjustments to nomenclature over time, and, as more data is generated we may continue to see modifications in terminology. The chicken genome assembly database GRCg6a has considerable information on the currently identified chicken chemokines and the naming of these orthologues as agreed by the chicken gene nomenclature consortium. Analysis of the chicken genome suggests that these chemokines are represented across a number of genes, however, there are particular clusters identified on chromosome 4, 19, and the Z chromosome (Fig. 9.8, Table 9.4).

TABLE 9.4 Chicken chemokines and chromosomal locations.

Name	Also known as	Gene ID	ENS	Chromosome
XCL1	SCYC1, lymphotactin	395914	15235	1
CCL1	CCLi6	395552	2329	19
CCL4	SCYA4; MIP-1 β	395551	32717	19
CCL4	K203	395468	34478	19
CCL5	ah294	417465	43603	19
CCL17	TARC	415652	24466	11
CCL19	CC motif chemokine 19	427406	28256	Z
CCL20	MIP-3 α	395082	3003	9
CCL26	CCLi10	417534	14585	19
CCL28	CC motif chemokine ligand 28	107049316	-	Z
CXCL12	SDF1	395180	41346	6
CXCL13	CXC motif chemokine 13	109648311	45607	4
CXCL14	JSC	395451	6346	13
CX3CL1	fractalkine	415651	26663	11
CCLi5	uncharacterized protein LOC417533	417533	-	19
CCLi7	CC motif chemokine 3	417535	49551	19
CCLi8	CC motif chemokine 3	771679	54196	19
CCLi9	ah221	417536	53121	19
CCL26L	CC motif chemokine 26-like	100857191	46192	Z
CCLL4	GGCL1, chemokine-like ligand 1	417464	53868	19
IL-8L1	IL-8; K60; interleukin-8	395872	11668	4
IL-8L2	IL-8; CXCL8; EMF-1; CXCLi2	396495	26098	4
CXCL1L	CXC motif chemokine ligand 1-like	422654	46160	4
CXCL13L2	CXC motif chemokine ligand 13-like 2	422510	10338	4
CXCL13L3	CXC motif chemokine ligand 13-like 3	422511	29532	4

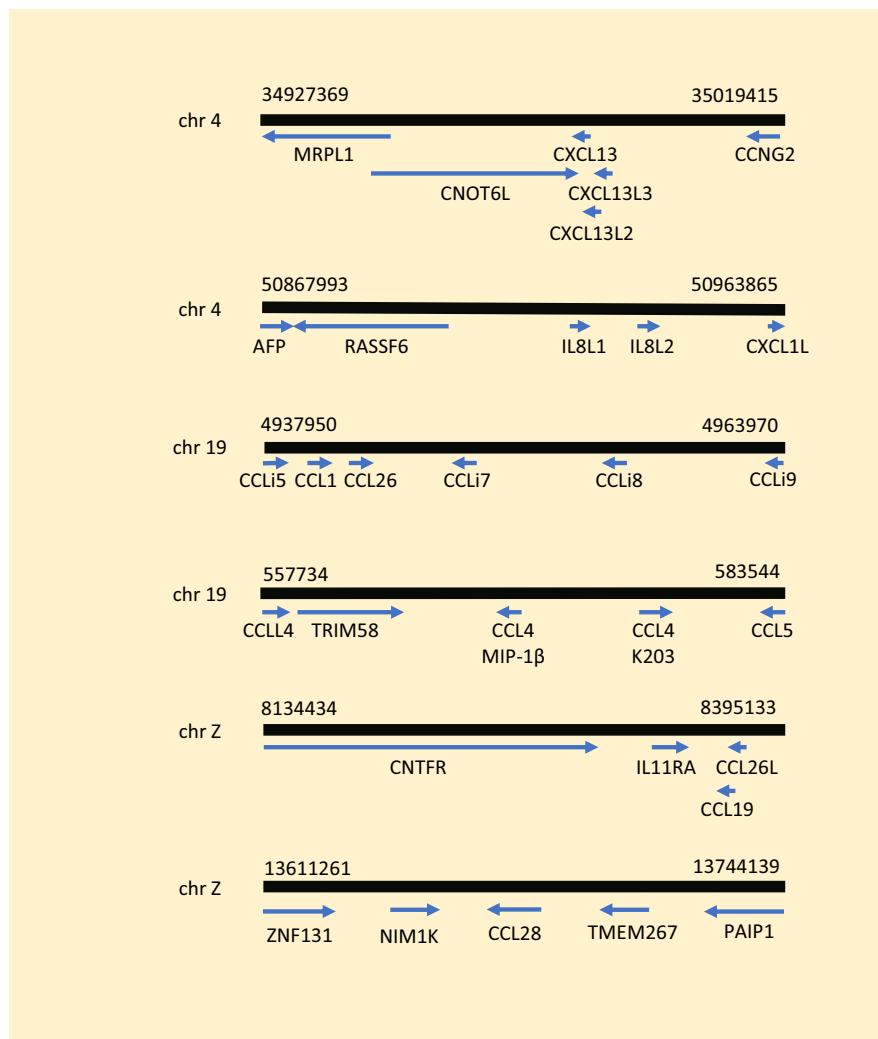


FIGURE 9.8 Representation of the genomic organization of selected chicken chemokine family gene clusters (not all are represented here). The information is from the assembly database GRCg6a. Positions on the chromosomes (chr) are indicated and arrows show transcriptional orientation.

9.7.1 XC and CX3C chemokines

The chicken orthologue of mammalian XCL, or lymphotactin, has been cloned and seems to act as a chemoattractant preferentially for splenic B cells [131] and is located on chromosome 1. The chicken genome sequence also contains the gene for fractalkine (CX3CL), however, there is little information on its biological activity. In mammals fractalkine has two functional forms, one is a membrane-anchored CX3CL1 and the other is a soluble CX3CL1. Soluble CX3CL1 acts as a chemoattractant for T cells and monocytes, while the cell-bound chemokine encourages adhesion of leukocytes to activated endothelial cells [132]. It would be interesting to see if this same activity is conserved in chickens, particularly as there are descriptions of commercially available recombinant ChCX3CL1. Additionally, a line of transgenic chickens has been developed that carry lacZ and express bacterial β -galactosidase, in which the viral insertion carrying the lacZ gene is within the CX3CL gene resulting in the

absence of CX3CL mRNA expression in the brain of homozygous individuals [133]. This may provide an opportunity to investigate the function of CX3CL.

9.7.2 CC Chemokines

There are a number of CCL chemokines, as well as CCL-like chemokines, encoded in the chicken genome and many of these are grouped on chromosome 19 in two clusters [27] (Fig. 9.8, Table 9.4). Previously, Kaiser et al. (2005) had proposed to name these chemokines ChCCLi1–10 (where “i” stands for inflammatory), since the relationship of these chemokines to those in human and mouse was somewhat unclear. The CCLi1–4 cluster was associated with the Macrophage Inflammatory Proteins (MIP) family and the CCLi5–10 were associated with the Monocyte Chemotactic Protein family. More recently the genome assembly database GRCg6a, which uses the current Chicken Gene Nomenclature Consortium

naming, indicates the current annotation for these genes. These clusters correspond to collections of genes on human chromosome 17 and mouse chromosome 11 [134]. It appears that the chicken has fewer chemokine representatives and the first genome assembly appears to contain 4 chemokines associated with MIP, major factors produced by activated macrophages and monocytes which are crucial for immune responses towards infection and inflammation. With regards to ChCCL4, there are ChCCL4/MIP-1 β [135,136] and ChCCL4/K203 [137,138] as well as a chemokine-like ligand 4 (ChCCL4) candidate, all closely situated on chromosome 19, as is also ChCCL5 [136,139,140]. The second chromosome 19 cluster contains six CCL chemokines [27] including ChCCL1. In mammals CCL1 is involved in inflammatory processes, such as leukocyte recruitment and is involved in tumoral processes [141]. In the chicken the gene for CCL1 was isolated from a hematopoietic cell cDNA library [135]. Nevertheless, there is still a paucity of information about the function of ChCCL1 protein activity. Located in close proximity to ChCCL1 is ChCCL26 and the 4 chemokines are currently annotated as CCLi5, 7, 8, and 9 (Fig. 9.8, Table 9.4). ChCCL20 is located on chromosome 9 and shows approximately 50% identity with human CCL20 [142]. Additionally, ChCCL17 is sited on chromosome 11 neighboring to ChCX3CL1, while ChCCL19 is located on the Z chromosome nearby ChCCL26L and ChCCL28.

9.7.3 CXC chemokines

Chickens appear to have fewer homeostatic CXC chemokines than mammals, with the absence of CXCL9, 10,

and 11. However, there are two orthologues of CXCL12 and CXCL14 [143,144] on chromosomes 6 and 13, respectively. There is an additional cluster of CXC chemokines located on chromosome 4. CXCL13, which is a single copy gene in humans and mice and is a B cell chemoattractant, appears as an apparent expansion in the chicken genome and there are three genes (CXCL13L1, CXCL13L2, and CXCL13L3). Furthermore, there are another 3 genes on chromosome 4 including CXCL1L and two ChIL-8 genes. The chicken appears to have evolved two different CXCL8-like chemokines, IL-8L1, and IL-8L2, in contrast to human CXCL8 (IL-8) [145]. ChIL-8 chemokine is expressed by fibroblasts, epithelial cells, endothelial cells, neutrophils, and macrophages and is a part of the inflammatory response. Intriguingly, the mouse has no CXCL8 orthologue. Of interest is that the MDV has assimilated a CXC chemokine into its genome. This gene was denoted vIL-8 due to its similarity to ChIL-8 [146], nevertheless, subsequent analysis has shown that this viral gene is an ortholog of CXCL13L1 [146].

9.8 Cytokine and chemokine receptors

Cytokine receptors can be grouped into families based on common structural features (Fig. 9.9) including the type I and type II cytokine receptors [which includes the IFN/IL10 families (Fig. 9.6)], the TGF- β and TNFRSF (Table 9.3), the chemokine receptors, and the IL-1 family receptors (Fig. 9.2).

The largest family is the type I cytokine receptor family. These are single-pass transmembrane proteins most

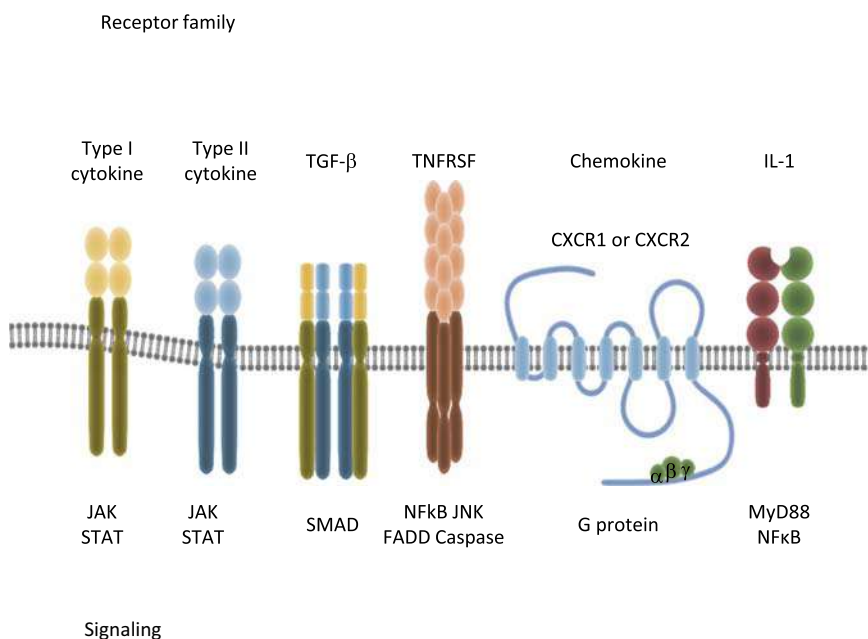


FIGURE 9.9 Schematic illustration of the different cytokine and chemokine receptors. Receptors for the various cytokines are often classified into families on the basis of conserved extracellular domain structures and signaling mechanisms founded on mammalian information.

of whose members form heterodimers, although some form homodimers or heterotrimers. Many form subfamilies, with one of the heterodimeric or heterotrimeric receptor chains, generally the signaling chain, being common to all subfamily members. For example, the IL-2 group of receptors (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21) share a common γ chain [147], the IL-6 group (IL-6, IL-11, CNTF, LIF, oncostatin M) share a common gp130 chain [148], and the GM-CSF group (GM-CSF, IL-3, IL-5) share a common β chain [149]. Typically type II cytokine receptors are heterodimers or multimers with a high and a low affinity component, while the TGF- β family members signal through the TGF- β type II receptor (TGF β RII) and the TGF- β type I receptor (TGF β RI) [150]. The TNFRSF members are single-chain receptors that form homotrimers and become activated when cross-linked by their ligands, which are also homotrimers. The chemokine receptors are all 7-transmembrane-domain, G-protein-coupled receptors and, like their ligands, are subdivided into XCR, CCR, CXCR, and CX3CR. The IL-1 family receptors (e.g., IL-1R and IL-18R) are heterodimers, formed of a cytokine-specific type I receptor and a receptor accessory protein (e.g., IL-1RI and IL-1RAcP; IL-18RI and IL-18RAcP) [151].

9.8.1 Type I receptors

For the Type I cytokine receptors, the ChIL-2 common γ chain [152], located on chromosome 4, the gp130 [153] located on the Z chromosome, and ChGM-CSFR β chain, on chromosome 1, have been identified in the chicken genome sequence. For the IL-2 group of receptors, CD25 (IL-2R α chain) has been characterized [154] and is on chromosome 1 and CD122 (ChIL-2R β chain) is located on chromosome 1. Likewise, the ChIL-15R α chain, also on chromosome 1, was cloned and characterized [155] and the ChIL-4R α , ChIL-9R α , and ChIL-21R α chain genes are syntenic and present in the genome on chromosome 14. The ChIL-7R α chain has been cloned and characterized [156] and is positioned on the Z chromosome.

For the gp130 group, the ChIL-6R α chain is on chromosome 25 [157] and the ChIL-11R α chain has been cloned and characterized [158]. ChCNTFR is syntenic with ChIL-11R α on the Z chromosome and, similarly, ChLIFR and ChOSMR are syntenic on chromosome Z. For the ChGM-CSF group, CD116 (ChGM-CSFR α chain) is present on chromosome 1 and CD125 (ChIL-5R α chain) is present on chromosome 12 and binds recombinant ChIL-5 but not recombinant KK34 [42].

The other Type I cytokine receptors (ChIL-12 and ChG-CSF) are present in the chicken. The two chains of the ChIL-12R are present in the genome (ChIL12R β 1, on chromosome 28 and ChIL12R β 2, on chromosome 8) and CD114 (ChG-CSFR) is present in the genome on chromosome 23.

9.8.2 Type II receptors

The Type II family cytokines includes the IFN and IL-10 family cytokines [159]. Type II receptors form a heterodimer and signaling is induced by binding of the cytokine to the receptor complex leading to Janus kinase (JAK) interactions. Analogous to Type I, the Type II receptor group includes both shared and cytokine-specific receptors. Nevertheless, a feature of Type II cytokine signaling is a single receptor can bind multiple cytokines and a cytokine can, in some cases, bind multiple receptors (Fig. 9.6) [160]. Members of the Type II cytokine receptor family consist of an extracellular region, containing two tandem fibronectin type III (FN3) repeats, except IFN- α receptor 1 (IFNAR1) which has four, a single pass transmembrane domain and an intracellular signaling region [161]. The Type II cytokine receptor family is composed of a number of distinct receptor complexes, which are used for signaling by the IFNs, IL-10, and IL-10 related proteins as well as factor VII (FVIIa) to mediate their biological activities [162]. The three IFN families use distinct receptors to signal, which results in slightly different signaling pathways and ultimately variation in their respective biological activities. IL-10 family members bind to the various IL-10R family molecules (Fig. 9.6) with IL-10 binding to a complex of two IL-10R1 and two IL-10R2 subunits. IL-19, IL-20, and IL-24 can engage with an IL-20R1 and IL-20R2 complex, and additionally IL-20 and IL-24 can also bind to an IL-22R1 and IL-10R2 heterodimer. IL-22 binds to a heterodimer made up of IL-22R and IL-10R2 whilst IL-26 engages with an IL-20R1 and IL-10R2 complex. Intriguingly, IFN- λ molecules bind to a complex of the IFNLR1 and IL-10R2. The type I IFNs signal through a IFNAR1 and IFNAR2 complex [163], type II IFN signals via IFNGR1 and IFNGR2 complex [164], whereas the type III IFNs bind to a unique complex consisting of IFN λ R1 along with the promiscuous IL10R2 subunit [165].

Many of the Type II cytokine receptor family genes have been identified, cloned and characterized in the chicken. The ChIFNAR1, ChIFNAR2, ChIFNGR2, and ChIL10R2 genes are syntenic and found to cluster on chicken chromosome 1 in a similar fashion to that seen in humans [166]. ChIFNGR1 is situated on chromosome 3 and is syntenic with ChIL20R1 and ChIL22BP. Whilst, ChIL20R2 is on chromosome 14 and ChIFN λ R1 and ChIL22R1 are on chromosome 23. Several studies have indicated that the JAK-STAT pathway is active in birds, suggesting a possible conservation of the downstream signaling pathways [167]. However, there remains the requirement to further investigate these molecules and their associated pathways in the chicken.

9.8.3 Transforming growth factor- β family receptors

The genes for TGF- β RI (chromosome 2), TGF- β RII (chromosome 2), and TGF- β RIII (chromosome 8) are all present in the chicken genome, nevertheless, more work needs to be carried out to fully characterize these molecules.

9.8.4 Tumor necrosis factor superfamily receptors

Table 9.3 highlights the information available for the ChTNFRSF. Many of the receptors have been identified, yet, there is the potential to identify further molecules given that a number of the ligands can be identified.

9.8.5 Chemokine receptors

Chemokine receptors are typical G-protein coupled receptors with seven transmembrane domains. A number of chemokine receptors have been identified in the chicken genome [27]. The genes for XCR are positioned on chromosome 2 near ChCCR5 and ChCCR2. Similarly, ChCX3CR is present on chromosome 2 near ChCCR4 and ChCCR8. Of the CCR genes so far identified in human and mouse, direct orthologues of most can be found with many located on chicken chromosome 2 along with ChCCR2, -4, -5, -8, -9, and ChACKR4. Additionally, ChCCR6 is on chromosome 3, while ChCCR7 and 10 are on chromosome 27 as identified is from the assembly database GRCg6a.

In humans and mice, several CCR (including ChCCR1–3 and ChCCR5 as well as the receptors for the MIP family chemokines) cluster together on chromosome 3 and 9, respectively. ChCCR6 [142] and ChCCR7 [168] have been cloned and characterized. In chickens fewer of the CXCR have been observed. ChCXCR1, 4, and 7 are situated on chromosome 7 and ChCXCR5 is on chromosome 24. Recently, ChCXCR4 and its ligand ChCXCL12 were shown to play an important role for the sequential migration events of B cells in the bursa [169,170] (see Chapter 4). ChCXCR5 does bind to all three CXCL13 isoforms (L1–L3) and in addition is also the receptor for the MDV encoded chemokine vCXCL13 (formerly vIL-8) [146].

9.8.6 Interleukin-1 family receptors

As described earlier, the chicken has the same repertoire of IL-1 family receptors as mammals (Table 9.1 and Fig. 9.2). The chicken type-I IL-1R was the first chicken cytokine receptor to be cloned and characterized [171]. Soluble type I ChIL-1R blocks ChIL-1 activity [172]. ChIL-1RL1/ST2 [173] was cloned and is located on chromosome 1 while ChSIGIRR [174] was cloned and is found on chromosome 5. All of the IL-1 family receptor

genes are readily identifiable in the chicken genome, with conservation of synteny for the ChIL-1 family receptor genes (IL-1R1, IL-1R2, ST2, IL-18R α , IL-18R β , IL-36, and TIGGR-2) encoded on human chromosome 2 and a locus on chicken chromosome 1 (Table 9.1).

9.9 The importance of regulation of cytokine responses

When the immune system is fighting pathogens, cytokines signal immune cells such as T cells and macrophages to migrate to the site of infection. In addition, they activate those immune cells, which in turn stimulate a cascade of additional cytokines which then bind to their highly specific cell surface receptors and trigger multiple functional activities on a variety of target cells. Furthermore, many cytokines share similar biological effects, an evolved redundancy feature so that in the absence of any one cytokine, others may fill the void. Cytokines are produced in response to stimuli via receptor induced pathways. Under normal circumstances cytokine secretion is a closely regulated and controlled process. However, in some instances regulation of this signaling becomes uncontrolled, leading to a state of cytokine dysregulation, whereby too many immune cells are activated in a single location. The precise reason for this is not entirely understood, but may be caused by an exaggerated cytokine response (referred to as hypercytokinemia or cytokine storm) when the immune system encounters a new and highly pathogenic invader, such as H5N1 influenza viruses [175].

A cytokine storm usually results in an unbridled immune system leading to the upregulation of a myriad of inflammatory mediators that has the potential to do significant damage to organs and tissues. As an example, highly pathogenic AI virus infection is associated with a pronounced elevation of cytokines, and this cytokine storm is a contributing factor for the fatal outcome of his disease. During a cytokine storm damage to host cells occurs through either hyperacute production or persistent chronic over production of an immune response and can be associated with a range of diseases. During AI infection a rapid degradation of organs, predominantly the lungs, systemic inflammation and intravascular coagulation are consequential of a cytokine storm. Hypercytokinemia and hemophagocytic syndrome, invokes the release of many different inflammatory mediators; primarily cytokines but, also free radicals and serum associated factors [176]. With these studies in mind it is clear that a better understanding of this complex interplay between cytokines, chemokines, and the inflammatory response to infection is needed. Knowledge of the interaction between cytokines and their mediators may give us insight into methods for controlling the appearance of complications and

reduce the tissue damage associated with hypercytokinemia and infection in chickens [175].

9.10 Therapeutic potential of chicken cytokines

9.10.1 Alternatives to antibiotic growth promoters

Antibiotics have been used to treat and control diseases in livestock and poultry for more than 50 years. Historically, in-feed antibiotics have been used at low levels to improve growth rate and feed conversion efficiency, primarily via controlling pathogenic bacteria and associated secondary viral infections. Thus in-feed antibiotics have been referred to as growth promoters. For many years problems with emergence of antibiotic resistant bacteria in humans have been linked to the overuse of antibiotics in livestock feed. The use of chemicals to control bacterial and parasitic diseases, such as coccidiosis in poultry, has also gained public attention and concern. For example, chemical resistance has emerged in certain strains of *Eimeria*, thereby rendering them difficult to treat [177]. There are also environmental concerns about chemical residues remaining in the meat and waste, and a risk of chemical contamination of rivers and lakes through water runoff. Public awareness over these issues resulted in a world-wide review of the practices involving the use of medication in livestock feed. As a consequence, many of these treatments are now highly regulated and their use severely limited, resulting in the general acceptance of the need for alternative preventions and treatments for pathogens in commercial poultry.

Alternatives to in-feed medication in poultry will need to be safe, easy to administer, cost-effective, and most importantly effective. They need to guarantee that poultry are able to achieve their growth potential without a concomitant increase in the food conversion ratios and higher costs to the farmer. A number of alternatives have been suggested, however, most of these will not compensate fully for the removal of antibiotics and chemicals.

Chicken cytokines are being examined as potential replacements for in-feed antibiotics in poultry, either as therapeutics or as vaccine adjuvants. To date, cytokine administration has proven successful in larger food production animals such as sheep, cattle, and pigs [178]. Cytokine modulation therapy has also been used to boost the immune response of human patients suffering from viral infections [179]. Under current intensive poultry rearing conditions, the use of cytokines as nonspecific natural potentiators of the immune system may be beneficial in protection against infectious diseases. Cytokine therapy is a practical solution that may have more public

acceptance than antibiotic therapy. Moreover, the outcomes of viral infections are costly in terms of human and animal health and welfare worldwide, particularly in the case of zoonotic pathogens. The observed increase in the virulence of some viruses and failure of many vaccines to stop these infections, or prevent viral shedding, has led to the need to develop new antiviral strategies. One approach to dealing with viral infection may be to employ the therapeutic administration of recombinant cytokines to act as antiviral therapies or “immune boosters” to assist in augmenting the host response to virus. There have been several studies that have demonstrated the potential use of cytokines in poultry. For example, infection by Infectious Bursal Disease virus causes depletion of B cells and leads to immunosuppression [180]. Consequently, an approach that can reinstate immune responses would be a potential treatment. Coincidentally, ChIL-7 promotes B and T cell immune responses and therefore antiviral activity. With this in mind, ChIL-7 might be a potential therapeutic agent for certain virus-induced immunosuppression diseases in chickens [181].

The identification of a number of Th1-type cytokines provides an opportunity to explore their potential use in enhancing antiviral treatment strategies in chickens. ChIFN- γ treatment has been researched in the context of Chicken Anemia Virus (CAV) infection. CAV infection results in thymocyte depletion and immunosuppression and the subclinical form of the disease is often associated with secondary complicating infections due to an immunocompromised state. The *in ovo* administration of ChIFN- γ was observed to impact the deleterious aspects of CAV infection, particularly the depletion of T-cell precursors. This suggests that ChIFN- γ could be further studied as a potential therapeutic reagent to modify virus infection and adjust the immunosuppression caused by CAV [182]. Coccidiosis is a serious enteric disease of chickens that requires treatment using ionophore antibiotics. Chickens treated with ChIFN- γ were found to be protected from weight loss and clinical signs following infection with *Eimeria acervulina* [183]. Furthermore, it has been observed that *Eimeria* stimulates IL-10 production in the gut which in turn leads to a reduced immune response allowing further replication of the coccidia. By feeding chickens transgenic corn expressing antibodies that neutralize IL-10 activity, *Eimeria*-challenged chickens showed improved body weight gain, lowered *Eimeria* lesion scores and reduced oocyst counts [184]. This shows that counteracting the undesirable properties of cytokines during infection may contribute to reducing the use of chemicals and antibiotics in poultry. These approaches highlight the potential use of cytokines to modify the chicken immune response for practical outcomes.

9.10.2 Potential use of cytokines as vaccine adjuvants

With the increased emphasis on the need of safer, yet more effective poultry vaccines, there is a growing opportunity for the development of improved adjuvants to augment vaccination strategies [185]. Current oil-based adjuvants induce adverse site reactions resulting in decreased meat quality and animal discomfort. At this time there are no suitable, cost-effective adjuvants for use in poultry, particularly broiler chickens (meat production birds). Since endogenous cytokines are potent regulators of immunological reactions, it is reasonable to believe that vaccine formulations which include exogenous cytokines might yield enhanced immune responses [182]. Several studies have demonstrated beneficial effects of a variety of cytokines, including ChIL-1 β , ChIL-2, ChMGF, ChIFN- α , and ChIFN- γ [12,183,186–188].

ChIL-1 β has been shown in proof-of-concept trials to increase antibody responses when administered *in vivo* as a recombinant protein compared to tetanus toxoid antigen alone [12]. Furthermore, combined administration of ChIFN- α , ChIFN- γ , and ChIL-1 β increased antibody responses to tetanus toxoid in an additive manner [12]. This supports the view that cytokines could be used as effective adjuvants and may produce a better response when used in combination. Similarly, the therapeutic and antiviral effect of ChIFN- α has been demonstrated by Karaca et al., (1998) [189] in which coexpression of NDV and ChIFN- α in a fowl pox virus (FPV) vector protected birds from challenge with NDV. When delivered by the FPV vector, NDV genes gave protection to birds after challenging with NDV, however, the chickens had lost weight due to the fowl pox virus. When ChIFN- α was coadministered with the NDV gene in the FPV vector, the birds did not lose body weight compared to nonvaccinated birds. This supports the concept that ChIFN- α treatment prevented disease through its anti-FPV activity whilst maintaining NDV vaccination potential.

Additionally, a number of cytokines have been tested as an adjuvant for DNA vaccines for protection against coccidiosis. Various ILs and other factors have been included as expression molecules in DNA vaccines against *Eimeria* with both ChIL-2 and ChIFN- γ showing promise [187,190,191]. Lowenthal et al., (1998) [192] have previously demonstrated the adjuvant ability of ChIFN- γ when chickens were injected with sheep red blood cells as an antigen. Treatment with ChIFN- γ had no significant effect on the primary antibody response but significantly increased magnitude and duration of the secondary (IgG) antibody response. Furthermore, ChIFN- γ allowed a 10-fold lower dose of antigen to be used to achieve the same antibody titer. These studies support that ChIFN- γ has an adjuvant activity and could be used

in vaccine formulations for better protection against disease. Until recently, the adjuvant function of chicken myelomonocytic growth factor (cMGF) was not characterized. The *in vivo* biological activity of cMGF delivered via a live FPV (fp/cMGF) had been demonstrated in a previous study. Birds that were highly susceptible to MDV were treated with fp/cMGF. The birds were then vaccinated with a commercial vaccine, herpes-virus of turkeys (HVT) on day 4 and challenged with a virulent (RB-1B) strain of MDV one-week post cytokine treatment. In this trial, nonvaccinated birds suffered 100% mortality after challenge. Birds vaccinated with HVT alone suffered 33% mortality whereas vaccinated birds that were also cMGF treated were all protected. These results showed improved protection and less tumor incidence in MDV challenged birds and clearly indicate the potential use of cMGF as vaccine adjuvant for vaccinal viral strains to increase protection against MDV [188].

Similar to the therapeutic use of cytokines, when considering cytokines as adjuvants in chickens, to achieve a cost-effective outcome it is important to consider the mechanism of cytokine administration. However, although the adjuvant effect of cytokines was significant in most of these studies, their wide use in poultry vaccines is not currently evident mainly because of their high manufacturing costs and additional practical hurdles. Coexpression of cytokine genes in FPV vaccine strains or other viral vectors might overcome most of these problems [183]. Nevertheless, there have been a number of reports recently [52,193–196] on the use of fowl pox vaccines expressing genes from various viruses (e.g., AI virus, infectious bronchitis virus, infectious laryngotracheitis virus, NDV) alongside several cytokine genes, including ChIFN- γ , ChIL-6, ChIL-12, and ChIL-18.

The commercial use of *in ovo* vaccination has increased in recent years and will likely become the preferred means of immunization in poultry, due to reduced labor costs and less animal handling [197]. *In ovo* administration involves the injection of vaccine/adjuvant into the egg shortly prior to hatch. Many studies have shown a number of vaccines can be safely and effectively administered *in ovo* [197]. Therefore the development of automated systems for vaccine and adjuvant administration on a commercial scale introduces a new method of delivery of cytokines as vaccine adjuvants or therapeutics for future research. Automated machines can safely inject large numbers of eggs ensuring accurate dosage and increased consistency. With this in mind, from an industry point of view, cytokines have the possibility to be administered to chickens *in ovo* along with other vaccines. *In ovo* therapeutic administration has been shown to reduce expenses associated with individually injecting newly hatched chickens. However, for this system to be viable within the poultry industry, large scale cytokine

production and delivery must be taken into consideration. Cytokines delivered as adjuvants *in ovo* could reduce the cost of vaccines by increasing the effectiveness of the vaccine, allowing lower doses of vaccine to be used and reducing labor costs. Therefore *in ovo* administration may provide a simple and effective delivery method and can be used to deliver cytokines to study their therapeutic potential in chickens [185].

9.11 Conclusion

Since the chicken genome sequence was sequenced almost 2 decades ago there has been an enormous amount of progress made in identifying chicken cytokines and their receptors (as well as other components of the immune system) and understanding the implications of their activity during infection and immunity. This knowledge has not only been important from a comparative immunology perspective providing information about the evolution of the immune response, but it has provided critical information about the role of cytokines in the host–pathogen interaction. Understanding the cytokine associated aspects of disease is critically important, as well as providing insights into developing new control approaches. Bioinformatics has vastly accelerated the identification of new genes for chicken cytokines and receptors and has helped to generate a huge catalog of sequences for of chicken orthologues of mammalian immune genes. The next challenge will be to verify and assess the biological function of these gene products, which entails production of recombinant proteins, development of biological assays and assessment *in vivo*. This ongoing work will crucially form the basis of developing alternative vaccine and targeted therapeutic strategies for improved programs for the control of poultry diseases.

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Immunogenetics and the mapping of immunological functions

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10.1 Introduction

Effective and coordinated functioning of the immune system is essential to maintain health, and the genetics of a bird defines its maximum achievable immune response. A comprehensive disease control program contains many components, including vaccination, biosecurity, sanitation, and host genetics. To meet consumer preferences and legislation, the use of antibiotics in poultry production is expected to decrease. Genetic enhancement of immunity has permanent and cumulative effects in a breeding population and is therefore a sustainable way to maintain health in poultry [1–3]. Some microbes with an asymptomatic presence in poultry are foodborne pathogens that cause disease in humans who consume improperly prepared poultry products [4]. Therefore reduction of the microbial burden in poultry is an important step in preharvest food safety. The identification of biomarkers associated with immune function can effectively enhance host resistance to pathogens and thereby reduce disease and pharmaceutical treatments in live birds as well as microbial contamination of poultry products [5–8]. In addition to maintaining health, some genes for immune response are associated with production traits such as growth, which reinforces the complex relationships of health and production [9].

The availability of the chicken genome sequence [10], the single-nucleotide polymorphism (SNP) map [11], and technical platforms to simultaneously genotype hundreds of thousands of SNPs [12], to sequence the entire genome [13], or to assess the whole transcriptome have opened a new era of understanding how genetic variation can impact immune responses, health, and response to pathogens. This information will enable more informed and effective use of genetic strategies to protect avian health

through genetic selection for improved immune responsiveness, the use of recombinant proteins to enhance immunity and vaccine efficacy, rational design of vaccines, and the production of specific pathogen-resistant transgenic bird lines.

10.2 Genetics and immunological traits in the chicken

Selection for immunological traits in the chicken has the immediate aim of understanding the genetic control of immune responses and the overall goal of enhancing flock health. After biomarkers associated with immune responses are identified, they can be applied in breeding programs for this purpose. The biomarkers may be of many types. Early studies were limited to easily assessed markers, such as serum antibodies and blood group antigens, and these biomarker types are still relevant because of the ease and economy of their use. Within recent decades, however, the advent of molecular genetics and its application in studies of farm animals have opened the door to DNA marker identification. Rapidly progressing research on gene expression in avian species is defining expression-based biomarkers for immunity and disease resistance.

It is feasible to genetically select for host responses to individual pathogens [6,14–16], or for innate or general immune response. The cost of pathogen challenge trials is very high and presents a biosecurity risk. In addition, with the notable exception of the ubiquitous Marek's disease virus (MDV), it is difficult to predict the specific pathogens that poultry may encounter in the field, regardless of whether they are raised in intensive or extensive management settings. This unpredictability complicates the

decision about which pathogens should be considered in genetic selection of breeding stock. Attention has therefore been directed toward elucidating the genetic control of immune responses, with the underlying assumption that they will provide protection against narrow or broad ranges of pathogens. The methodology for the identification of genes or biomarkers associated with immune responses or with disease resistance (pathogen response) is fundamentally the same.

Several large-scale studies on selection for immune responses in chickens have been conducted by measuring antibody levels to a defined antigen at a specific age and using that phenotypic information to select breeders. Although genetic selection may be based on single-trait, correlated responses in the complex immune system may alter many other immune functions. Several large, long-term studies have been conducted which collectively have generated much information about the genetics of antibody response as well as the correlated changes that accompany antibody response selection. These are reviewed in Lamont et al. [17]. Two major studies, at Virginia Tech University in the United States and at Wageningen University in the Netherlands, implemented long-term, divergent, single-trait selection for antibody response to sheep red blood cells (SRBC). Although not an infectious agent, antibodies to SRBC are efficiently measured with simple hemagglutination assays and they are not an infection hazard to chickens or researchers. Another study, at the Hebrew University in Israel, selected for antibody response to *Escherichia coli* vaccine to assess responses to pathogen-derived antigens. Still other studies have been designed to reflect the complexity of the total immune response by incorporating multiple immune response traits, either combined in a single selection index (Iowa State University, United States) or selected individually in separate selection lines (INRA, France). Major lessons from these long-term studies are described here.

The heritabilities of immune response traits indicate that genetic selection is quite feasible, especially for antibody levels, which have moderate (generally ~ 0.2 – 0.3) heritabilities [18–21]. For nonantibody traits, such as cell-mediated response to phytohemagglutinin or phagocytic response measured by carbon clearance, the estimated heritabilities are lower (0.05 – 0.15), suggesting that these traits would be more difficult to alter by genetic selection [17,19]. The genetic correlations among the various types of immunity measures are generally low, which indicates that each is independently controlled and therefore can be independently altered by genetic selection.

Changes in allelic frequencies correlated with immune response selection provide suggestive evidence that a gene is associated with the selected trait and therefore identifies a candidate gene for more intensive study to

verify its role in immunity. In the aforementioned selection experiments, marked alterations in major histocompatibility complex (MHC) genes were consistently evident, although the fraction of phenotypic variation explained was not large. This suggests that the selected immune traits were under polygenic control [22–25].

Correlated changes that occur concomitantly with genetic selection for immunological traits are important in the context of overall flock performance. Selection for immune responses is conducted with the assumption that it will improve resistance to disease. Indeed, this was verified by testing of immune response–selected lines; divergent lines generally exhibited divergent resistance properties to several diseases [17]. A cautionary note, however, comes from studies of the Virginia Tech lines: the high-antibody line was relatively more resistant than the low-antibody line to many, but not all, tested pathogens [26–28]. The specific relationship (beneficial or detrimental) of immune response traits for resistance to various pathogens must be defined before undertaking a commercial genetic selection program with the intent to improve disease resistance.

To be effectively implemented in a breeding program for improved bird health, genetic selection for immune response traits must not unduly compromise other important traits. In this context, the relationship between antibody response and growth is important. In both of the single-trait selection experiments for antibodies to SRBC, there was a negative correlation between antibody level and body weight or growth [17]. This was postulated to have arisen from competition for limited nutrients among the different physiological systems, such as protein deposition in muscle and secretion in antibodies [29]. However, the benefits or drawbacks of genetic enhancement of antibody production for growth might differ between high-hygiene environments, such as breeding companies and research facilities, and lower hygiene environments with significant environmental antigen challenges, such as might be found in typical commercial production. There is evidence for this type of environmental interaction with genetic control of production traits for several genes of the immune system [9]. The nutritional and metabolic “cost” of genetically programming an animal for high-antibody production might be beneficial in a low-hygiene environment in which protection against pathogens is essential to maintain health. The specifics of the nutrient requirements of an activated immune system and the details of the genetic correlations between immune and production traits in a variety of environments remain to be elucidated [30].

To further elucidate the genetic architecture of immune response traits, genome-wide association studies (GWAS) have been performed to identify genomic regions associated with immune response. The Animal

Genome QTL Database in January 2020 reported 739 quantitative trait loci (QTL) across 46 disease susceptibility traits in the chicken, and 91 QTL for other health traits [31].

Many of the identified immune response QTL have been identified for antibody responses because of the ease of measuring this trait, moderate heritability, and the availability of divergently selected lines to form appropriate resource populations for study. QTL have been associated with production of antibodies to SRBC [32,33], *Brucella abortus* antigen [32], and *E. coli* vaccine [34]. Also, many QTL associated with response to specific pathogens have been identified, including MDV [35,36], *Eimeria* [37], Newcastle disease virus (NDV) [38–40], avian influenza virus (AIV) [41], and *Salmonella* [42–47]. The results of independent studies have often revealed different marker–trait associations. These differences in detected QTLs may arise because of biological differences such as different QTL alleles existing in the tested populations, or because of technical issues such as marker spacing and coverage or the size of the tested population limiting the power of QTL detection.

Recently, interest has risen in the levels of natural antibodies of IgM and IgG isotypes, due to their association with survival and moderate heritability. Multiple SNPs in genes of interleukins *IL10*, *IL19*, *IL6*, *TRIM33*, and *HSP90AB1* were associated with natural antibody levels in layers [48]. Further studies showed that the genomic region containing Toll-like receptor (TLR) genes has a strong association with total IgM antibody levels [49], and that lines divergently selected for low and high feather pecking differed in IgM and IgG levels [50]. There is, however, a potential trade-off between increased immune response and some production traits [51].

A study by Zhang et al. (2015) reported 9 SNPs significantly and 30 suggestively associated across six immune traits (total serum IgY level, numbers of and the ratio of heterophils and lymphocytes, and antibody responses against AIV and SRBC) in Beijing-You chickens [52]. Five of the nine significant SNPs were located on chromosome 16, the microchromosome bearing the MHC region. Regions associated with primary and secondary response to SRBC measured by immunoglobulin M and Y and total Ig levels were found on chromosomes 3, 4, 19, 24, 25, and linkage group 64 in the regions of genes representing biological pathways, including antigen processing, immunodeficiency, phagosome, and MHC [53]. Knowledge of genomic regions associated with immune traits can increase understanding of the biology of these important traits. However, a long-term (39 generations) divergent selection for antibody response in chickens has shown differentiation in up to 20% of the genome between the low and high lines [54], which suggests that

immune response has a very complex genetic background.

10.3 Key gene loci for immunological traits

Although most immunological traits are polygenic, meaning controlled by multiple genetic loci (QTL), many individual genes and families of genes have major effects on immune responses and/or responses to pathogens in avian species. Identification of many immune system genes was initially hampered by their rapid evolutionary rate and limited genome sequence similarity to mammalian sequences [55], given that the chicken genome has several features that are unique in comparison to those of mammals [56]. However, analysis of large expressed sequence tag sets identified almost 200 putative immunity-related gene sequences [57]. Use of the genome sequence, especially in a species-comparative manner, contributed to the identification of chicken cytokines and their receptors [58]. For many of the genes identified through in silico (bioinformatic) methods, the task still remains to demonstrate their unique role(s) in avian immune responses through functional genomic analysis and immunological assessment.

The best known of the key gene loci for immunological traits in the chicken is the MHC, which was initially characterized as a blood group antigen locus [59] and thereafter was demonstrated to be the region containing the genes controlling tissue histocompatibility [60]. The chicken MHC represents the longest term application of marker-assisted selection (MAS) in animal production, with decades of commercial breeder selection in layer chickens for B blood group antigens linked to the MHC alleles that confer resistance to Marek's disease.

The avian MHC is described in detail in Chapter 7. The MHC of the chicken is considered to be the “minimal essential MHC” because of the compact and gene-dense nature of its genomic organization [61]. The plethora of immune-related genes that are located within the MHC may explain the varied and pleiotropic immune responses associated with this region, including those with many pathogens [62–64]. The high SNP frequency of the MHC-bearing chromosome 16, in comparison to the genome as a whole [11], is proposed to be a mechanism for the MHC genes to encode highly polymorphic proteins that allow a wide repertoire of antigen recognition and cellular interaction [65]. Comparative genomics with the MHC of other birds, such as the quail, lends insight into possible evolutionary MHC mechanisms and functions [66].

Cluster of differentiation (CD) antigens make up a large family of cell-surface proteins serving diverse roles in immune responsiveness, with most of them being

limited to expression on specific cell types and/or at differentiation stages. Characterization of two CD1 genes within the chicken MHC region suggests an evolutionary origin for them by extensive sequence divergence from classical MHC genes, while they are still genetically linked to the MHC [67].

The immunoglobulin (Ig) genes encode nonspecific and specific antibodies, which are crucial for humoral immune defense against many types of pathogens (see Chapter 5). Because antibodies are easily measured and highly heritable, they have long been the subject of immunogenetic studies which have clearly demonstrated the ability of genetic selection to alter the level, kinetics, and persistence of antibody production, as described earlier in this chapter. Genetic selection to modulate antibody production, which presumably acts primarily through genes that modulate the expression of Ig genes, can enhance poultry populations in many ways. These alterations can include more efficacious responses to vaccination and higher levels of specific antibodies, as well as greater affinity and/or more persistent production. It is also of interest to define the genetics controlling deposition of maternal antibodies into the egg, both for the protection of offspring via passive antibody (or, conversely, interference with vaccination) and for the identification of birds that will deposit large quantities of Ig into the egg for use of hens in production of recombinant proteins.

Cytokines, chemokines, and their receptors serve as an extensive and redundant system to ensure effective intercellular communication and coordination in the immune system. Most avian cytokine and chemokine genes were described only since 2000 [58,68] (see Chapter 9). Identification of the cytokine and cytokine receptor genes, and production of the recombinant proteins, opens up possibilities for genetically selecting breeding populations for more effective cytokine responses and for using cytokines as vaccine adjuvants [69] or as biomarkers for disease resistance [1,7,70]. Many studies have reported the association of cytokine expression levels with responses to diseases [71–74].

Additional genes of the innate immune response have been characterized in chickens. The TLRs serve as cell-surface receptors, with different TLR being relatively specific in binding various classes of antigens. TLR genes are described in detail in Chapter 8. The genomic region containing the TLR genes has been associated with IgM total antibody levels and with KLH-binding IgM natural antibodies [49]. The TLR expression levels are associated with response to pathogens [75,76]. The avian beta-defensins, or gallinacins, are small antimicrobial peptides that have a broad range of activity against Gram-negative and Gram-positive bacteria. Genetic variation in chicken beta-defensin genes has been associated with antibody titers to *Salmonella enteritidis* vaccine [77,78]. Although

it was once thought to be relatively passive, the innate immune system's importance as a first line of defense, and as a key component in the orchestration of an effective acquired immune response, is now more fully appreciated.

The Mx gene is of interest for its potential role in genetic resistance to AIV, based on activity against the virus in mice [79]. In transfected cell lines, a mutation at amino acid position 631 in the carboxy terminus of the chicken Mx gene is essential in determining antiviral specificity of the Mx protein against vesicular stomatitis virus [80]. Skewed frequencies of a favorable allele in native Chinese breeds, compared to commercial populations, suggest that this allele may respond to natural selection pressure from the environment [81]. Several Egyptian breeds and strains were found to have a high frequency of the resistant allele [82]. The Asn631 variant of chicken Mx1 was shown to be associated with reductions in mortality, morbidity, and viral shedding after high-path avian influenza infection in one study [83]. However, in another study, no associations were detected [84]. Thus the role of Mx in viral infection in birds does not appear to be as clear as it is in mice.

Many additional gene loci that are not currently identified as biological candidate genes for immune function in poultry are likely to be identified in the near future as studies expand to investigate genes in a global fashion and are not limited to the “known” immune function genes. These studies include transcriptomics, the use of genome-wide high-density SNP panels, and integrated use of multiple approaches [85,86].

10.4 Detecting quantitative trait loci

Over the decades, many experimental approaches have been developed for the use of anonymous genetic markers to identify chromosomal regions harboring genes (QTL) that control quantitative traits [87–89]. The same methods can also be used to identify genes or chromosomal regions that control immunological traits and disease resistance [90]. All methods rely on identifying statistical associations of genotypes at marker loci with phenotype—for example, by contrasting the mean phenotype of individuals that have alternate genotypes at the marker locus or at a set of neighboring marker loci. A difference in mean phenotype (e.g., pathology score or mortality) indicates that the marker is linked to a QTL or, in the ideal case, that it represents the causative mutation for it (a direct marker). However, this does not mean that every marker linked to a QTL is expected to show a mean difference in phenotype; besides linkage, the second condition needed to create a difference in mean phenotype between alternate marker genotypes is the presence of linkage disequilibrium (LD) between the marker and the

QTL. The concept of LD is important for both QTL detection and the use of QTL in genetic selection. This concept is explained in the following section.

10.4.1 Linkage disequilibrium

Consider a marker locus with alleles *M* and *m* and a linked QTL with alleles *Q* and *q*. The alleles at the two loci are arranged in haplotypes on the two chromosomes of a homologous pair that each individual carries. For example, an individual with genotype *MmQq* could have the following two haplotypes: *MQ/mq*, where the slash separates the two homologous chromosomes. Alternatively, it could have the marker–QTL linkage phases *Mq/mQ*. The arrangement of alleles in haplotypes is important because a progeny inherits one of the two haplotypes that a parent carries, barring recombination.

The presence of LD relates to the relative frequencies of alternative haplotypes in a population. In a population that is in linkage equilibrium (LE), alleles at two loci are randomly assorted into haplotypes; that is, chromosomes or haplotypes that carry marker allele *M* are no more likely to carry the QTL allele *Q* than are chromosomes that carry marker allele *m*. In this case, there is no value in knowing an individual's marker genotype because the marker genotype provides no information on the QTL genotype. If the marker and the QTL are in LD, however, there will be a difference in the probability of carrying *Q* between chromosomes that carry allele *M* versus allele *m*, and therefore we would also expect a difference in mean phenotype between individuals that have alternate marker genotypes. This situation makes the marker an effective predictor of the phenotype associated with the QTL.

Although several measures to quantify the amount of LD that exists between pairs of loci in a population have been developed, the most useful measure for biallelic markers is *r*-square [91]. This measure is defined as the square of the correlation between the alleles present on chromosomes at the two loci when alternate alleles at each locus are coded 0/1. Thus, similar to regression, *r*-square represents the proportion of variation at one locus (e.g., a QTL) that can be explained or predicted based on the allele that is present at the other locus (e.g., a genetic marker). This measure thereby quantifies a marker's ability to identify or predict variation present at a QTL.

The main factors that create LD in a population are mutation, selection, drift (inbreeding), and migration or crossing (e.g., Goddard and Meuwissen [92]). The main factor that breaks down LD is recombination, which can rearrange the haplotypes that exist within a parent during each meiosis. The rate at which the LD that exists between two loci decays depends on the rate of recombination between the loci—that is, on their genetic distance on the chromosome. For tightly linked loci, any LD

created will persist over many generations, but for loosely linked loci (recombination rate > 0.1), LD will decline rapidly over generations. Some regions of the genome—especially those containing similar genes in tandem, as is the case for several families of immune-related genes—are more prone to recombination. This is seen, for example, in the microchromosome that bears the MHC [10].

Although a marker and a linked QTL may not be in LD across the population, LD will always exist within an individual family, even between loosely linked loci. This fact can be effectively used to design and analyze studies to identify marker–QTL associations. Consider a double heterozygous sire with haplotypes *MQ/mq*. The genotype of this sire is identical to that of an *F*₁ resulting from a cross of inbred lines. This sire will produce four types of gametes: nonrecombinants *MQ* and *mq* and recombinants *Mq* and *mQ*. Because the nonrecombinants will have a higher frequency, depending on the recombination rate between *M* and *Q*, the sire will produce gametes for which these two loci will be in LD, which will extend over a large distance because it has undergone only one round of recombination. This specific type of LD, however, will exist only within this family; the gametes produced by an *Mq/mQ* sire will also show LD between these two loci but the LD will be in the opposite direction because of the different marker–QTL linkage phase. On the other hand, *MQ/mQ* and *Mq/mq* sire families will not be in LD because the QTL does not segregate in them. When pooled across families, these four LD types cancel each other out, resulting in these two loci being in LE across the population. Nevertheless, within-family LD can be used to detect QTL, provided the differences in linkage phase are considered in experimental design and statistical analyses.

10.4.2 Experimental designs to detect quantitative trait loci

Using the alternate types of LD just described, the main experimental strategies that have been used to detect QTL in domestic animal populations are summarized in Table 10.1 and are described further in the following sections (see also Refs. [87,93,94]).

Line or breed crosses

Crossing two breeds that differ in allele and, therefore, haplotype frequencies creates extensive LD in the *F*₁ generation. Much of this LD will still extend over large distances in the *F*₂ generation because it has undergone only one generation of recombination. This enables detection of QTL across the genome, which differ in frequency between the two breeds with only a limited number of markers spread over the genome (approximately every

TABLE 10.1 Strategies for quantitative trait loci (QTL) detection.

Population type	Line or breed crosses			Outbred population		
	F2 or backcross	Advanced intercross		Half- or full-sib families	Extended pedigree	Non-pedigreed population sample
Marker type	LD		LE		LD	
Genome coverage	Genome-wide		Genome-wide		Candidate gene regions	Genome-wide
Marker density	Sparse	Denser	Sparse	Denser	Few loci	Dense
LD used	Population-wide		Within-family		Population-wide	
Number of generations of recombination used for mapping	1	>1	1	>1	>> 1	
Extent of LD around QTL	Long	Smaller	Long	Smaller	Small	
Map resolution	Poor	Better	Poor	Better	High	

LD, linkage disequilibrium; LE, linkage equilibrium.

15–20 cM). This is the basis for the extensive use of F_2 or backcrosses between breeds or lines for QTL detection when microsatellites were the main source of genetic markers available [32,87,88,95]. The extensive LD that is present in an F_2 population enables the detection of QTL that are at some distance from the markers, but this also limits the accuracy with which the position of the QTL can be determined. The latter can be overcome by using advanced intercrosses (e.g., F_6 or higher). In such populations, the extent of LD at greater distances is eroded by repeated recombination, so LD will span much shorter distances around a QTL. Detection of QTL in advanced intercrosses requires a denser marker map but also enables more precise QTL positioning [96].

Within-family linkage disequilibrium in outbred populations

Because linkage phases between the marker and the QTL can differ from family to family, use of within-family LD for QTL detection requires marker effects to be fitted on a within-family basis, rather than across the population. Similar to F_2 or backcrosses, however, the extent of within-family LD is extensive and so genome-wide coverage is provided by a limited number of markers. However, similar to F_2 crosses, significant markers may be some distance from the QTL. Many examples of successful applications of this methodology for QTL detection for immune response, disease resistance, and other traits in poultry are available in the earlier QTL detection literature [97–100], as summarized in Abasht et al. [101] and available online with up-to-date information in the chicken QTLdb (<http://www.genome.iastate.edu/cgi-bin/QTLdb/GG/index>) [102].

Population-wide linkage disequilibrium in outbred populations

The amount and extent of LD that exists in populations that are used for research and/or for genetic improvement are the net result of all forces that create and break down LD. Thus the pattern of LD in these populations is the result of the breeding and selection history of the population, along with random sampling. On this basis, in populations that have been closed for many generations, loci are expected to be in LE, except for closely linked loci. Therefore in those populations, only markers that are tightly linked to QTL may show an association with phenotype. However, a pair of closely linked loci is not guaranteed to be in LD in closed populations because of the chance effects of random sampling. Such closed populations include noninbred lines kept for experimental studies at research institutions and lines used for commercial breeding.

Capitalizing on the short-range LD in these populations, there are two strategies to identify markers that are in population-wide LD with QTL in these populations:

- candidate gene analysis: evaluating markers that are in, or close to, genes that are hypothesized to be associated with phenotype for the trait of interest and
- GWAS: evaluating a large number of markers across the genome for association with the trait phenotype.

The candidate gene approach utilizes knowledge from species that are rich in genome information (e.g., human and mouse), from the effects of mutations in other species, from previously identified QTL regions, and/or from knowledge of the physiological basis of traits to identify genes likely to play a role in the trait's physiology [103]. After mapping and identification of polymorphisms, the association of genotype at the candidate gene with disease or immune phenotype can be estimated in a closed breeding population.

The GWAS approach has been made possible recently by the development and availability of high-density SNP panels, and it is now the method of choice for identifying QTL regions in outbred populations [89]. Currently available panels allow the evaluation of up to 650,000 SNPs across the chicken genome [12]. GWAS allow QTL regions to be identified directly in breeding populations of interest without requiring a specific family structure, because the search for associations of SNP with phenotype is based on LD expected to exist across the population between tightly linked loci. In fact, the preferred design for GWAS is to genotype a large number of individuals (at least 500) from the population with extensive phenotypic information on the traits of interest. This information can consist of not only the individual's own phenotype (e.g., Wolc et al. [104]) but also average progeny performance (e.g., survival of progeny after pathogen challenge). When resources for genotyping are limited, an efficient design is to genotype all males used for breeding and use average progeny performance as the phenotype in the association study.

10.5 Statistical procedures for quantitative trait loci detection

As previously mentioned, QTL detection is based on the identification of statistical associations between phenotype and the inheritance or presence of specific chromosomal regions or marker genotypes. Several statistical methods have been developed to detect QTL in the context of the experimental or population designs described earlier [88]. These include the following:

- least squares regression
- variance component mixed model approaches

- complex segregation analysis using maximum likelihood or Bayesian approaches
- whole-genome analysis using Bayesian approaches for genomic prediction [89,105]

The use of marker genotype information in these models includes:

- simply fitting marker genotypes as fixed effects in the model of analysis (e.g., Hassen et al. [106]);
- fitting probabilities of inheritance of specific parental alleles at putative positions on the chromosome for the QTL derived from marker data—as in least squares regression interval mapping [107];
- the use of marker genotypes to quantify covariances between putative QTL effects of pairs of individuals based on identity by descent probabilities derived from marker genotypes (e.g., George et al. [108]); and
- simultaneous fitting of all SNPs across the genome using random effects (e.g., Goddard and Hayes [89] and Meuwissen et al. [105]).

For GWAS, a common procedure is to evaluate each SNP separately by fitting the SNP genotype as a fixed effect in the model of analysis. Results are presented in so-called Manhattan plots [109] as the absolute value of the estimated effect for each SNP across SNP position in the genome. Alternatively, the negative of the logarithm of the p-value, or the proportion of variance explained by the SNP or by a window of neighboring SNP [104], can be plotted.

An important consideration in GWAS is population structure or population stratification [110]. Population stratification refers to the presence of subpopulations due to family structure, crossing, or migration. Differences in allele frequencies at SNPs and at QTL between these subpopulations can result in population-wide LD between loci that are not closely linked or even reside on different chromosomes. If unaccounted for in the model of analysis, population structure can lead to extensive false-positive associations of SNPs with phenotype. In populations with extensive pedigree information, as is the norm for populations of interest in commercial poultry breeding, a proper way to account for population structure is through the use of an animal model, which fits a random polygenic breeding value effect for individuals included in the analysis, with genetic relationships between animals quantified based on pedigree or genomics.

An alternative strategy for GWAS is to fit models developed for whole-genome breeding value prediction, also referred to as genomic prediction or genomic selection (GS) [105]. In these models, all SNPs are fitted simultaneously and their effects are fitted as random to allow for the number of SNPs fitted to be larger than the number of records included in the analysis. A range of

alternative models for genomic prediction have been developed [111,112], including so-called Bayesian variable selection models implemented with Monte Carlo Markov chain sampling. In the latter models, the effects of a certain proportion of SNPs are set to zero in each iteration of the chain, thereby allowing effects of SNPs that have strong associations with phenotype to be identified more clearly [113]. In contrast to single-SNP models, by fitting all SNPs simultaneously, whole-genome prediction models avoid the double counting of QTL effects when multiple SNPs within a genomic region are in LD with the QTL. However, this same phenomenon can result in the QTL effects being spread over multiple SNPs around the QTL in whole-genome prediction models. Thus identification of QTL regions for these models should be based on results across a region. A common statistic is the variance explained by a window of, say, 5–10 SNPs or by a 1-Mb region [104,114,115].

Genomic best linear unbiased prediction (GBLUP) is the standard model used for estimation of breeding values using high-density genotypes [116]. GBLUP is equivalent to the standard animal model method for the estimation of breeding values based on pedigree, but using a genomic relationship matrix derived from marker genotypes [117] rather than a pedigree-based relationship matrix. Although this method fits the breeding value of animals, rather than effects of individual markers, under certain assumptions for the distribution SNP effects, these methods are equivalent [118] and Sun et al. [119] showed how GBLUP can be used to back solve for marker effect and, therefore, for GWAS.

Further development of GBLUP called single-step GBLUP allows combining information from genotyped and nongenotyped animals for further improvement in accuracy of genomic prediction and GWAS [120]. Similarly, Bayesian variable selection models for genomic prediction and GWAS have been extended to allow the inclusion of information on nongenotyped individuals [121].

For most quantitative traits, the standard statistical methods based on a normal phenotype distribution can be used. However, many traits associated with disease do not follow a normal distribution but are recorded on a binary scale (e.g., the presence or absence of pathogen or pathology) or on a survival scale (e.g., age at mortality). Although linear models can in principle be used to analyze phenotypes that do not follow a normal distribution, more appropriate statistical methods are available for such analysis that can also be applied to the analysis of marker and phenotypic data for QTL detection [122]. Examples of such applications include survival analysis [95,123], logistic regression for disease incidence data [124], and Bayesian GS models [125].

10.6 Strategies to use molecular data in genetic selection

10.6.1 Marker-assisted selection

Traditional strategies for the use of molecular data for genetic improvement involve a two-step approach, leading to MAS [126]. In MAS, the first step is to use phenotypes and genotypes to identify genetic markers associated with the trait, employing the approaches described earlier. Then, in the second step, these “significant” markers are incorporated in genetic evaluation and selection procedures by estimating their effects on phenotype. The resulting estimates can be used to assign a “molecular score” to each selection candidate which can be used both for prediction of the genetic value of the individual and for selection. The constitution and method of quantification of the molecular score depends on the type of LD used and on how the marker will be used in selection [126]. Methods for incorporating marker data in genetic evaluation procedures are described in Fernando and Totir [127].

Based on the previously described QTL detection methods, three types of observable genetic loci in QTL detection and MAS can be distinguished, as described by Dekkers [128]:

- direct markers: loci that genotype the functional polymorphism for a QTL,
- LD markers: loci that are in population-wide LD with a QTL, and
- LE markers: loci that are in not in high population-wide LD with the functional mutation but that can be used on a within-family basis.

The three types of molecular loci differ not only in methods of detection but also in methods of application in genetic selection programs. Whereas direct and, to a lesser degree, LD markers allow selection on genotype across the population, LE markers must allow for different linkage phases between markers and QTL from family to family. In addition to a molecular score, individuals can obtain a regular estimate of the breeding value for the collective effect of all other genes (polygenes) that affect the trait. Thus, although the molecular score can be used for preselection of animals for further testing, at the time of selection, generally both molecular and phenotypic information is available. The following three selection strategies can then be distinguished:

- selection on the molecular score alone,
- tandem selection, with selection on molecular score followed by selection on phenotype-based genetic evaluation, and

- selection on an index of the molecular score and the regular phenotype-based evaluation.

Selection on molecular score alone ignores information that is available for all other genes (polygenes) that affect the trait, and it is expected to result in the lowest response to selection, unless the molecular score captures a large proportion of the genetic variance for the trait. Nevertheless, this strategy does not require additional phenotypes other than those needed to estimate marker effects, and it can be attractive when the phenotype is difficult or expensive to record, as is the case for disease and survival traits.

If both phenotypic and molecular information is available on selection candidates, index selection is expected to result in greater response than tandem selection. The reason for this is similar to why two-trait selection using independent culling levels is expected to give lower multiple-trait response than index selection: two-stage selection does not select individuals for which a low molecular score may be compensated for by a high phenotype-based evaluation.

The optimum choice of selection strategy (and other alternatives) also depends on factors such as market and cost. It is often difficult to estimate economic values to enhanced genetic resistance to disease because the extent of exposure of production population to pathogens may be unpredictable, and even asymptomatic levels of pathogens can reduce production efficiency. In addition, it is difficult to assign an economic value to the ethical aspects of improved animal health and well-being that result from genetic enhancement of disease resistance.

10.6.2 Whole-genome prediction

Despite a large number of reports of significant QTL and genetic markers associated with important traits, prior to 2010 the application of resulting QTL or markers in livestock breeding programs was limited. The main reasons for the limited use of molecular data prior to that time are described in Refs [128,129]. These include the limited amount of genetic variation that the significant markers explained, the limited number of genetic markers available and their relatively high genotyping costs, poor reproducibility of marker or QTL effects in populations of interest (particularly experimental discovery populations), and the need to estimate marker or QTL effects on a within-family basis, making it more difficult to incorporate them into breeding programs. However, by 2020, many of these limitations were lifted by advances in molecular technology, in particular genome sequencing, the identification of large numbers of SNPs across the genome, and cost-effective high-throughput genotyping of

tens of thousands of SNPs. The ability to effectively use this large number of SNPs for breeding value estimation, however, has required a paradigm shift in statistical models for estimation of SNP effects, which has taken us from MAS to GS [105].

In GS, often and more appropriately referred to as whole genomic prediction, the association of each SNP with phenotype is estimated using sophisticated statistical and quantitative genetics models without prescreening markers based on significance. The genomic estimated breeding value of an individual is then the sum of the estimated effect at each SNP summed across all SNPs [105]. This is in contrast to the two-step approach employed in traditional MAS, which uses only markers that are significant for estimation of breeding values. By using all markers across the genome, GS allows QTL with small effects to be included in breeding value estimation. Further, by fitting the effect of each SNP as random, estimates of the association of each SNP with phenotype are shrunk toward zero, depending on the amount of training data available and the prior distribution assumed for these effects [105].

During the past decade, many models that assume different priors for the distribution of marker effects have been developed and employed for GS [105,111,112]. However, in most applications, a model in which genetic variance is assumed to be equally distributed across all SNPs in the genome has been shown to result in an accuracy of estimated breeding values as high as that of more complex models [130]. This model is often referred to as GBLUP, as it has been shown to be equivalent to the standard animal model BLUP procedure that is extensively used to estimate breeding values in livestock [118] but with use of a genetic relationship matrix, based on marker genotypes rather than pedigree, to quantify how closely individuals are related [131]. GBLUP and its extension to enable inclusion of nongenotyped individuals greatly facilitates whole-genome prediction because of its technical similarity to pedigree-based animal model BLUP. Whole-genome prediction has been successfully implemented in many dairy cattle breeding programs, and other livestock species [132,133], including poultry [134].

One reason for the success of GBLUP is that many of the quantitative traits of interest are highly polygenic, with no or only a few SNPs or QTL with major effects. This may not be true for all traits of interest, however. If QTL with large effects are detected, there may be value in incorporating only those markers in selection programs through MAS.

A major challenge for GS is that large training data sets with up to several thousands of genotyped and phenotyped individuals are needed to obtain genomic estimated breeding values (GEBV) with substantial accuracy [89]. However, even with limited training sizes, genotypes

were shown to be partially predictive of outcomes of avian influenza and MDV infection [135,136]. The costs of genotyping can still be reduced substantially using low-density SNP panels to genotype selection candidates, combined with imputation on nongenotyped SNPs, as described by Habier et al. [137] and shown to provide good accuracy of imputed genotypes by Wang et al. [138]. This approach or the use of medium-density SNP chips (~60,000 markers) are now implemented in most GS strategies. There is also ongoing research on utilizing low-pass, whole-genome sequencing instead of SNP chips based on similar genotype imputation approaches. Strategies and issues associated with the use of GS in breeding programs are reviewed in Dekkers [139].

10.7 Systems biology

High-throughput technologies, especially next-generation sequencing, have been recently widely used to catalog genes, proteins, RNAs, genetic variations, and small molecules in many organisms, including farm animals. The tremendous data generated in the “omics” (henceforth used without quotation marks) era have generated many more questions than they have answered. Host immune responses are a function not only of the host but also of the pathogens, the environment, and host–pathogen interactions. This biological interaction consists of a complex set of integrated responses involving dynamic interplays of thousands of molecules. The complexity of the host–pathogen paradigm necessitates a systems biology approach, which provides comprehensive views of a biological system along with its components and their interactions, each of which is more than a simple sum of a series of the system’s discrete components [140,141]. With high-throughput approaches, the genome, transcriptome, proteome, microRNAome, epigenome, microbiome, and metabolome of biological systems in the omics era can all be analyzed. The genome, transcriptome, epigenome, proteome, and microRNAome are of particular interest within a particular physiological, developmental, or disease state.

Transcriptomics is one of the most powerful methods for understanding avian immunology on a genome-wide scale. In theory, the expression profile of an organism’s complete transcriptome can be evaluated, and by comparing a diseased state to a normal state, or the progress of an immune response over time, information on genes that play a role in the response can be obtained. The microarray was a major technology for profiling mRNA gene expression around the year 2000, until the development of next-generation sequencing technology around 2010. Recently, single-cell transcriptome analysis by RNA-seq has been developed to capture genetic information of

expressed transcripts in single cell [142] which represents the future of transcriptomics.

Many transcriptome studies have been reported on economically important poultry pathogens or food safety-related pathogens, including AIV [143,144], infectious bursal disease virus (IBDV) [145,146], NDV [147–152], MDV [153–155], Salmonella [156–160], Campylobacter [161–163], and *Eimeria tenella* [164–166], in the past decade. Two major questions have been addressed in these studies: which genes and signaling pathways or biological processes were associated with (1) host response to the pathogen and (2) with disease resistance to these pathogens in variety of immune-related tissues at different stages of infection. To address the first question, a general experiment design consisting of an infected group and noninfected group was used. For the second question, pathogen-specific relatively resistant and susceptible chicken lines from either experimental lines or commercial lines were utilized with both infected and noninfected groups.

Because mRNA expression does not always reflect expression at the protein level (posttranscriptional, translational, and protein degradation regulation are the major factors in cellular protein abundance), proteins that are expressed in a specific cell or tissue at given time point provide another perspective on host response to pathogen infection. In contrast to RNA-Seq for mRNA identification and quantification, the difficult nature of protein separation and solubilization, as well as the lack of comprehensive protein databases for mass spectrometry spectra, has limited wide application of high-throughput proteomics. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) are the primary methods for proteomic analysis. Both have been employed to identify cellular and MDV proteins and a predominant integrin and extracellular signal-regulated kinase/mitogen-activated protein kinase pathway in MDV-transformed cell lines [167,168]. Differentially expressed proteins associated with MDV infection have been revealed in chicken embryonic fibroblast cells [169–171], and in spleens of genetically MDV-resistant B21 and MDV-susceptible B19 lines of chickens [172–174]. Both genetic and epigenetic differences were detected in the thymus of chicken lines that differ for MDV susceptibility [175]. Proteins associated with virulence of infectious bronchitis virus (IBV) in chicken trachea and kidney [176], and with IBDV infection in bursa, were identified [177]. A chicken-specific peptide array focusing on kinase proteins has been developed [178,179] and has been used to interrogate immune function to Salmonella in chickens [180].

In the past decade, microRNAs (miRNAs) have been recognized as one of the essential regulators of gene

expression at the posttranscriptional level. miRNAs are small noncoding regulatory RNA molecules of about 19 to 25 nucleotides in length. When mature, they recognize their target mRNAs by base pairing their seed sequence (2–8 bp) with complementary nucleotides in the target mRNAs' 3'-UTR. Each miRNA has hundreds of evolutionarily conserved targets and maybe more nonconserved targets [180].

An estimated 30% of all human genes may be regulated by miRNAs [181,182]. Next-generation sequencing has been used to investigate miRNA signatures in response to chicken pathogen infection. Using RNA-seq, researchers have identified differentially expressed chicken miRNAs in lung and trachea induced by AIV [183,184], MDV [185–187], NDV [188], avian pathogenic *E. coli* [189], IBV [190], and necrotic enteritis [191,192]. Furthermore, specific miRNAs were functionally examined to assess their roles on the regulation of pathogenesis of IBV [193], and inhibition of tumorigenesis of MDV [194]. These studies have provided novel insights into the molecular mechanisms of host response to pathogen infection and have paved the way for new discoveries in avian immunology.

Recent studies have revealed that variations on cis-regulatory elements such as promoters and enhancers have significant contributions on phenotypic differences by regulating expression of genes associated with complex traits such as immune response. Efforts of the Functional Annotation of Animal Genomes (FAANG) on the identification and annotation of regulatory elements of chicken genome will form the foundation for identifying causative variants within regulatory regions of genes [195,196]. Epigenomic assays such as chromatin immunoprecipitation (ChIP)-seq targeting histone modification are one of the major methods to identify regulatory elements such as promoters and enhancers in chicken genome [197]. Significant differences in chromatin accessibility and gene expression are due to posttranslational histone modifications. Assays for histone H3 lysine 4 trimethylation (H3K4me3), H3 lysine 27 trimethylation (H3K27me3), H3 lysine 27 acetylation (H3K27ac), and H3 lysine 4 monomethylation (H3K4me1) are most informative of chromatin states [198]. An abundance of H3K4me3 correlates with the promoters of active genes [199], while increased levels of H3K27me3 are associated with promoters of inactive genes [200]. H3K27ac is a mark of active regulatory elements and may distinguish active enhancers and promoters from their inactive counterparts [201]. H3K4me1 is a mark of regulatory elements associated with enhancers and other distal elements, but also enriched downstream of transcription starts [199]. Differential H3K27me3 and H3K4me3 marks were observed in MD-resistant and MDS-susceptible inbred lines with MDV infection [202,203]. A study on the four

mentioned histone modification marks on host response to NDV infection under heat stress in two genetically distinct inbred chicken lines revealed significant epigenomic regulation due to the infection and between the two lines, which may shed novel insights on the understanding underlying mechanism of disease resistance to NDV infection in chickens [204]. It is expected that there will be a new wave of studies in the arena of epigenetics.

High-throughput omics analyses are not the end point of an experimental design but a conduit to developing testable hypotheses. Their inherent strength is that they generate unbiased results and frequently provide unexpected insights. Thus, although they are not a substitute for hypothesis-driven biological investigation, they are destined to become a prerequisite for much future research. Genes, genetic variations, proteins, or miRNAs identified by omics analysis as being important in the experimental paradigm under study should be further characterized in a variety of ways, including gene knock-down, knockout, knockin, and other functional assays. Ultimately, their roles can be evaluated or verified in knockout, knockin, siRNA assays, or transgenic animals. In addition, recent advances in clustered regularly interspaced short palindromic repeats (CRISPR) allow more systematic and genome-wide screening and validating of functions of genes and variants through CRISPR interference (CRISPRi) and gain-of-function CRISPR activation (CRISPRa) approach [205].

10.8 Transgenic animals

A transgenic animal is one in which there has been a deliberate modification of the genome. A foreign gene, termed the “transgene,” is introduced such that it will be transmitted through the germline and expressed either in every cell in the mature animal or in selected tissues or at selected times in development. The development of agriculturally relevant transgenic animals has the potential to accelerate conventional breeding programs for improvements in desirable production traits [206].

Relevant to avian immunology is the possibility of developing disease-resistant birds [207]. In theory, this can be accomplished in myriad ways. For example:

- By enhancing expression of genes that are fundamental to the innate immune response, birds might display resistance to pathogens.
- By targeting of key viral proteins of each virus, birds can potentially reduce virus shedding and prevent the spread of infection.
- By inactivating viral receptors, birds can become resistant to viral pathogens.

However, because many viral receptors have other physiological functions in the host, transgenic animals

with alterations in viral receptor activity should be carefully evaluated for any unintended consequences.

The technology for the development of transgenic chickens has lagged behind that for mammals, in large part because of differences in reproductive biology. In mammals, the single-cell oocyte is the starting place for the introduction of the transgene, and this ensures that after cell division and embryo formation, all subsequent cells will harbor the introduced gene. Because of the intricate processes involved in laying avian eggs, the single-cell chicken oocyte is not available for manipulation. An egg is certainly readily accessible after it is laid, but the embryo represents more than 50,000 cells at this stage. This vast difference between mammals and aves hampered progress until the development of retroviral vectors and especially transposon-based piggyBac and Tol2 [208,209], and the advancement of programmable site-specific nucleases such as transcription activator-like effector nuclease [210] and CRISPR [211] for the efficient introduction of transgenes and the establishment of transfectable chicken primordial germ cells (PGCs).

Expression of virally introduced transgenes has been improved through the use of lentiviral vectors. Lentiviruses are a complex retrovirus subfamily that can incorporate foreign DNA into both dividing and nondividing cells and produce high titers of viral particles [212]. Lentiviral vectors can accommodate up to 9000 nucleotides of foreign genes and produce stable and sustained gene expression. Based on high success rates in the production of transgenic mice, lentiviral vectors have been used successfully to create transgenic chickens [213]. Transgenic chickens suppressing influenza virus A were generated using an RNA expression cassette expressing a short-hairpin RNA molecule, decoy 5, that inhibits and blocks influenza virus polymerase and thus prevents virus replication although transgenic birds were still susceptible to direct infection of highly pathogenic AIV [214]. This suggests potential great promise in breeding disease-resistant chickens by permanently introducing transgenes into founder lines for subsequent mass production of a resistant production population.

The lentivirus system has also been refined for tissue-specific expression. Lillico et al. [215] used lentiviral vectors derived from equine infectious anemia virus to produce transgenic chickens expressing functional recombinant therapeutic protein, humanized ScFv-Fc mini-antibody (known as miR24), and human interferon- β -1a specifically in the oviduct of laying hens as a component of egg white. This approach is an important advance in that it will allow tissue-specific expression of desired transgenes or directed knockout of deleterious genes [216]. Transgene birds were developed expressing 3D8 scFv that has a hydrolytic activity against both DNA and RNA viruses, and significant lower viral load of low

pathogenic AIV in the cloaca and oropharynx was observed in the transgenic birds compared to nontransgenic birds [217]. Another study utilized an avian sarcoma-leukosis virus (replication-competent avian retrovirus, RCAS)-based gene transfer system to constitutively express chicken overexpressing interferon-stimulated gene 58 (ISG58) [218]. The ISG58 transgenic chickens were protected from challenge with low dose of both highly pathogenic AIV and NDV with reduced histopathological lesions and viral shedding; however, they were not protected from high dose of challenge from both AIV and NDV [218].

Unlike mammalian PGCs, chicken PGCs, the lineage-restricted stem cells for the germ cell population, can be propagated continuously *in vitro* without loss of germ cell properties; moreover, they have been proven highly resistant to genetic modification [219]. For these reasons, PGCs can serve as a unique stem cell model for generating genetically modified chickens. Still, despite stable electroporation or viral transduction to achieve transgenic birds, many obstacles exist for practical applications because of potential epigenetic silencing as well as safety issues related to retroviral vector use. A method of direct transfection of PGCs using combined Lipofectamine 2000 with Tol2 transposon has significantly reduced the time; however, germline transmission in G0 was not significantly increased [220]. The CRISPR/Cas9 system is more efficient, cheap, and simpler tool for gene manipulation by delivering Cas9 and gene-specific single-guide RNA. The CRISPR/Cas9 system has been widely used in chicken knockout experiments [221,222].

The high efficiency of germline transmission via CRISPR/Cas9 in chicken PGCs has demonstrated great potential in a large-scale mutational screen of gene function in the chicken, although a novel delivery technology of gesicles (derived nanovesicles) was recently developed to address the shortfalls of current delivery systems such as off-target effects and potential cell toxicity [223]. Such recent successes, especially in CRISPR/Cas9, in producing transgenic birds now mean that this technology is likely to become routinely effective in modifying the bird genome. Omics studies can lead to the identification of potentially important target transgenes. This technology opens new frontiers for poultry science with the potential of using chickens as bioreactors for “pharming,” elucidating functions of genes, and as models for disease-resistant breeding.

10.9 Future directions for systems biology in avian immunology

With the rapid development of techniques and the significant reduction in sequencing (especially next-generation

sequencing) cost, the tools for omics analysis are now readily available and we can expect a wave of omics studies in avian immunology in the near future. There are several technologies available for next-generation sequencing. Based on the method of sequencing and detection, they can be further classified as second-, third-, and now fourth-generation [224]. For example:

- For second-generation sequencing, Illumina (San Diego, CA) (NovaSeq, HiSeq, NextSeq, and MiSeq) uses reversible terminator sequencing by synthesis with a read length of 2X250 bp.
- MGI Tech (a subsidiary of the Beijing Genomics Institute Group) (Shenzhen, China) has developed new sequencer based on DNA nanoball technology (MGISEG-2000, MGISEQ-T7).
- Pacific Biosciences (Menlo Park, CA) has developed a third-generation sequencer using a real-time, single-molecule method with a read length of 5000–10,000 bp.
- Ion-Torrent (Life Technologies, Grand Island, NY) uses ion-sensitive field effect transistors to eliminate the need for optical detection of sequencing with a read length of 100–600 bp.
- Oxford Nanopore (Oxford, United Kingdom) offers a fundamentally different sequencing technology using Nanopore exonuclease and aims to deliver ultra-long read-length single-molecule sequence data (fourth-generation).

Each technology has its advantages and limitations in terms of cost per megabase, read length, run time, and error rate. Because of dramatic throughput increases and the development of multiplex labeling, the cost per sample becomes a major factor in choosing the platform, particularly for transcriptome, epigenome, and miRNAome analyses, in which multiple samples can be indexed into a single lane without sacrificing sufficient depth of coverage [225].

Another challenge for avian immunology research is to integrate omics data of both host and microbes to understand cellular processes at a global scale during host–microbe interplay. To date, most studies related to host–microbe interaction have been performed either in hosts or in microbes because of the complexity of the system and the difficulties of data set modeling. In addition, many such studies have focused only on a single microbial species, in which the interaction with other microbial species *in vivo* is virtually ignored. Future work needs to take the complexity of the multispecies environment into account [226,227]. Furthermore, a dynamic viewpoint of host response to microbial species is crucial in immunology. The immune system implements its response in stages: first, it responds to infectious agents by recognizing antigens that are unique to the pathogen (innate

immunity) but are absent from self (tolerance); then a secondary response gives rise to immunological memory (adaptive immunity). Data at multiple tissues, time points, or conditions must be integrated to conduct a more comprehensive and robust analysis.

Recent studies have revealed that host–microbe interaction is a complex network of interconnected pathways with activities related to many factors, especially posttranscriptional regulation by miRNAs and DNA methylation and posttranslational modification (e.g., phosphorylation, acetylation, and ubiquitination). To gain novel systems-level insights, a comprehensive integration of omics data in their proper biological context is needed. Such a systems biology approach will enable a further leap forward in our understanding of many aspects of host–microbe interaction that are presently not well characterized.

Finally, due to the complexity of immune system, which involves a large number of cell types with multiple cell lineages and states, phenotypic variations in the form of gene expression in a tissue could be significantly masked because of the nature of biologically cell heterogeneity. With the development of cell-specific surface markers, many immune cells can be distinguished by flow cytometry. However, not all immune cell types can be fully identified [228], especially for avian immunology with limited cell-specific surface markers available. Recent advances in single-cell RNA-sequencing (scRNA-seq) has allowed the interrogation of immune system at the individual cell level [229]. In addition, emerging development of whole-transcriptome spatial mapping at the single-cell resolution will make it possible to construct an atlas of cellular interaction in chicken tissues similar to that in humans [230]. There is no doubt that single-cell omics application in immunology will have unprecedented impact on our understanding of regulatory mechanisms of cellular immunity and virulence mechanisms of pathogens.

The integrated application of omics to the study of host–microbe interaction is still in its infancy. In the coming years, a fruitful outcome and eventually new insights are expected from multiple high-throughput technologies, especially in single-cell sequencing technology. However, each of these technologies requires specific expertise and is also costly and therefore often not feasible for individual research groups. A large consortium of researchers who can collaborate closely by sharing their expertise and knowledge and using their resources efficiently is a necessity to accelerate scientific discovery in avian immunology, such as developing the chicken immune atlas. The ultimate goal of systems biology is to model in silico an immune system that can predict the effects of a perturbation at both tissue and cell levels, such as pathogen infection, gene knockout, knockin, or other external stimuli. In the future, systems biology will

deepen our understanding of the avian immune system and provide the functional information needed to improve annotation and understanding of the role of immune function genes in biology.

Next-generation technologies have already revealed new system components and new regulatory mechanisms, and they have generated important hypotheses. Their use in avian immunology, however, is still limited. The vast quantities of data generated from next-generation sequencing as well as the complexity of the omics data pose significant challenges to bioinformatics systems because heterogeneous data sets at different levels and from both host and pathogen must be integrated for a comprehensive systems biology analysis. The success of systems biology analysis requires sophisticated data mining, deep curation, knowledge database integration, neural network modeling, artificial intelligence, and machine learning. The integration of discoveries from transcriptomics, epigenomics, proteomics, and transgenics, with assessment of structural variation in the genome and association with immunological traits, is expected to increase our fundamental understanding of the genetics of avian immunology. This research will also provide tools to enhance avian health by genetic selection of breeding stock, individual gene modification, and improved vaccines and immunomodulators.

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The mucosal immune system

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With very few exceptions (e.g., vertically transmitted pathogens such as *Salmonella pullorum* and exogenous avian leukosis virus), avian pathogens enter the host by breaching the barrier between the external and internal milieus. This barrier consists of specialized tissues characterized by two main components: an externally located epithelium and the underlying connective tissue. The nomenclature for the epithelium and connective tissues depends on the surface covered by the barrier. For instance, the epithelial tissue covering the external body surface is referred to as the epidermis and its underlying tissue the dermis; both together form the skin. Some of the immune protection mechanisms associated with the skin are described in Chapters 8.1 and 16. The surface layers involved with the digestive, respiratory, and reproductive tracts are referred to as epithelium that secretes mucus but also exerts numerous immunological functions [1], the underlying loose connective tissue as the lamina propria, and a thin layer of smooth muscle called the muscularis mucosa. The combination of epithelium and muscularis mucosa forms the mucosal membrane layer, which is discussed in Chapters 11.1–11.3.

The avian skin, including the feather pulp, is also an important part of the immune system (reviewed in [2]). Many viruses replicate in, or are associated with, the feather pulp and/or the feather follicle epithelium—for example, Marek's disease virus [3,4], avian leukosis virus [5], and chicken infectious anemia virus [6,7].

The ease of collecting repeated feather samples from the same bird and the importance of the feather pulp for several viruses makes the study of immune responses in the feather pulp highly attractive. Recently, the growing feather was established as an accessible test site to monitor immune responses to a range of compounds including cytokines, chemokines, and pathogen-associated molecular patterns [8,9]. A more detailed discussion of immunological responses in the feather pulp is provided in Chapter 14.

The anatomy of other mucosa-associated tissues including the eye-associated lymphoid tissue, the Harderian gland, and the conjunctiva of the lower eyelid are described in Chapter 2, whereas the bursa of Fabricius is discussed in detail in Chapters 2 and 4.

The combined mucosal surfaces of the gut, respiratory, and reproductive tracts represent by far the largest surface area in contact with the external milieu. They can also be considered the largest organ system in vertebrates, with each tract involved in diverse physiological functions such as digestion and gas exchange. These functions come along with the continuous movement of external substances—nutrients and air, respectively—and the need to transport or exchange essential molecules across the mucosal surface. Hence, there is a continual challenge from foreign materials and microorganisms, including pathogenic, which pass through the system.

To prevent the entry of pathogens through the mucosal tissues, a wide variety of protective mechanisms has evolved. These range from barrier functions (e.g., keratinized skin, ciliated cells in the trachea, mucus secretion, secreted immunoglobulins) to highly specialized immune cells (e.g., Langerhans cells in the skin) and organized lymphoid structures. In particular, the mucosal immune systems of the respiratory, gut, and reproductive tracts have highly organized lymphoid tissues such as bronchus-associated lymphoid tissues and gut-associated lymphoid tissue along with Peyer's patches, and cecal tonsils (Fig. 11.1).

In addition, the mucosal barrier harbors a wide range of immune cells, such as intraepithelial and lamina propria lymphocytes, macrophages, and dendritic cells. In mammalian species, the gut contains more lymphocytes than all secondary lymphoid tissues collectively. It is likely that this is also the case in avian species (reviewed in [10]), particularly since birds lack draining lymph nodes. Finally, the epithelial cells of the mucosal surfaces

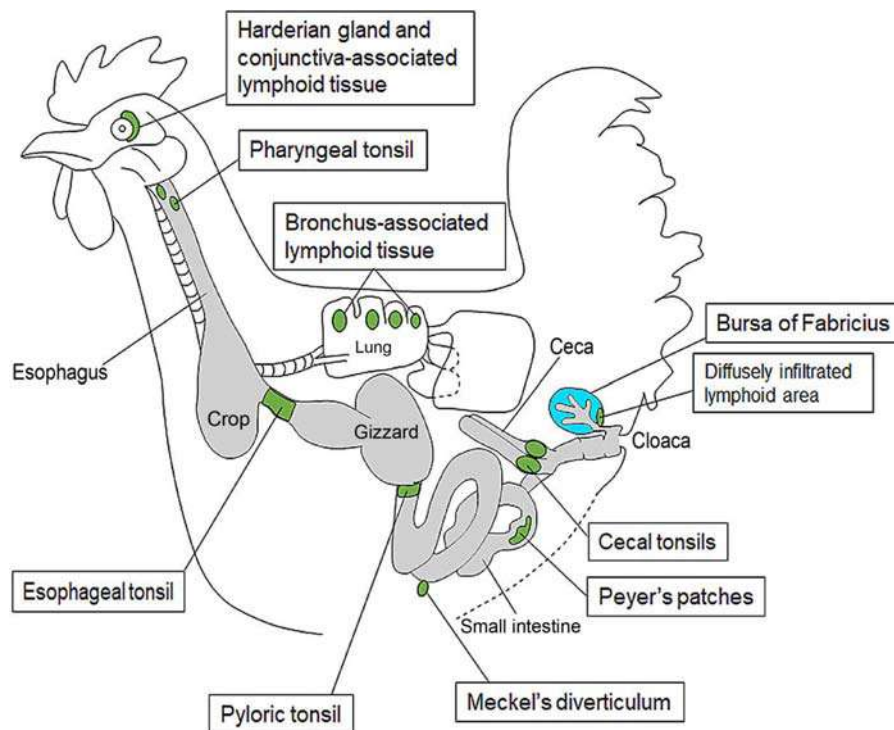


FIGURE 11.1 Location of organized lymphoid structures (text boxes) in mucosa-associated lymphoid tissues in the chicken. Illustration kindly provided by Dr. Nándor Nagy, Department of Anatomy, Histology and Embryology, Semmelweis University, Budapest, Hungary.

can sense pathogens and actively shape the response of the immune cells underneath [1].

For many years, research in mucosal immunology has focused on the question of how these cells become activated and interact to protect the host from invasion and dissemination of pathogenic microorganisms. Immunologists only recently began to appreciate that the gut harbors, and is in constant contact with, trillions of commensal microorganisms, which closely interact with the mucosal immune system [11]. This microbiome not only plays an important role in nutrient digestion; it is also a critical player in the development and function of the immune system and maintenance of intestinal integrity [12]. Current research on the avian microbiome and host–microbiome crosstalk is discussed in Chapter 12.

Under physiological conditions, the mucosal immune system must remain tolerant of the microbiome to avoid chronic mucosal inflammation while at the same time being able to respond quickly and appropriately to pathogenic challenges. Therefore, dysregulation of the crosstalk between the microbiome, the epithelium, and the mucosal immune system may result in local and systemic diseases. While research on epithelial barrier biology has been a major topic in mammalian immunology in recent years, comparative studies in birds are only now being initiated [13–15].

The mucosal immune systems have many features in common; however, the very special features of the digestive, respiratory, and reproductive tracts are far too diverse to be dealt with in a single chapter. Each is therefore described separately in the substantive chapters that follow.

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The avian enteric immune system in health and disease

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11.1.1 General considerations

The intestine is a structurally complex organ that has a primary role in the acquisition of nutrients and water, with physiologically and structurally distinct regions specializing in different digestive and/or absorptive functions. The digestive/absorptive functions of different parts of the intestine are outwith the remit of this chapter, but those interested in gut immune function will find it useful to become familiar with these aspects of intestinal physiology. The gut is a major site of development, residence, and portal of entry for pathogenic microorganisms into the deeper body tissues; therefore any perturbation of gut physiology often results in substantial clinical consequences. For this reason, an effective immune capability in the gut is essential to combat the plethora of pathogenic microorganisms that reside in, or pass through, this tissue.

Various aspects of gut physiology are important for limiting the establishment or growth of pathogens, including the acidity of the stomach and the activity of digestive enzymes or bile constituents. However, many microorganisms also use gut physiological features as triggers for development and maturation. For example, the excystation or hatching of protozoa and helminths from the transmission stages (oocysts, cysts, or eggs) is triggered by conditions such as temperature, short exposure to acidic environments, the physical disruptive capacity of the gizzard, and/or exposure to enzymes and bile components. Similarly, these environmental changes trigger physiological events in many bacterial species that facilitate their survival and colonization of the intestine.

Although the positive effects of the immune system in combating pathogens are often a focus for the infectious disease immunologist, the etiology of enteric disease is

often linked to inappropriate immune activity. Indeed, with many infections it is difficult to separate the physical damage associated with the direct action of the pathogen from the damage associated with the immune response. The intestine is highly sensitive to damage mediated by ongoing immune responses, and in many cases the immune response is the major cause of intestinal pathogenesis in mammals and birds (e.g., [1]). Classical signs of an ongoing immune response in the gut include increased leukocyte infiltration of the *lamina propria* and changes in gut structure such as villus atrophy and hyperplasia of enterocytes in the crypts of Lieberkühn.

One of the most important gut-specific features affecting immune function is the requirement not to respond to components of food or the “commensal” microorganisms that inhabit the gut. Indeed, the etiology of inflammatory bowel diseases in humans is a dysregulation of gut immune responses, especially those driven by the constituents of commensal organisms or food. Mechanistically, there are at least two groups of effects that contribute to the control of potentially harmful responses against nonpathogen-derived antigens in the gut; the first is a failure to respond because of a lack of exposure to a particular antigen (oral ignorance) and the second is due to some form of active prevention or modulation of a response (oral tolerance). The requirement not to respond to the majority of foreign material presented in the gut is a feature that marks this immune environment as unique.

In the following sections, we will deal with the structure and function of the gut immune system of birds with particular reference to development and function of the immune response against major groups of enteric pathogens (including viruses, bacteria, and parasites). Unfortunately, with many avian pathogens the immune responses induced by

infection and those that operate to kill the invading pathogen are not well defined. Hence, much of our fundamental knowledge has been gained from studies on a small group of selected pathogens that infect the chicken. The work on avian enteric pathogens has been dominated by studies on the enteric bacteria (principally *Salmonella enterica*) and with protozoan parasites of the genus *Eimeria*, which cause enteric coccidiosis. However, the increased prominence of avian influenza (AI) has stimulated more studies on immunity to enteric viral infections.

11.1.2 Gut structure and immune compartments

The structure and organization of intestinal immune tissue vary along the length of the gut, and most studies have focused on structures associated with the small and large intestine. The basic organization of immune compartments in the gut is given in Fig. 11.1.1. Essentially, the gut is a tubular structure enclosed by a single layer of polarized epithelial cells affixed to an extracellular matrix known as the basement membrane. The epithelial cells are held in tight juxtaposition by complex intercellular structures, collectively known as “tight junctions” (reviewed in [2]). To increase the functional surface area, many parts of the gut are convoluted to form protruding villus structures interspersed by indentations known as crypts. The villus/crypt unit is highly zonal in organization and enterocytes of many different types, or stages of differentiation, are found at distinct locations within the

villus or crypt. The proliferative zone lies within the crypt unit and the epithelial stem cells divide to form daughter cells that differentiate during migration along the villus structure or deeper into the base of the crypt. In an elegant set of studies using the mouse, it was shown that enterocytes derived from each crypt are responsible for feeding enterocytes into four villi, and each villus is formed from cells derived from four crypts [3]. The enterocytes derived from the crypts migrate along the villus structure in a spiral path and survive for between 48 and 96 hours (dependent upon the length of the villus and age of the chicken) before being lost by apoptosis from the villus tip [4–7]. In contrast to the simple crypt/villus structures found in the small intestine, the structures found in the chicken ceca are more complex. The villus-like outgrowths, or *plica circularis*, contain multiple folds that have a ruffled architecture, with active crypts found within each folded structure and at the base of the overall multifolded unit.

Interactions between pathogens and the intestinal tract may be initiated by interactions occurring at the most proximal sites associated with the mouth and buccal cavity; the small lymphoid aggregates in these tissues [8,9] may be important to prime responses at more distal sites in the gut as well as to perform a local protective function. The proventriculus (glandular stomach) and gizzard are sparsely populated with immune cells, and little information is available documenting the ability to mediate immune responses in these tissues. In contrast, numerous studies have examined the function of the immune system

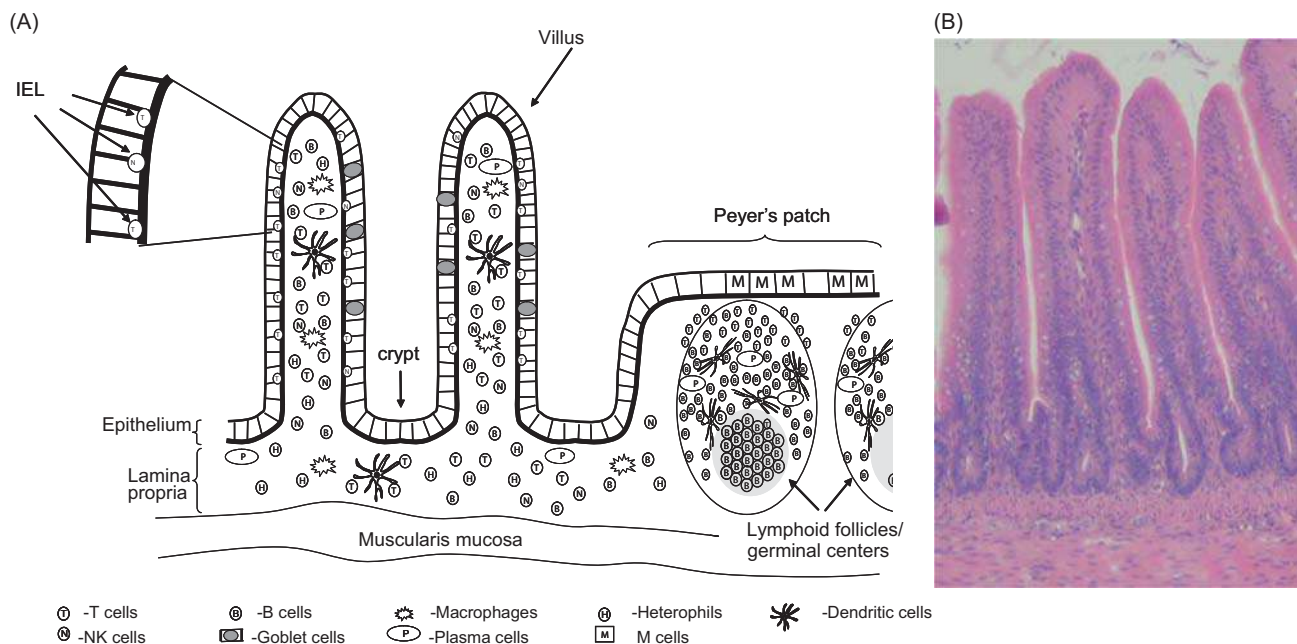


FIGURE 11.1.1 Schematic of the organization of immune cell compartments in the small intestine (A) and photomicrograph of the chicken ileum depicting intestinal villi and crypts (B). IEL, Intraepithelial lymphocyte; NK, natural killer.

in the small intestine (duodenum, jejunum, and ileum) and in the large intestine, including the ceca. It is important to note that the standing immune compartment and subsequent immune responses can be very different according to the region of the gut being examined. It is therefore inappropriate to extrapolate information gained from analyses of a specific region to other parts of the gut.

The villus/crypt structures in many regions of the intestine pose an additional constraint on the function of the immune system, increasing the physical area and complexity of the tissue. The gut-associated lymphoid tissues (GALT) form an organized series of structures that comprise part of the more extensive mucosa-associated lymphoid tissues (described in Chapter 2) that also include nasal, lung, and reproductive tract tissues (see other sections of Chapter 11). The homing of immune cells is governed by interactions between molecular addressins on tissue vessel walls (e.g., high endothelial venules) with immune cell-expressed integrins and adhesins as well as the ability of the immune cells to respond to particular chemokines and other chemoattractant molecules (described in Chapter 9). The nature of these interactions links different areas of the mucosal immune system, and cells stimulated in one mucosal site migrate through other mucosal sites. Although many of the important molecules have not been functionally identified in birds, the *Gallus gallus* genome sequence (http://www.ensembl.org/Gallus_gallus/Info/Index) is a key resource for those interested in the repertoire of processes that might operate in the chicken.

11.1.2.1 Chicken gut-associated lymphoid tissue structures

The GALT comprises lymphoid cells residing in the epithelial lining and distributed in the underlying lamina propria as well as specialized lymphoid structures located at strategic sites along the gut. The definable structures include lymphoid aggregates located within the lamina propria, Meckel's diverticulum, Peyer's patches (PP), and cecal tonsils (CT) (see Figure 11.1 and Figure 2.12 of the second edition of Avian Immunology).

The GALT is a key immunological system estimated to comprise more immune cells than any other tissue [10], with the associated structures forming a site to promote co-localization of the many immune cell types required to initiate and mediate immune function. There is also considerable cellular traffic between different gut immune structures and the systemic sites, including the bone marrow and spleen. Many of the organized GALT structures are sites of immune induction [11–13], providing conditions necessary to induce appropriate immune responses [e.g., immunoglobulin (Ig)A production by B cells].

Chickens lack structured lymph nodes, like those found in mammals, but have a number of distinct lymphoid aggregates that line the length of the gut (see Chapter 2). In their simplest form, these consist of a specialized epithelium containing microfold cells (M cells) that sample the gut lumen contents and deliver them to underlying macrophages and dendritic cells (DC) [14–17]. In close proximity to these cells, and with varying degrees of organization, are follicles rich in B and T lymphocytes. The chicken foregut is considered to have relatively little organized lymphoid tissue compared with the hindgut [18], although esophageal tonsils have been described at the junction of the esophagus and the proventriculus [9]. Up to eight tonsillar units have been described, each consisting of a crypt surrounded by dense lymphoid tissue organized into B and T cell regions. Organized lymphoid follicles are also located throughout the entire lamina propria of the proventriculus above the glandular units [11].

The unique avian lymphoid structure, Meckel's diverticulum, develops at the junction of the duodenum and jejunum in the young chick [19]. Although the function of this lymphoid tissue is not well defined, it is formed from the lining of the vitelline duct and has been suggested that it may be important for preventing pathogen transmission from the yolk sac in the newly hatched chick (discussed in Chapter 2). Several lymphoid structures with the characteristics of mammalian PP can be found scattered throughout the intestine [18] comprising a dome of specialized epithelium (M cells) overlaying structured follicles with clearly defined T and B cell areas. Although not evident at hatching, chicken PP become visible to the naked eye in the second week [18,20] and increase in number with age. The PP anterior to the ileocecal junction is the most prominent and is enlarged after many gut infections. Within the large intestinal region, the CTs are located in the neck region of each of the ceca and represent the most studied structures of the avian GALT. The CTs are large lymphoid aggregates, structurally similar to the PP, that contain multiple follicles and are overlaid by M cell-rich epithelium [17,21]. Structurally less well-defined, isolated lymphoid follicles are present throughout the small and large intestine, with greatest density in the apical region of the cecum [21–23]. The colon is less well endowed with lymphoid structures, although follicles have been observed in the proctodeal and urodeal regions of the cloaca [18] and are particularly abundant around the bursal duct [24].

The bursa of Fabricius, a lymphoepithelial outgrowth from the proctodeum, is one of the primary lymphoid organs of birds, primarily responsible for B lymphocyte development (reviewed in [25]). However, the antigen-sampling capacity of the bursal tissue is well established [26,27], and this, in addition to the presence of T cells, has led to the suggestion that the bursa could also play a role as a secondary lymphoid organ [24,28]. The process

of retrograde peristalsis, usually considered as a means of increasing water conservation from kidney secretions entering the cloaca, could also be the means by which bursal follicles sample hindgut bacteria [24] and cloacally applied antigens enter the bursa and induce specific B cell responses in the peripheral tissues [29] (this is described in Chapter 2). The influence of gut-derived antigens or products of the microflora that act as B cell mitogens in normal posthatch B cell development is considered elsewhere [25].

11.1.2.2 Cellular composition of the avian gut-associated lymphoid tissues

The avian GALT comprises a diverse set of cell subsets, distinct from that seen in systemic tissues, but includes representatives of each of the major cell populations found at other sites. Overall, the gut is populated with heterophils, macrophages, DC, and natural killer (NK) cells, as well as B and T lymphocytes, although the proportions of each cell type differ according to locality, microbial status, and age. Other factors that affect the composition and surface phenotype of the gut-associated immune cell populations include the natural intestinal microbiota, diet, host genetics, and exposure to pathogenic microorganisms. In addition to the cell populations found in distinct gut structures, the epithelial layers of the gut are populated with a highly specialized group of lymphocytes collectively known as the intraepithelial lymphocytes (IEL).

The composition of the small intestinal IEL population in a healthy adult bird includes major subsets of NK cells and T cells bearing the $\gamma\delta$ or $\alpha\beta$ form of the T cell receptor (TCR) [30,31]. In contrast to other tissues, B cells are almost entirely absent from the IEL population and the T cells predominantly express the CD8 co-receptor with smaller populations of TCR $\alpha\beta$ ⁺ CD4⁺ and CD4⁺CD8⁺ cells [32,33]. Moreover, within the CD8⁺ IEL population the majority express CD8 $\alpha\alpha$ homodimers rather than the CD8 $\alpha\beta$ heterodimer commonly expressed on classical CD8⁺ T cells found at systemic sites [32–34]. The proportions of IEL belonging to each subpopulation differ according to age, genetics, and environment (including infection). Very few heterophils are present in the epithelial compartment of healthy chickens but infection can lead to translocation of these cells into, and across, the epithelial barrier [13,35–37].

The *lamina propria* underlies the epithelial layer and is highly populated with a wide range of different leukocytes, including granulocytes, macrophages, DC, and lymphocytes of B and T cell lineages. Numerically, B and T cells are the most common lymphocytes (~90%), the remainder being of the NK cell phenotype (CD3[−] Bu-1[−]). In contrast to the IEL population, the T cell population of the *lamina*

propria contains a smaller proportion of $\gamma\delta$ -T cells (~10%); the much larger $\alpha\beta$ -T cell population is dominated by CD4⁺ T cells, with a less prominent CD8⁺ cell population. Many of the B cells in the *lamina propria* have undergone class switching to the secretory IgA isotype; IgA is present at high concentrations in intestinal fluids and bile (3.5–12 mg/mL) compared with IgM (<100 μ g/mL [38–40]). In serum, IgA is predominantly in monomeric form, whereas in secretions it is found in a polymeric configuration [39]. Directional transport of IgA across epithelia is mediated by secretory component interactions with the polymeric Ig receptor [41–44]. The cellular composition of the PP and the CT is essentially similar to that found in the *lamina propria*.

Chickens have three families of TCR $\alpha\beta$ ⁺ cells expressing either V β 1 (identified by the anti-TCR2 monoclonal antibody), referred to as the TCR $\alpha\beta$ 1 subfamily, or those expressing V β 2 (identified by anti-TCR3 monoclonal antibody), referred to as the TCR $\alpha\beta$ 2 subfamily as well as the more recently described but almost completely unstudied V β 3 [45] (see Chapter 6 for a focused discussion of avian T cell biology). Interestingly, TCR2⁺ cells predominate within the IEL compartment and are relatively abundant in the intestinal *lamina propria* (in contrast, both TCR2⁺ and TCR3⁺ populations are abundant in the spleen). Depletion of TCR2⁺ T cells led to a deficiency in IgA production in the gut but did not affect systemic IgM or IgY responses. This suggests that some functions of TCR $\alpha\beta$ ⁺ T cells may differ according to TCRV β usage [46,47]. A high proportion of the IEL in the chicken are TCR $\gamma\delta$ ⁺ (identified by the anti-TCR1 antibody), a feature also shared with mammalian IEL. In contrast to mouse and human (but in common with some other mammals such as cattle and pigs), high numbers of TCR $\gamma\delta$ ⁺ T cells are also found in the blood and spleen of chickens [48]. In the periphery, most TCR $\gamma\delta$ ⁺ T cells do not express either CD4 or CD8, whereas those in the gut are predominantly CD8⁺ [49]. While the biological functions of avian TCR $\gamma\delta$ ⁺ T cells remain unclear, *in vitro* studies have demonstrated that they produce cytokines and interferons, are capable of cytotoxic activity [50], and may also have immunoregulatory functions [51]. Purified chicken TCR $\gamma\delta$ ⁺ T cells respond poorly to stimulation with mitogens or TCR crosslinking [48,52]. TCR $\gamma\delta$ ⁺ T cell proliferation can be achieved by coculture with TCR $\alpha\beta$ ⁺ T cells [53] or by providing an exogenous source of interleukin-2 (IL-2) or other growth factors [10,54].

11.1.2.3 The enterocyte as part of an integrated gut immune system

The enterocytes are often overlooked when considering the function of the gut immune system. It is clear that these multifunctional epithelial cells play an important

role in the organization and function of the enteric immune system. Studies using murine systems have shown the importance of enterocyte-expressed chemokines in the recruitment and retention of IEL cell populations [55,56]. In a more direct manner, enterocytes form the physical barrier between the host and the intestinal contents. Specialized enterocytes, called goblet cells, are responsible for the production of mucus that forms a protective layer overlying the epithelial cells. Mucus is largely comprised of heavily glycosylated mucin proteins. Mucins can be divided into two distinct functional groups; those that are secreted (gel-forming) and those that are membrane-bound. Nine candidate mucin genes have been identified in the chicken; *MUC1*, *MUC2*, *MUC4*, *MUC5ac*, *MUC5b*, *MUC6*, *MUC13*, *MUC16*, and the bird-specific *OVOMUCIN* [57]. The gel-forming mucins (*MUC2*, *MUC5ac*, *MUC5b*, and *MUC6*) are located as a cluster on chicken chromosome 5, with *OVOMUCIN* lying in the center of the cluster [57]. Of these, *MUC2* is the major mucin produced in the large intestine of humans [58] or chickens (Powers and Smith, unpublished data). In mammals, *MUC2* forms two distinct layers, with the layer closest to the enterocytes devoid of bacteria and the outer layer containing populations of commensal bacteria [59]. Knockout mice, rendered deficient in *MUC2*, experience larger numbers of bacteria within intestinal crypts compared with intact mice [59], indicating a role for this mucin in maintaining the balance between the host and commensal microbes.

In mammals, the specialized enterocytes in the crypts of Lieberkuhn that contain prominent cytoplasmic granules are called Paneth cells [60]. Mammalian Paneth cells secrete lysozyme and phospholipase A₂, as well as the α - and β -defensins, which all have antimicrobial activity in vitro against bacteria [61], fungi [62,63], and some enveloped viruses [64,65]. Paneth cells are reported to be absent in ostriches [66] and we have been unable to visualize these cells in the chicken intestinal tract, although this is a site of defensin production (Powers et al., unpublished). A lysosome-containing rod-shaped enterocyte has been described in the chicken although it is dispersed throughout the intestinal villi rather than being located in the crypt [67].

The chicken defensin repertoire is restricted to β -defensins, which has led to the proposal that other defensin subfamilies (α and θ) evolved from duplication of ancestral β -defensin gene clusters [68–70]. Fourteen β -defensin genes have been identified in the chicken, located in a single cluster on chromosome 3, and these are called avian β -defensins (AvBD) [71]. The AvBD are produced by heterophils, macrophages, and at mucosal surfaces [68,72–74]; and the AvBD are active against a wide range of pathogens [72–77]. Defensins interact directly with pathogen membranes, leading to the

formation of pores that disrupt pathogen metabolism or structural integrity [78–81]. Although in-depth studies evaluating pore formation have not been performed with AvBD, these peptides are active against a wide range of pathogens [72–77].

The microfold or M cells represent a specialized enterocyte type that overlies many of the lymphoid follicles, PP, and CT and has a primary function of sampling the gut lumen and delivering antigens to classical antigen-presenting cells (see Chapter 8.1). Isolated M cells are also found dispersed through many areas of the intestinal epithelia.

Enterocytes can influence the induction of enteric immune responses by virtue of their expression of pattern recognition receptors (PRR), including the Toll-like receptors (TLR) and NOD-like receptors (see Chapter 8.6). Discrimination between molecular patterns (agonists) associated with pathogenic microorganisms and those found on “commensal” microorganisms is, at least in part, mediated by the polarization of PRR or their ability to respond to agonists (reviewed in [82]). Cultured chicken enterocytes responded to exposure with bacteria-derived PRR agonists by increased transcription of a variety of immune genes (e.g., [67]). These include inflammatory cytokines, avidin, lysozyme, and the polymeric immunoglobulin receptor (PIgR). The upregulation of PIgR is particularly interesting since this could represent a local inflammatory-driven increase in IgA transport into the lumen.

Enterocytes express major histocompatibility (MHC) class I molecules and can be induced to express MHC class II molecules (especially under inflammatory conditions). Hence, enterocytes can interact with both CD8⁺ and CD4⁺ TCR $\alpha\beta$ ⁺ T cells. Similarly, direct interactions between epithelial cells and TCR $\gamma\delta$ ⁺ T cells may be mediated by nonclassical MHC molecules, or by the expression of a variety of stress molecules at times of epithelial damage. Unfortunately, there is little evidence of these interactions in the chicken but studies in the mouse indicate a potential role in regulating epithelial homeostasis [83] and the response to enteric challenge [84,85].

11.1.3 Development of the enteric immune system

During the first 5 days after hatching extensive enterocyte proliferation leads to the formation of the basic structure of the gut (e.g., the crypt-villus unit in the small intestine [7,86]). Enterocytes also mature during this period, increasing in size and adopting a columnar phenotype with defined microvillus structures on the luminal face by 24 hours posthatching [24,86]. Differentiation of enterocytes into the mucus-producing goblet cells occurs prior

to hatching but their numbers increase rapidly in the post-hatch period [87]. Moreover, the mucin composition of goblet cells also changes during the early posthatch period with increasing amounts of neutral mucins (acidic mucins predominate prehatch). The stimulus for these changes is unknown but may involve the colonization of the gut with microbial flora and/or components of food. Indeed, chickens reared under germ-free conditions exhibit reduced numbers of goblet cells compared with those reared under conventional conditions (Powers et al., unpublished data).

Structurally, the chicken gut continues to develop for at least 3–4 weeks after hatching with increased complexity of intestinal epithelium (e.g., villus length), with more defined lamina propria, PP, and CT structures [6,24,32,88]. The size and cellular complexity of lamina propria and IEL compartments also increases with age [18,32,89] and is dependent upon the presence of enteric microflora [90,91] (Powers, Beal, and Smith unpublished data). The PP and CT are present at hatch but become more prominent with age ([18,32] and Chapter 2) as do smaller lymphoid aggregates that develop posthatch.

Changes in enterocyte turnover rates affect the structure of the intestine and these rates vary according to age. For example, the time taken for enterocytes to migrate along the length of the villus increases with age (and villus length) from 50 hours in 2-day-old chicks [4,92] to greater than 90 hours in the jejunum or ileum of 2- or 6-week-old birds [5,7]. One of the most dramatic responses to inflammation in the gut is the increased production of enterocytes in the crypt and the increased loss of enterocytes from the villus tip. This response results in shortened enterocyte life-span, crypt hyperplasia, and villus atrophy and represents an important mechanism for removal of infected or damaged enterocytes. In murine models of intestinal inflammation the epithelial cell response is dependent upon the activity of T cells [1].

Profound changes in the number and cellular composition of all gut-associated immune cell populations occur just prior to and shortly after hatching [18,32,89]. This is partly a consequence of the export of T cells from the thymus and B cells from the bursa. Dunon et al. [93] demonstrated that the process of thymic T cell export could be described as a series of three waves, each wave beginning with the export of TCR1^+ cells followed 2–3 days later by the export of $\text{TCR}\alpha\beta_1^+$ and $\text{TCR}\alpha\beta_2^+$ cells (described in Chapter 3). The first wave of $\text{TCR}\gamma\delta^+$ cells is exported to the periphery at 15–17 embryonic incubation days (EID) with TCR2^+ and TCR3^+ cells produced at 18–20 EID. A second wave of T cell export occurs around the time of hatch for $\text{TCR}\gamma\delta^+$ T cells and at 2–4 days posthatch for $\text{TCR}\alpha\beta^+$ T cells. A third wave of T cells is initiated at 6–8 days posthatch, again with $\text{TCR}\gamma\delta^+$ T cells preceding the $\text{TCR}\alpha\beta^+$ T cells by ~2 days. The export of B cells from the bursa begins at around 18 EID [94,95].

At hatch the intestinal tract is populated with few lymphoid cells [13] and within the epithelium the number of lymphocytes increases with age, with peak numbers present by approximately 8 weeks posthatch [34]. In studies by Bar-Shira et al. [12], measurements of the expression of specific mRNA produced by different cell subsets has been used to monitor age-related changes in lymphocyte populations in different parts of the gut. Large increases in CD3^+ cells were detected at 4 days posthatch in all parts of the gut. The number of B cells also increased significantly, initially in the small intestine (4 days posthatch) and then in the large intestine and CT (6 days posthatch). At hatch and shortly afterward the level of B cell-specific mRNA expression was greatest in the CT. These authors also suggested that GALT lymphocytes are functionally immature at hatch, as indicated by the levels of mRNA for IL-2 and interferon- γ (IFN- γ). Functional maturity of both B and T cells appears to occur in a biphasic manner, with the first stage occurring during the first week after hatching and the second stage during the second week [12]. Many of these changes are related to the colonization of the gut with microbial flora [91,96] and see Chapter 12). Further evidence of the impact of gut microbes on the gut immune system is evident from the repertoire of $\text{TCR}\beta_1$ and $\text{TCR}\beta_2$ in chickens reared under conventional or germ-free conditions [97]. Chickens reared under germ-free conditions contained polyclonal $\text{TCR}\alpha\beta^+$ T cell repertoires with no evidence for large clones of T cells. Those reared with a complex microflora displayed clonal expansions in $\text{TCR}\beta$ that were spatially restricted to regions within the intestine, whereas monocolonized chickens experienced clonal expansions that could be detected throughout the intestine. These data illustrate the relationship between the diversity of gut microflora and the complexity of T cell clonal expansions in the intestine. Moreover, these responses and changes in repertoire indicate that the host responds specifically to “commensal” microbes present in the gut (see Chapter 12) for a focused discussion of microbiome–immune interactions.

During the first 4 weeks after hatching there are also important changes in the proportions of different lymphocyte subsets within the different gut compartments. For example, there is a shift toward increased numbers of $\text{TCR}\gamma\delta^+$ cells in the IEL compartment and also an increase in the expression of $\text{CD8}\alpha$ [98]. In the lamina propria there are increases in the proportion of lymphocytes expressing $\text{TCR}\alpha\beta_1$ [98]. The lamina propria of 1-day-old chicks is poorly developed, containing little stroma (capillaries, lacteals, reticular, and muscle fibers) and few lymphocytes [99]. By 17 days after hatch the lamina propria has changed significantly with increases in stromal tissue, lymphocytes, mononuclear cells, and polymorphonuclear cells (e.g., heterophils).

Cells of the innate immune system develop earlier than T and B cells, with large numbers of heterophils and monocytes being present in the blood at hatch [100,101]. During the first 2 weeks after hatch there are further increases in polymorphonuclear cells in all parts of the intestine [13].

11.1.3.1 Development of immune responses to model antigens

Systemic immune responsiveness to immunization with bovine serum albumin differed following in ovo (16 or 18 EID) compared to posthatch administration (at 1, 7, or 12 days after hatching) [102]. Antigen-specific IgM and IgY were detected only in those chicks immunized 12 days after hatching, peaking at 7 (IgM) and 10 (IgY) days postimmunization. Chickens immunized at 7 and 10 days old were capable of an anamnestic response after a second immunization, however, those immunized at 1-day-old were not. These data suggest that young chicks (<1 week old) are not capable of mounting an effective antibody response to antigen. Similarly, oral administration of BSA or cloacal administration of haemocyanin only evoked a specific antibody response in chicks older than 8 days of age [12]. Other studies by Klipper et al. [103,104] demonstrated that orally administered antigens induced systemic tolerance in chicks less than 3 days of age. Klipper et al. [103] hypothesized that the presence of maternal antibodies at hatching may help prevent tolerance against antigens derived from pathogens. They suggested that maternal antibodies block interactions between the immune cells involved in generating tolerance and pathogen-derived antigens. The progeny of hens immunized with BSA were compared with those from nonimmunized hens and their capacity to produce BSA-specific antibodies was measured after oral immunization at days 1–6 posthatch with an oral boost at 2 weeks of age. Chicks with high levels of maternal anti-BSA antibodies produced high levels of endogenous anti-BSA antibodies after immunization, whereas the progeny of nonimmunized hens (with no specific maternal antibody) were tolerized by the oral immunization schedule at 1–6 days.

11.1.3.2 Immunity to enteric pathogens

The gut is a site constantly challenged by infectious agents, where a wide range of immune responses are induced and active against viral, bacterial, and parasitic pathogens. When considering the mechanisms of gut immunity it is important to consider the biology of the pathogens in relation to the host tissues. For example, some pathogens localize predominantly in the gut lumen and cause little damage (e.g., nematode, cestodes, and some enteric bacteria) but can persist for extended periods, while others reside in intracellular niches and can cause acute disease (e.g., *Eimeria* spp.). Other

pathogens use the intestine as a portal of entry into the tissues and, although residing in the gut for relatively short periods of time, the local mechanisms that limit entry into deeper tissues can be very important. Responses that control pathogens such as *S. enterica* serovars Gallinarum or Pullorum include those active at the gut and systemic sites. When considering the immune mechanisms that operate to limit infection in the gut there are general features that are relevant to many infections. First, there are constitutive barriers to infection (e.g., mucus layers), the composition of which may also change as a consequence of inflammation. To induce an inflammatory response the host first has to detect the presence of a pathogen (and to discriminate between pathogen and commensal) and many cell types in the gut express PRR including the TLR [105]. PRR expression is not restricted to immune cell types and the role of epithelial and stromal cells in pathogen detection should not be overlooked. Once alerted, a wide range of responses are induced in the gut with combined activities of innate and adaptive cell types resulting from infectious challenges.

The infiltration of heterophils often accompanies the early phase of infection in the gut (e.g., [36,37]) and these cells can both kill invading pathogens and contribute to recruitment of other immune cell types. Indeed, heterophils are well-equipped for pathogen detection expressing a wide spectrum of TLR [106]. Similarly, NK cells, macrophages, and TCR $\gamma\delta^+$ T cells often play a significant role early in infection, limiting establishment and spread of infection. Later during infection, TCR $\alpha\beta^+$ T cells and B cells are activated and infiltrate the lamina propria and IEL compartments; cell types of the adaptive immune system are often involved in the resolution of infection. However, infectious challenge often induces the activity of responses that are ineffective at controlling infection, and at times these can be damaging to the host's tissues. Unfortunately, detecting a response does not indicate effectiveness or involvement in controlling infection and "response" data should be interpreted with considerable care.

In the following sections we will describe some of the general concepts in gut immunity with reference to different pathogens in the chicken. As indicated above, our knowledge of immunity to enteric infection in the chicken is dominated by studies on a restricted range of pathogens, most notably the enteric *S. enterica* serovars and the protozoan parasites of the genus *Eimeria*. A more comprehensive understanding of the mechanisms of immunity in the gut will be supported by studies with other infections, including AI and the gut helminths.

11.1.3.3 Development of immunity to enteric pathogens

The gut is a dynamic immune compartment and substantial development occurs after hatching (as discussed

above), hence the mechanisms that operate to limit infection can be very different in young chicks compared to those in older chickens. The enteric immune system of the young chick is poorly developed and matures rapidly up to 4–6 weeks after hatching.

Perhaps the most dramatic example of age-dependent development of resistance is that seen with the enteric serovars of *S. enterica* (Enteritidis and Typhimurium). Infection of young chicks (<3 days of age) leads to severe systemic disease and high mortality, whilst infection of older chickens is largely restricted to the gut and associated with almost no clinical signs [107,108]. The dramatic difference in infection biology reflects the development of gut immune competence during the first week after hatching [7,86].

Salmonella enterica serovar Typhimurium has also been used to define more subtle changes in gut immune competence that occurs between 1 and 6 weeks after hatching. Oral challenge of chicks at 1, 3, or 6 weeks did not result in overt clinical signs but the time taken to clear enteric infection differed markedly [109]. Indeed, irrespective of the timing of challenge, all three groups of chicks cleared infection at about the same age, around 9–11 weeks, which suggests that mechanisms operating to clear enteric *Salmonella* develop over a considerable period of time. Interestingly, chicks inoculated at 1-week-old also had reduced antigen-specific antibody and splenic T cell proliferation compared with those infected at 3 or 6 weeks of age and were less well protected against rechallenge infection at 15 weeks. Moreover, the influence of host genetics on the time taken to clear infection in 6-week-old chickens was not evident with 10-day-old chicks, which had much weaker T cell, antibody, and cytokine responses [110]. These results have implications for transmission of salmonellosis to humans via contamination of meat from broiler chickens, and the likely success of vaccines applied to young chicks.

An age-dependent increase in resistance was also evident with reovirus infection that also correlated with the capacity to produce antigen-specific antibody responses [111]. Chicks infected on the day of hatch were incapable of producing significant levels of virus-specific IgA, contrasting with the more resistant 3-week-old chickens that produced a substantial IgA response. Age-dependent resistance is less evident in other infection models, for example, with the intracellular protozoan, *Eimeria tenella* [112].

11.1.3.4 Maternal antibody and protection of the young chick

The maternal/progeny relationship is important in the protection of young animals, particularly against endemic infections. With mammals the transfer of maternal

antibodies occurs by both transplacental and colostral routes but with birds all antibodies are transferred via the egg. Both IgM and IgA are secreted into the albumin and can be taken up by the developing chick but IgY is the major maternal antibody transferred via the egg yolk (see Chapter 5). High titers of specific IgY are deposited into the yolk of the developing egg and have a transient, but essential, role in protecting chicks from pathogens such as *Eimeria*, *Cryptosporidium*, *Salmonella*, and *Campylobacter* [113–117]. In challenge experiments, when the progeny of immunized, or recently infected, hens were compared with the progeny of control hens, the level of infection was reduced. The importance of maternal antibody declines with the age of the chick and is considered most important during the first 3 weeks of age. Transfer of protective antibody into the yolk requires high levels of circulating antibody in the hen and naturally occurring maternal protection is probably limited to those infections current in the environment at the time of lay. For example, the transitory nature of maternal protection was evident with *E. maxima* where significant protection occurred only in chicks obtained from the eggs laid between 17 and 30 days postinfection [113]. Nonetheless, the maternal protection route has been exploited in the control of coccidiosis [111] and a commercial vaccine (CoxAbic) has been developed, based upon the maternal transfer concept. The potential for exploiting maternal immunization is attractive in many ways, since large numbers of offspring can be protected by intensive immunization of a relatively few hens. Although this route of immunization deserves further exploitation, protection is transient and maternal transfer of antibody (either from natural ongoing infection or immunization) may also interfere with direct immunization of the chick.

11.1.4 Viral infections of the gut

Unfortunately, there is a paucity of data on the immune mechanisms that operate against enteric viral infections of chickens, although antigen-specific antibody is readily detected after infection [111,118–125]. For example, an antigen-specific IgA response can be detected after rotavirus infection but elimination of this response by embryonic bursectomy had little impact on the course of infection [124].

Microarray analysis of the lungs and spleen of chickens infected with AI H9N2 induced upregulation of a wide range of immune-associated mRNAs [126]. AI viruses replicate readily in the gut of chickens [127] and fecal contamination of the environment is likely to be important in transmission of infection. The relative importance of gut and lung tissues in the immunobiology of AI has not been established but gut immune responses induced by exposure to low pathogenicity viruses may be important in protecting chickens from the lethal effects of

high pathogenicity strains. The AI virus is a member of the *Orthomyxoviridae*, a family of enveloped, segmented, single-stranded RNA viruses that are subtyped according to their expression of serologically distinct hemagglutinin (HA) and neuraminidase (N) surface proteins (for general information see Refs. [128–130]). A wide variety of vaccine strategies has been used to protect chickens against AI. Many of these prevent the pathological consequences of infection but are less effective at preventing infection itself or, more importantly, transmission (e.g., Refs. [119–122,125,131–141]). The risks associated with partial protection with a vaccine (or by natural exposure to a low pathogenicity virus) occur at both the individual bird and at flock level. These risks include (1) the selection of immune escape variants and (2) environmental contamination with high pathogenicity virus, both of which can be devastating to the overall effectiveness of AI control strategies [141,142].

The activity of avian type I IFN in limiting viral replication is well-documented [143,144] and likely to be important in the very earliest stages of infection. Indeed, with many high pathogenicity strains an overwhelming infection of broad tissue specificity develops very rapidly and death of the chicken can occur within 3 days. The adaptation of high pathogenicity isolates to different avian species may be due in part to the ability of these viruses to evade innate immune mechanisms, including the activity of type I IFN [145,146].

Pathogen recognition is crucial to the host response to infection. Examination of the avian TLR repertoire revealed intact functional TLR3, TLR4, and TLR7 [all implicated in recognition of viral PAMPs (see Chapter 8.6 for detailed descriptions)] but that TLR8 is disrupted and entirely nonfunctional in galliform birds [147,148]. Although chTLR7 is functional, exposure of chicken splenocytes or the HD11 macrophage-like cell line to TLR7/8 agonists failed to reproducibly elevate production of type I IFN mRNA [147]. However, IFN activity was detected (using an antiviral assay) when S28828 (structurally related to R848) was administered orally to chickens, which may indicate the importance of specific cell types in the avian TLR7-induced IFN response [149]. In contrast to the TLR7 agonists, exposure of chicken splenocytes to the TLR3 agonist, synthetic double-stranded RNA (poly-inositol-cytosine) induced significant upregulation of IFN- α and IFN- β mRNA [147].

The activity of antibody against AIV is well established, with high-titer antibody responses detected against the HA and N proteins that inhibit viral entry into cultured cells [118–123,125]. The importance of anti-HA antibody-mediated immunity was evident by the abrogation of immunity (generated by an HA-vaccinia vaccine construct) after chemical bursectomy and the ability to passively transfer protective antibody to naïve recipients

[120]. Although antibody is clearly an important mechanism for limiting viral infection it is often insufficient to completely protect against viral replication. Indeed, in many immune responses to viruses both CD4⁺ and CD8⁺ TCR $\alpha\beta$ ⁺ T cells are important for controlling infections by mechanisms that include: help for B cell production of antibody, production of antiviral cytokines (e.g., type I and type II IFNs), and direct cytotoxicity of virus-infected cells. The importance of T cells has been best demonstrated with studies of heterologous immunity between H9N2 and H5N1 viruses [150,151]. Adoptive transfer of CD8⁺ T cells from H9N2-vaccinated birds protected recipient birds challenged with H5N1 virus in terms of survival and reduced viral load. By contrast, CD4⁺ T cells, B cells, or cells from naïve donor chickens were unable to confer immunity [150]. Using a depletion protocol with monoclonal antibodies TCR1 (TCR $\gamma\delta$), TCR2 (TCR $\alpha\beta$ 1), and TCR3 (TCR $\alpha\beta$ 2) and immunization with H9N2, only the anti-TCR2 treatment resulted in an exacerbated infection after challenge with H5N1 [151]. Similarly, anti-CD3 or CD8 treatment, but not anti-CD4 treatment, exacerbated challenge infection [151], and IFN- γ -containing CD8⁺ T cells were detected in the spleen and lungs of the H9N2-immunized birds. The impact of the high pathogenicity AI virus H5N1 has stimulated considerable interest in understanding the immune response to AI infections in the chicken. We look forward to the impact of these studies on fundamental avian gut immunology.

11.1.5 Bacterial infections of the gut

11.1.5.1 Salmonella

The outcome of infection with *S. enterica* differs according to serovar, chicken age, history of exposure to salmonellae, and, to a lesser degree, chicken genotype. Challenge with the highly host-restricted serovars, Gallinarum and Pullorum, causes more typhoid-like diseases in chickens of any age and with considerable mortality. In contrast, infection with broad host range serovars, Typhimurium and Enteritidis, results in a long-lived infection of the gut (with little systemic infection) in chickens greater than 2–3 days of age. However, with young chickens *S. Typhimurium* and *S. Enteritidis* cause substantial mortality with high numbers of bacteria replicating in the systemic organs.

Both *S. Gallinarum* and *Pullorum* are aflagellate and thought to enter systemic sites via enterocytes and lymphatic tissues such as the PP and CT [152]. Once they cross the gut these serotypes are carried to systemic sites, probably within cells of the reticuloendothelial system, and can then be isolated from sites such as the liver, spleen, ovaries, and bone marrow. Very little

inflammation occurs in the gut after infection with *S. Pullorum*, where there is little heterophil infiltration [35]. Immune responses to these serovars are characterized by high titers of specific antibodies and T cell proliferation [153]. Those chickens that survive the acute phase of *S. Pullorum* infection remain persistently infected with low numbers of salmonellae present in splenic macrophages [154]. Persistence of *S. Pullorum* in chickens may be related to an ability to divert chicken CD4+ T cell responses from a Th1/Th17 type response toward a Th2-type response perhaps by manipulating the infected macrophage [155]. Hormonal changes at point of lay may lead to a substantial transient depression in immune responsiveness and recrudescence of infection [153].

Differential resistance/susceptibility of chicken lines to systemic salmonellosis has allowed identification of host loci associated with disease outcome. The *SALI* locus was mapped to chicken chromosome 5 and is thought to be involved in survival of salmonellae within macrophages [154,156]. The *SALI* locus has been refined to encompass only 14 genes and a microRNA [157]. Additional candidate loci involved in resistance to systemic salmonellosis in the chicken include regions containing the MHC, *Nramp1*, and TLR4 [156–160].

In contrast, genetic resistance of chickens to enteric colonization with *S. Typhimurium* and *S. Enteritidis* does not associate with the *SALI* or MHC loci [161]. Some loci have been associated with reduced numbers of *Salmonella* in the ceca (resistance) and include the genes *NRAMP-1*, *TGF- β 2*, *TGF- β 4*, *IAP1*, *PSAP*, *CASPI*, *iNOS*, and *IL-2* [162,163].

Evidence for acquired immunity to challenge with *S. Gallinarum* led to the development of a live-attenuated vaccine strain known as 9R, which is used to control fowl typhoid in countries where biosecurity measures have failed to control disease. A recent report has indicated that cross-serovar protection may occur, since an attenuated live *S. Enteritidis* vaccine TAD *Salmonella* Vac E (Lohmann Animal Health) was also reported to protect against virulent *S. Gallinarum* challenge [164].

Enteric salmonellosis is caused by colonization of the chicken gut with serovars such as *S. Typhimurium* and *S. Enteritidis*. The drive to control enteric salmonellosis is because of the ability of these serovars to infect and cause disease in humans as a food-borne zoonosis following contamination of chicken meat products and eggs. In chicks aged more than a few days old these serovars colonize the gastrointestinal tract (predominantly the ileum and cecum), where they can persist for many weeks, with few bacteria invading the epithelium and entering systemic sites such as the spleen and liver [165]. Indeed, although there is an early, transient inflammatory reaction in the gut, lesions are microscopic and clinical disease is only associated with infection of chicks less than 3 days

old. Age-associated changes in disease susceptibility are well defined for the enteric *Salmonella* serovars (discussed in detail above) and probably reflect the development of gut immune competence. Such changes first reduce bacterial translocation and restrict the majority of salmonellae to the gut with the rate of clearance from the gut increasing in older birds. Contamination of eggs with *S. Enteritidis* is thought to be associated with its prolonged survival in systemic sites, translocation to the oviduct occurring around the onset of lay, which may be associated with transient immunosuppression as reported for *S. Pullorum* [153].

In the gut, early host responses to infection include a localized inflammatory response with an associated influx of heterophils [166]. Interestingly, a role for TLR5-flagellin interactions in restricting *S. Typhimurium* to the gut lumen was proposed after more rapid translocation to systemic sites was observed with an aflagellate *flhM* *S. Typhimurium* mutant [167]. Despite greater uptake, the aflagellate salmonellae induced much less IL-1 β and IL-6 in the gut and a reduced heterophil infiltrate [167]. A wide range of cell types (heterophils, macrophages, B cells, fibroblasts, and epithelial cells) respond strongly to exposure to live *Salmonella* or products of this bacterium including the well-defined TLR agonists LPS, flagellin, and CpG-containing DNA [106,168–171]. Live *Salmonella* readily invade cultured heterophils and macrophages but are then killed by these cells, a response enhanced by prior exposure of cells to pro-inflammatory cytokines such as IFN- γ or IL-2 [172,173]. The level of responsiveness of heterophils and macrophages to *Salmonella* or their extracts correlates with the level of susceptibility of chickens and this has led to the proposal that these cells are, in some way, involved in protection [169,174,175].

Chickens infected with enteric *Salmonella* serovars mount a substantial immune response typified by high levels of antigen-specific antibodies (IgM, IgY, and IgA) (reviewed in [176]), strong T cell responses [109,110], and increases in expression of mRNA in the spleen and gut encoding an array of cytokines and chemokines [109,110,177]. Evidence for an effective host response includes the age-dependent ability to clear the *Salmonella* from the gut and the more rapid clearance after secondary challenge [109,178,179]. The timing of clearance in 6-week-old chickens correlates with the peak antibody and T cell responses and, considering the luminal location of the *Salmonella*, the specific IgA response is a prime candidate effector mechanism. However, the role of B cells and antibodies in protecting the chicken against enteric *Salmonella* infections is less clear-cut. Desmidt et al. [180] rendered chickens B cell deficient by a combination of cyclophosphamide and testosterone propionate treatments and concluded that the prolonged persistence of *S.*

Enteritidis in these birds indicated a prominent role for B cells in the clearance phenotype. More recent studies, comparing different methods of producing B cell-deficient chickens, demonstrated that surgical bursectomy at EID17 had no effect on the capacity of chickens to clear either a primary or secondary *S. Typhimurium* infection [181]. By contrast, chickens treated with cyclophosphamide posthatch (usually termed chemical bursectomy) and challenged at 6 or 15 weeks of age took longer to clear a primary infection. These data suggest that a cyclophosphamide-sensitive (and nonrecoverable), non-B cell mechanism is responsible for clearance of the primary infection. Extensive literature on cyclophosphamide-treated chickens indicates a transient suppression of non-B cell compartments with recovery by ~4 weeks post-treatment [95,182–190]. These data with *Salmonella* suggest one or more, as yet unidentified, mechanism(s) is involved, and important for mediating clearance. Interestingly, both surgically bursectomised chickens and cyclophosphamide-treated chickens were capable of clearing a secondary infection at the same rate as intact chickens. These data suggest that either different non-B cell mechanisms are involved in the more rapid clearance evident after secondary challenge or that primary infection stimulates the rescue of an effector mechanism in cyclophosphamide-treated chickens. The lack of a requirement for B cells and antibody in enteric salmonellosis must be considered surprising and highlights the need for careful interpretation of the role for immune mechanisms, based upon a detected response.

The role of T cell responses in clearance of enteric *Salmonella* infections has not been proven but, in the absence of an essential role for B cells and with faster clearance of infection at secondary challenge (evidence for immune memory), these responses are likely to be important. Cellular responses induced by infection include antigen-specific delayed-type hypersensitivity responses and changes in the distribution of T and B cells, antigen-specific T cell proliferation, and T-dependent class switching in B cells [109,110,179,191,192]. Strong cellular and humoral immune responses correlated temporally with clearance of *S. Typhimurium* from the gut following primary infection, although these responses were less intense following rechallenge [109,179]. Immediately prior to clearance of primary infection significant increases in mRNA encoding IL-1 β , IFN- γ , and TGF- β were observed. The global gene expression profiles of the gut in two lines of chicken with differential susceptibility to infection revealed upregulation of a wide range of mRNAs, in particular, some suggesting pronounced T cell infiltration or activation [193]. Chickens have relatively high numbers of TCR $\gamma\delta^+$ T cells and these clearly respond after oral infection with *S. Enteritidis*, especially with those expressing CD8 (either as a CD8 $\alpha\alpha$

homodimer or CD8 $\alpha\beta$ heterodimers) [194]. A range of responses were assessed using inbred chicken lines that differed in susceptibility to infection at 6 weeks of age but not at 10 days old; only the level of antigen-specific T cell response correlated with the rate of clearance in both [110]. Various vaccination strategies have been employed with *S. Enteritidis* and *Typhimurium*, including administration of heat-killed and various live attenuated strains (reviewed in [195]). Although successful, the level of immunity does not match with that induced by previous exposure to wild-type salmonellae [178]. Priming with either *S. Typhimurium* or *S. Enteritidis* activates a strong cross-reactive T and B cell response and considerable protection to challenge with either serovar [196] suggesting conservation of protective antigens.

11.1.5.2 *Campylobacter*

Campylobacter spp. are currently the most common causative agents of bacterial human food-borne disease (particularly *C. jejuni*); however, in the chicken they do not generally cause significant disease [197]. Morphologically, *Campylobacter* spp. are Gram-negative rods, which are curved, spiral, or “S” shaped, with uni- or bi-polar flagella making them highly motile. *Campylobacter* are microaerophilic and thermophilic bacteria that colonize the mucus layer, most commonly in the cecum, where they can be found at up to 10⁹ cfu/g [197–200]. Although *C. jejuni* are predominantly found in the gut of chickens, small numbers have been isolated from other tissues [201,202]. Natural infection of chickens is rarely detectable by culture methods before about 3 weeks of age (and often much later in the production period), concurrent with reducing amounts of maternal antibody [117]. Transmission between chickens is thought to occur horizontally, with little or no vertical transfer [203]. Recent research using molecular methods has demonstrated that *Campylobacter* is present in very young, broiler chicks, a finding which may necessitate reconsideration of the routes of entry into broiler flocks [204,205]. With most strains of *Campylobacter* colonization is long-lived, although there are differences in the level of colonization associated with host genetics [206].

A few studies have examined the host response to *Campylobacter* infection in the chicken; of these most have concentrated on examining resistance, vaccination using inactivated cultures, or by measuring *Campylobacter*-specific antibody production. Vaccination with formalin-inactivated *C. jejuni* afforded a modest degree of success in experimental trials [207] but the small reductions in bacterial load in the ceca or the small increase in specific antibody were not statistically significant compared with the unvaccinated controls. A small number of more defined targets have been tested as subunit vaccines (reviewed in [208]) including some

delivered using *Salmonella* or *Eimeria* as live vaccine vectors [209–211]. The fact that some of these reduce the numbers of *Campylobacter* in the intestine indicates that immunity can operate against this infection. Significant increases in antigen-specific IgA antibodies were detected in the gut and serum following infection with *C. jejuni* [198,212,213]. Significant responses were directed against the flagellin and outer membrane proteins as revealed by Western Blot analysis. Biliary antibodies were purified and when added to the inoculating dose reduced the ability to initiate colonization [213] indicating that IgA can have a detrimental effect on *C. jejuni*. The production of high levels of antibody indicates that the host is responding to infection and therefore must be inducing a pro-inflammatory response greater than that expected from commensal organisms.

Exposure of cultured chick kidney cells or the macrophage-like cell line HD11 to *C. jejuni* led to increased levels of nitric oxide and proinflammatory cytokine production [214]. Infection of 1-day-old or 14-day-old chicks with *C. jejuni* led to increased expression of a range of pro-inflammatory chemokines, IL1 β and IL6 mRNA [215]. The changes were more marked in 14-day-old chicks and were associated with an increased number of heterophils in the intestinal tissue [215]. Differential expression of mRNA encoding a range of inflammatory mediators was associated with differences in the level of colonization of chickens at 48 hours postchallenge [216].

At a functional level, treatment of birds with cyclophosphamide (with a schedule designed for chemical bursectomy and challenge at 21 days old; see discussion of this treatment in Section 11-1.5.1) did have some effect on *C. jejuni* infection dynamics, particularly on the time taken to clear infection [217]. Hence it is likely that antibody and B cells or a cyclophosphamide-sensitive nonrecoverable element of the immune response can play some role during *Campylobacter* infections.

11.1.5.3 Necrotic enteritis

Necrotic enteritis (NE) lesions in chickens are often associated with *Clostridium perfringens*, a bacterium naturally found in the gut of 75%–90% of poultry. High clostridial load does not directly correlate with NE incidence and disease is influenced by factors such as coinfection, intestinal pH, and diet [218]. Pathogens that damage the small intestine, such as *E. maxima* and *E. acervulina*, are thought to provide an environment suitable for differentiation and proliferation of *C. perfringens* [219]. *Clostridium perfringens* is a Gram-positive anaerobic bacterium that can produce toxins and enzymes that cause enteric lesions. The strains of *C. perfringens* can be classified by the toxins that they produce into five groups (A–E). Only strains A and C are associated with disease in the chicken [220]. The α -toxin (produced by

A and C strains) is a phospholipase C sphingomyelinase that hydrolyzes phospholipids leading to disruption of the host cell's membrane and stimulation of the arachidonic cascade which stimulates production of inflammatory mediators [221]. The precise mechanism of action of the β -toxins (produced by strain C) is still unknown although they are also thought to disrupt cell membranes.

Earlier studies successfully experimentally induced acute NE by infecting chickens with *Eimeria* and then either providing them with feed contaminated with *C. perfringens* [222] or infecting them with *C. perfringens* broth cultures [223]. Edema, dilation of the surrounding blood vessels, and desquamation of epithelial cells in the gut mucosa occurred rapidly [224]. Within 3 hours the mucosa turned greyish and became thickened, a marked edema and a detachment of the epithelial layer from the lamina propria were apparent. Necrosis of the epithelial layer and lamina propria was evident by 5 hours, with congested blood vessels and an infiltration of both mononuclear cells and heterophils. By 8–12 hours there was massive necrosis of the villi with necrotic zones reaching down to the crypts and changes in the liver, heart, kidney, and bursa.

The subclinical form of the disease can be initiated experimentally by infecting birds with *C. perfringens* cultures that have been washed and resuspended in PBS [225]. By comparison, infecting chickens with either bacteria isolated from broth or the broth itself usually results in the acute form of disease. The subclinical disease usually affects the gut only in localized foci and may be associated with hepatitis and cholangiohepatitis [226].

Very few studies have examined the host immune response or the capacity to vaccinate against disease, probably due to the difficulty in reproducing the disease under experimental conditions. However, a recent study conducted by Thompson et al. [227] investigated the use of wild-type and attenuated *C. perfringens* to protect against a wild-type rechallenge. Chickens immunized with either wild-type virulent strains or strains that were deficient in the α -toxin had lower mean lesion scores following a wild-type challenge than the unvaccinated controls. Whilst these results demonstrate the capacity for chickens to mount immune responses that protect against NE, the components underpinning the protective immune response are still not understood.

11.1.6 Parasitic infections of the gut

A wide range of protozoan and helminth parasites infect the gut of the chicken, the most important of these for the poultry industry being the intracellular protozoan parasites of the genus *Eimeria*. Many poultry production practices have limited the impact of other parasitic diseases but with increased free-range farming these are likely to become more prevalent.

11.1.6.1 *Eimeria* spp

Infection with the eimerian parasites causes enteric coccidiosis which results in a wide spectrum of disease, dependent upon the site of infection, age and strain of chicken, and the biology of the infecting *Eimeria* spp. Chickens are challenged by seven species of eimerian parasite with the most important being *E. tenella*, *E. maxima*, *E. necatrix*, and *E. acervulina*. The biology of eimerian infections has been reviewed extensively, including immunity and vaccination strategies (e.g., Refs. [228–234]). In this section the discussion will be restricted to the major mechanisms of immunity and for more comprehensive coverage of the topic of antieimerian immunity the reader is encouraged to refer to the reviews indicated above.

The eimerian life cycle involves oral acquisition of infection, serial invasion of enterocytes by the zoite stages, development of stages within cells (schizonts and gametocytes), fertilization and release of oocysts into the environment. The most important features of the life cycle, with reference to immunity, are the phases of intracellular development (avoiding the action of antibody), the short duration of the infection cycle (meaning that any immune effect must be rapid), and the level of immunity generated by primary exposure to small numbers of parasites (hence vaccination works). Immune mechanisms operate to limit the magnitude of primary infection and, depending upon the *Eimeria* spp. and immunization/challenge schedule, can completely prevent oocyst production at secondary challenge.

The specificity of protection induced by prior exposure is restricted to the *Eimeria* spp. used to prime the birds (reviewed in [235]) and can be strain-specific with some species, such as *E. maxima* (e.g., Refs. [236,237]). The level of immunity conferred by primary infection is dependent upon the *Eimeria* spp. and is influenced by parasite dose, host age, and host genetics (e.g., Refs. [238,239]). With the most immunogenic *Eimeria* spp. (e.g., *E. maxima* in the chicken or *E. vermiformis* in the mouse), a very small priming infection leads rapidly to the establishment of complete immunity (i.e., no oocysts produced). Priming with similar numbers of less immunogenic *Eimeria* spp. (e.g., *E. tenella* in the chicken or *E. pragensis* in the mouse) induces substantial immunity to rechallenge infection but some oocysts are produced. Complete immunity to the less immunogenic *Eimeria* spp. can be established by multiple priming infections. In the case of the fully immune host, the majority of parasites are killed very rapidly with essentially no parasites remaining after 48–72 hours postchallenge (e.g., [240,241]).

Infection of chickens induces a wide variety of responses in the gut including heterophil infiltration, NK cell activation, antibody production, T cell activation, and upregulation of many cytokines and chemokines

(reviewed in [229,232–235]). It is clear that many of the responses induced by infection with the *Eimeria* spp. are at best irrelevant to protection and at worst contribute to the damage caused by infection [84,242]. Here we will focus on the responses that have been shown to contribute to protection using studies with avian and murine *Eimeria* spp. to illustrate the types of response that kill the eimerian parasites.

The short duration of the life cycle influences the mechanisms of immunity that can contribute to limiting the magnitude of primary infections and differ according to the *Eimeria* spp. and the genetics of the host. The genetics of the host can affect the level of parasite replication during primary infection with many *Eimeria* spp. (e.g., Refs. [242–246]) and differential resistance has been correlated to the rate of immune induction in the gut [247–249] or to the basal level of IL-10 production [250].

Mechanistically, T cells have been implicated in limiting primary infections in both murine [84,251–253] and avian hosts [234,251,254,255]. With the murine parasite, *E. papillata*, immune-mediated resistance to primary infection is mostly associated with the activity of NK cells [256] compared with their minor role with *E. vermiformis* [257,258]. The differential roles for NK and T cells in the control of these infections are probably related to the very short duration of the *E. papillata* life cycle which is completed before T cell-mediated immunity can develop.

In adult animals, immunity to *E. vermiformis* (considered a model for *E. maxima* in the chicken) is dependent upon the rapid activity of CD4⁺ TCRαβ⁺ T cells restricted by MHC class II and mediated by the production of IFN-γ [84,252,259–262]. However, in the absence of TCRαβ⁺ T cells the TCRγδ⁺ T cell population can mediate a protective effect [252]; this subset of cells can be important in young animals [85]. In chickens, the numbers of TCRγδ⁺ T cell IEL are altered by infection, suggesting a response, but the role for these cells has not been defined [30,263,264]. Partial depletion of CD4⁺ T cells by administration of monoclonal antibody against the CD4 antigen prior to primary challenge led to exacerbation of infection with *E. tenella*, but not *E. acervulina*, indicating that the mechanisms of resistance to primary infection may differ between the avian *Eimeria* spp. [254], as has been reported for the murine parasites (see above). Depletion of CD8⁺ T cells led to a decreased oocysts output with both *E. tenella* and *E. acervulina* [254] and this was attributed to depletion of cells involved in transport of sporozoites.

With both *E. vermiformis* and *E. maxima* there seems to be little requirement for B cell activity or antibodies in control of primary infection [253,259,265]. However, it is possible to interfere with parasite invasion by

administration of large amounts of specific antibody [266] and this has led to the development of a maternal antibody-based approach to protect chicks by immunization of hens [267].

Infection of chickens with *Eimeria* spp. induces upregulation of numerous cytokines in the intestine but only IFN- γ has been shown to mediate an antieimerian effect. Using anti-IFN- γ treatment or IFN- γ knockout mice, a prominent role for IFN- γ in limiting infection has become well established with murine *Eimeria* spp. [253,256,261]. Treatment of cultured cells with recombinant IFN- γ (from the appropriate host species) prior to infection with various *Eimeria* spp. inhibits sporozoite invasion and subsequent development [268–271]. Moreover, administration of recombinant chicken IFN- γ reduced the magnitude of infection in vivo [272].

Primary infection with all *Eimeria* spp. leads to substantial immunity to secondary challenge and the fully immune host terminates infection very rapidly. In the immune animal the intracellular sporozoite represents the main target for immunity (reviewed Refs. [229,235]), although where the level of immunity is insufficient to kill all invading parasites it is likely that immune-mediated attrition of parasites occurs throughout the life cycle. Mechanistically, it is clear that TCR $\alpha\beta^+$ T cells are essential for immunity [253] and studies with athymic mice and rats indicate that these cells are thymus-dependent for their development [243,265]. With *E. vermiformis*, where the host is completely immune to secondary challenge, it is clear that there is substantial redundancy in the requirement for immunity and many immunodeficient strains of mice are completely, or near completely, immune [253]. Mice deficient in all TCR $\alpha\beta^+$ T cells (i.e., TCR $\beta^{-/-}$) remain highly susceptible to infection, whereas those deficient in MHC class II expression produce few parasites and those deficient in MHC class I expression ($\beta 2m^{-/-}$ or TAP1 $^{-/-}$) or IFN- γ produce no oocysts [253]. Treatment with monoclonal antibodies against CD4, CD8 α , or IFN- γ had no, or little, impact on immunity after infection with *E. vermiformis* [260,262]. Very low numbers of oocysts were produced after anti-CD8 α treatment of *E. vermiformis* immune mice [262] and this is similar to experiments in chickens infected with *E. tenella* or *E. acervulina* [254]. Although TCR $\gamma\delta^+$ T cells can mediate protective immunity in young animals in the mouse, there is no evidence for the development of immune memory with this cell population [252]. With *E. papillata*, where primary infection induces partial immunity, secondary challenge of MHC class II-deficient mice was exacerbated indicating a role for CD4 $^+$ T cells in immunity [272]. In summary, the mechanisms that serve to protect the immune or partially immune animal are TCR $\alpha\beta^+$ T cell-dependent and involve both CD4 $^+$ and CD8 $^+$ T cell activities.

Commercially, vaccination is a realistic alternative to the prophylactic use of anticoccidial drugs and is largely based upon the use of carefully controlled numbers of wild-type or attenuated parasites (reviewed in [229]). The need to incorporate examples of each species (and multiple strains with *E. maxima*) in live vaccines has driven interest in developing subunit vaccine approaches. Some success has been achieved using various recombinant antigens (reviewed in [229]) but the degree of protection is often unsatisfactory. The reasons for this may be due to the inclusion of the wrong antigens or to the delivery systems employed being suboptimal for protection in the gut. Some studies have used live vector systems, such as *Salmonella*, to enhance delivery to the gut and others have used DNA vaccination technologies with enhancement by the inclusion of avian cytokines (e.g., [273]) but more work is needed to further improve the delivery of vaccine antigens. Identification of the appropriate antigen for vaccination is not a trivial task since many of the responses induced by infection are not protective. Over recent years an approach based upon parasite genetics with two strains of *E. maxima* has been developed and this has identified that only six regions of the parasite genome are under selective pressure from the protective immune response [274]. Two protective antigens have been identified using the genetic mapping approach, one that represents a homolog of apical membrane protein-1 that represents protective genes in a wide range of apicomplexan parasites. The second protective antigen, immune mapped protein-1 (IMP-1), was completely untested prior to the demonstration of an effect with *E. maxima* [275]. Interestingly, homolog of IMP-1 have been identified in *Neospora caninum* and *Toxoplasma gondii* genomes [275–277], and these homolog are protective in murine challenge models [276,277]. Identification of the antigens encoded in the remaining four regions of the genome may help identify more generally the characteristics of eimerian antigens that confer protection. The *E. tenella* genome sequencing project (http://www.sanger.ac.uk/Projects/E_tenella/) and other genome resources (e.g., EmaxDB; <http://www.genomemalaysia.gov.my/emaxdb/>) could impact on future vaccine development against coccidiosis, especially where they are used in conjunction with appropriate methods for selection of antigens targeted by effective immune responses.

11.1.6.2 Other parasitic infections

A wide range of noneimerian parasites have been reported to infect the chicken gut including various helminths (e.g., *Heterakis* spp., *Ascaridia* spp.) and other protozoa (e.g., *Cryptosporidium* spp., *Histomonas* spp.). Unfortunately, very little work has been published on the immune responses induced by infection and those that mediate protective immunity.

Most descriptions of responses induced by noneimerian intestinal parasites are with the protozoan *Cryptosporidium baileyi* and the nematode *Ascaridia galli*. Infection with either of these organisms induces a variety of responses including antigen-specific antibodies, lymphocyte proliferation, and cytokines [116,278–283]. Infection with *A. galli* induced increased levels of IL-4 and IL-13 mRNA but not IFN- γ mRNA in the intestine of chickens at 14 days postinfection [283], supporting the hypothesis that Th2-biased responses predominate during enteric nematode infections. The effectiveness of host immune response in controlling enteric parasites is evident by the clearance of primary infection and the enhanced clearance or reduced level of infection seen at challenge of previously exposed or immunized individuals [116,278–282,284,285]. The ability to resolve primary infection and immunity to secondary infection with the intracellular protozoan *C. baileyi* is dependent upon T cells rather than B cells as evident by studies with partially thymectomised and bursectomised chickens [73]. However, maternally derived antibody appears to confer partial protection against infection of the progeny of hens immunized by three large doses of parasites [116] probably by interfering with zoite invasion.

The course of infection with *Histomonas meleagridis* differs considerably between chickens and turkeys with the former being relatively resistant to the devastating pathological consequences of infection in the liver that typify the disease process in turkeys. Antigen-specific IgM, IgY, and IgA antibody responses can be detected in chickens or turkeys exposed to infection with *H. meleagridis* [286,287]. Interestingly, in the CT of chickens infected with *H. meleagridis* the levels of mRNA encoding a range of cytokines (e.g., IL1 β , IL6, IL10, IL13), CXCLi2 and IFN γ were increased compared with uninfected birds [288]. In turkeys the levels of mRNA encoding these mediators were either unchanged or decreased [288]. These data are consistent with the early recognition of infection in the CT of chickens which may restrict infection to the intestine, whereas the lack of an early intestinal response in turkeys may allow the *H. meleagridis* to replicate and migrate to the liver tissue. In chickens at least some of the IFN γ signal can be attributed to CD4 + T cells and an undefined subset of CD4/CD8 β negative cells (which may be TCR $\gamma\delta$ or NK cells) [289].

11.1.7 Concluding remarks

Our aim has been to describe the development and function of immune mechanisms in the gut in broad terms, especially with reference to infectious challenge. Unfortunately, this has necessitated a selective approach to illuminate the major mechanisms of gut immunity and we recommend that the reader supplements the information in this chapter by making use of the literature. The gut is a complex immunological

tissue and the immune mechanisms that operate to protect this site from pathogen challenge are diverse. One important point is that the immune system is a responsive system and the induction of a response does not necessarily indicate a protective effect. It is much more difficult to examine the mechanisms of immune protection than to measure a response, but there is a need to develop approaches where functional evidence can support description of the events that occur during infection. The development of transgenic (especially knockout) chickens has recently been reported and this technology has the potential to revolutionize our understanding of which parts of the immune system are essential for the control of infections in the chicken. These technologies need to be applied and interpreted with care and our advantage is that we can learn a great deal from the work with murine systems. Since the broiler chicken is short-lived, and most development of the gut immune system occurs shortly after hatching, much avian immunology research that is commercially relevant attempts to understand a system in a state of flux. Hence, subtle differences in age, health status, microbial environment, or genetics can have profound effects on the mechanisms available for immune protection.

Immune-mediated control of infectious disease by enhanced resistance or by classical vaccination is accepted and well-used in the commercial poultry sector. However, induction of immunity in the gut is more difficult than with other systemic sites, often requiring targeting to mucosal surfaces. Indeed, inducing immunity in the gut is affected by chick development, oral tolerance, and the immunomodulatory environment within the gut. The most successful enteric vaccines are based on live pathogens or are vectored by live pathogens and a greater understanding of how the gut immune system is stimulated, and which mechanisms operate against specific gut pathogens, will provide the foundation for immune control and vaccination in the future.

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The avian respiratory immune system

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11.2.1 Introduction

To perform its primary function, the delivery of oxygen to and the removal of carbon dioxide from the body, the respiratory system continually ventilates the lung. Therefore particles and particle-associated microorganisms are inhaled as unavoidable constituents of the tidal air. Intensive management systems for poultry are frequently associated with high loads of dust and pathogens in the environment and therefore pose a particular stress to the respiratory system [1,2]. Furthermore, antigens can be intentionally delivered to the respiratory tract in the form of aerosols and this vaccination method is highly effective (see Chapter 18). The respiratory immune system has developed strategies to remove inhaled particles and to adequately respond to those microorganisms that succeed in crossing the epithelial barrier, so maintaining its integrity and functions. Here we will discuss the major features and functional aspects of the defense system of the avian respiratory tract. Since the avian respiratory system differs significantly from that of mammals, this chapter will begin with a brief introduction to its unique anatomy—for more detailed descriptions the reader is referred to Refs. [3–6].

Besides the respiratory tract the paraocular Harderian glands (HG), the conjunctiva-associated lymphoid tissue (CALT), and the paranasal organs encounter aerosolized particles and microorganisms (see Chapter 2). These structures harbor organized lymphoid follicles as well as scattered lymphocytes, indicating that they may play a role in immuno-protection of the head-associated mucosal surface [7]. The secretion of the HG lubricates the nictitating membranes and is further discharged via the lacrimal ducts into the mouth where it can be swallowed, inhaled, or expectorated. In contrast, the lateral nasal gland secretions discharge into the nasal cavity from where they can be inhaled. Via this route antimicrobial factors and secretory antibodies of the HG and nasal glands reach the mucosal surface of the head and trachea.

11.2.2 Anatomy of the respiratory tract

The morphology of the avian respiratory system, with its air sac system, rigid lung structure, and the continuous air flow rather than tidal ventilation, is considerably different from that of other vertebrates. The upper airway originates at the nares and at the mouth. During passage through the nasal cavity, the air passes an expanded surface area, the mucosa-covered conchae which contain organized lymphoid structures called the nasal-associated lymphoid tissue (NALT) [8]. Airflow can take either of two pathways: through the nasal or the buccal cavities, continuing to travel through the larynx, which primarily prevents the entry of foreign bodies to the trachea. The trachea, which is of variable length in different avian species, connects the larynx with the syrinx, the organ of voice production in birds. The tracheal surface is covered by a ciliated epithelium that contains mucus-producing goblet cells [9]. Lymphoid follicles and scattered lymphocytes are found in the lamina propria. The two primary bronchi originate from the syrinx. They each have a short extrapulmonary section before entering the lung from the ventromedial aspect and crossing its entire length before they finally open into the abdominal air sac (Fig. 11.2.1). From the intrapulmonary section of the primary bronchus a highly organized tubular network branches out and connects to nine air sacs filling most of the residual body cavity not occupied by the other organs.

The avian lung is a relatively rigid structure that does not expand or contract appreciably during the respiratory cycle [10]. This feature, together with the absence of a diaphragm, indicates that ventilation is accomplished by a strikingly different mechanism compared to that of mammals. Ventilation is achieved by the action of inspiratory and expiratory muscles that increase or decrease the body volume and consequently the volume of the air sacs. Thus the air sac system acts like a bellows that forces air

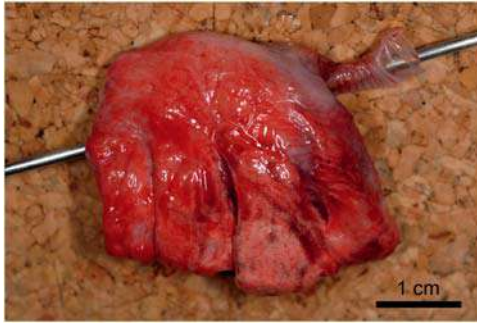


FIGURE 11.2.1 Right chicken lung with a blunt probe indicating the localization of the primary bronchus. The syringe with the primary bronchus is shown on the right.

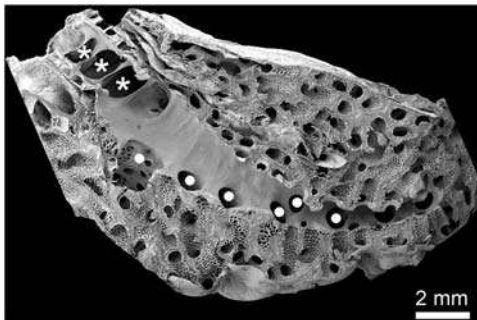


FIGURE 11.2.2 Longitudinal section of a primary bronchus. Openings to the medioventral (*) and mediodorsal (●) secondary bronchi are indicated (SEM preparation).

through the gas exchange tissue during both the inhalation and expiration phases of the cycle. During inhalation, air flows through the primary bronchus, bypassing the cranially located openings of the medioventral secondary bronchi and flows into the caudal air sacs or through the mediodorsal and lateroventral secondary bronchi (Fig. 11.2.2). This caudal-to-cranial flow pattern is also evident during expiration (Fig. 11.2.3) and provides the basis for continuous ventilation and the high efficacy of gas exchange in the avian lung [4,11]. A functional consequence of this flow pattern is that particles are primarily deposited in the caudal regions of the lung, as was first described for soot deposition in pigeons captured at train stations [12]. This observation may explain the absence of organized bronchus-associated lymphoid tissue (BALT) in the cranial part of the lung and its presence at the junctions of the primary bronchus and the caudal secondary bronchi in some avian species, such as the chicken [13].

From the secondary bronchi many “tertiary bronchi” called parabronchi originate, which connect the mediodorsal and lateroventral with the medioventral secondary bronchi [5,9]. They represent the functional unit of gas exchange in the avian lung and are organized in a parallel series separated from each other by a connective tissue that contains large blood vessels and lymphoid follicles (Fig. 11.2.4). Inhaled air flows through the parabronchial lumen and then centrifugally through the atria and infundibula into the air capillaries (Fig. 11.2.5). The air–blood

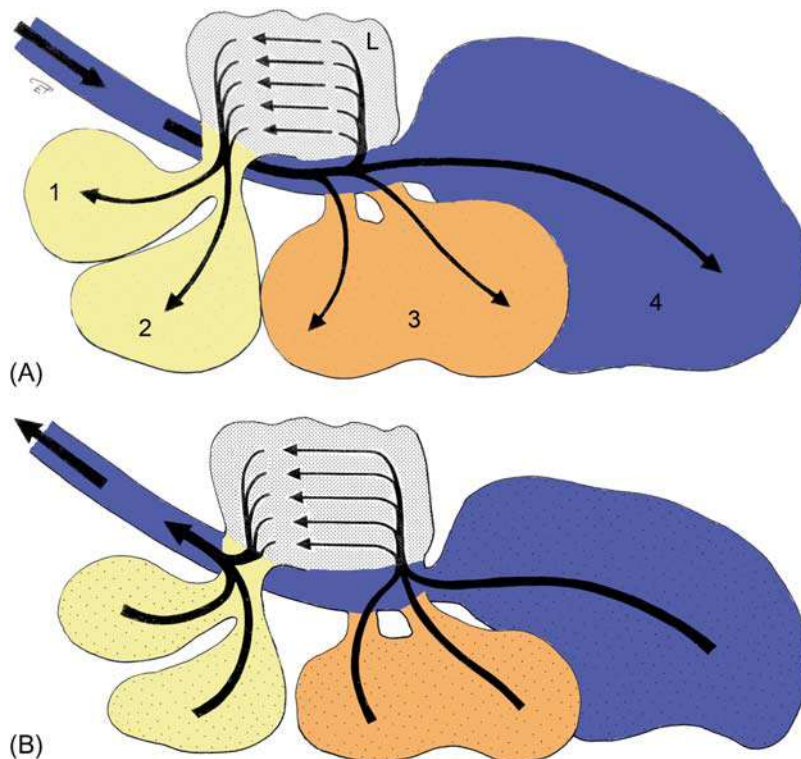


FIGURE 11.2.3 Air flow pattern during (A) inspiration and (B) expiration in the avian lung. L, Lung, 1. clavicular air sac, 2. cranial thoracic air sac, 3. caudal thoracic air sac, 4. abdominal air sac.

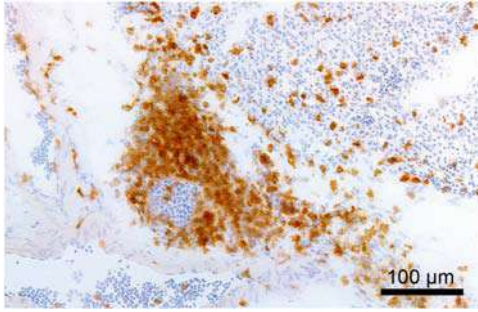


FIGURE 11.2.4 Organized lymphoid aggregates in the connective tissue between tertiary bronchi. Immunohistological staining with an anti-CD3 monoclonal antibody (CT3).

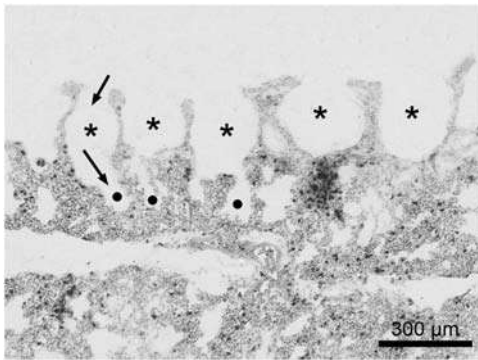


FIGURE 11.2.5 Section of a parabronchus. The air flow from the atrium (*) through the infundibulum (•) into the gas exchange area is marked by arrows. Immunohistological preparation is stained with an anti-CD4 monoclonal antibody (CT4).

capillary interface essentially consists of an epithelium, a basal membrane, and the blood vessel endothelium and is approximately 60% thinner than that of mammals. This provides a highly efficient gas exchange system but may also predispose the avian lung to injury from environmental irritants and pathogens [6].

While the air sacs are essential for ventilation of the lung, they do not participate in gas exchange. In most avian species, there are nine air sacs, grouped into cranial and caudal air sacs (Fig. 11.2.3). They are poorly vascularized, and their thin walls are covered by a squamous or cuboidal epithelium with few ciliated cells [5,14].

11.2.3 The paraocular lymphoid tissue

The HG represents the best-characterized part of the head-associated lymphoid tissue in the chicken. A detailed description of its anatomical structure can be found in Chapter 2. Briefly, the glandular lymphoid tissue is organized in two histologically distinct compartments [15], in which the lymphoid structure in the head of the gland with its follicle-associated epithelium (FAE), high endothelial venules, and germinal centers (GC) represents

a BALT-like secondary lymphoid organ; while the plasma cell-filled body resembles a tertiary lymphoid structure, maintaining classical immunosurveillance.

B cells in the HG are bursa-dependent. This has been demonstrated in several experiments involving bursectomy, which markedly reduces the number of plasma cells in the HG [16] as well as the lacrimal immunoglobulin (Ig) concentration [17]. Interestingly, the effects of bursectomy seem to depend on the method used. While surgical bursectomy of the day-old neonate had hardly any influence on Ig production of any isotype, chemical bursectomy using cyclophosphamide slightly influenced IgM concentrations, significantly reduced the amounts of IgY, and almost completely abrogated IgA secretion [17]. Bursa dependency of HG plasma cell numbers is also supported by studies in which the infection with infectious bursal disease virus significantly reduced plasma cell numbers in the HG [18]. From these results, it was concluded that bursa-derived B cells migrate to the HG before hatching and that IgM could be produced by B cells of nonbursal origin [16,19–21].

The widely accepted concept for B cell differentiation in human and mouse implies that during a GC reaction some activated B cells differentiate first into plasmablasts and then into plasma cells. Both plasmablasts and plasma cells secrete high levels of Ig and are therefore referred to as antibody-secreting cells (ASC). While plasmablasts represent a highly proliferating intermediate stage of differentiation, plasma cells are nondividing terminally differentiated cells [22,23]. Gallego and Glick showed [^3H]-thymidine uptake in the HG not only for plasmablasts but also for mature plasma cells [24], while Scott and Savage observed basal bromodeoxyuridine uptake by HG plasma cells in young chicks; both studies indicating an unexpected proliferation capacity for these plasma cells [25]. This proliferation rate was further increased after temporary plasma cell depletion by treatment with the protein synthesis inhibitor emetidine hydrochloride [25–27]. For a long time, it has been assumed that factors derived from the HG stroma influence plasma cell proliferation. This hypothesis has been supported by the demonstration of soluble stroma-derived factors which increase the proliferation rate of bursal cells [25]. New insights might be obtained using the recently characterized chicken homologs of B cell-activating factor [28,29] and CD40L [30]. These tumor necrosis factor family members are known to be important regulators of B cell development and function and therefore could be good candidates for those cytokines acting on B cell differentiation in the HG.

Mansikka et al. have shown that local immunization with tetanus toxoid vaccine leads to the production of high amounts of antigen-specific IgA and IgY antibodies but very low amounts of IgM [31]. These findings are consistent with those of Burns, who demonstrated bovine

serum albumin (BSA)-specific antibodies with the IgA and IgY isotypes but not of IgM isotype [32]. These results indicate that the HG can mount an immune response, in which plasma cells rapidly undergo isotype switching from IgM to IgY and IgA, respectively. Further support for this conclusion comes from the demonstration of rearrangement events [31]. However, thus far neither gene conversion nor somatic hypermutation has been demonstrated in GC of the HG.

Gene expression profiling of the HG using microarray and quantitative PCR techniques revealed regulation of genes involved in Ig synthesis and downregulation of B cell receptor signaling components [33], but so far nothing is known about activation-induced deaminase expression in the various stages of differentiation of HG B cells, which could address the question of gene conversion.

Various antigens were used to investigate antigen-specific antibody production in the HG, including sheep red blood cells [24,34], *Brucella abortus* antigen [34], *Salmonella* O antigen [35], tetanus toxoid vaccine [31], and BSA [32]. The route of antigen application seems to influence the outcome of the immune response. Regardless of whether antigen was applied by eye drop or injection into the third eyelid, antigen-specific IgY antibodies increased both in the lacrimal fluid and serum. In contrast, IgA levels showed a slight increase in the serum in both groups but IgA levels in the tear fluid were significantly higher after injection into the nictitating membrane [35]. From these results, together with those from Survashe and Aitken—who observed higher serum antibody levels after eye drop application in birds whose HG had been removed—several authors have concluded that parts of the lacrimal IgY have an extra-glandular origin and are transported from serum to tears [17,36]. The idea of a common mucosal system in the chicken has been addressed by Jayawardane and Spradbrow, who showed that, after oral application of a Newcastle disease vaccine, increases in HG plasma cell numbers and hemagglutination-inhibition antibodies can be detected in the lacrimal fluid [37]. An RNAseq analysis showed that in the HG of leghorn chickens in response to Newcastle disease virus (NDV) infection, at 6 days post-infection, both the cell-mediated and humoral arms of their adaptive immune response were activated and a large quantity of differentially expressed genes were connected to a T cell response [38]. Together, these data suggest that the lymphoid tissue of the HG can be considered to be immunocompetent and mount both T-dependent and T-independent immune responses.

Comparatively little is known about the other part of the eye-associated lymphoid tissue, the CALT. Fix and Arp (1991) observed particle uptake by the lymphoepithelium of the CALT and several other groups have described the immunological development of the CALT:

from randomly distributed lymphocyte aggregates in 1-week-old chicks to a BALT-like structure with organized GC and plasma cells from 4 weeks onward [39,40]. The cellular composition of CALT was like that of the HG including B cells, γ/δ T cells, and CTLs. Immunization experiments revealed the presence of antigen-specific IgA-secreting cells. Expression of the polymeric Ig receptor in CALT further indicates that IgA is secreted into the lacrimal fluid [41]. Hence it can be assumed that the CALT also contributes to the avian mucosal immunity.

11.2.4 Nasal-associated lymphoid tissue

The mucosal tissue of the nose is the first to encounter aerosols or microorganisms during inhalation. While particles and microorganisms may be removed mechanically by the mucus, invasive pathogens need to be controlled by the immune system. Lymphocytes are found in the nasal mucosa, the lateral nasal glands, and their secretory ducts [42]. In the nasal cavity, lymphocytes are primarily present in, or below, the respiratory epithelium and are relatively infrequent in the vestibular region at the nasal entrance and in the olfactory region [43]. While $CD8^+$ cells are distributed in the epithelium and the lamina propria, $CD4^+$ T cells are largely confined to organized lymphoid structures which develop in the subepithelial layer. These structures are called the NALT [43] and are considered to be part of a common mucosal immune system, as defined for several mammalian species [44]. At hatch, lymphoid aggregates are absent in the nasal cavity but start to appear in 1-week-old birds. In 5-week-old birds fully developed lymphoid follicles are present which subsequently increase in size and numbers [42].

The major characteristics of chicken NALT are the formation of circumscribed B cell areas occasionally displaying GC, which are covered by a cap of $CD4^+$ T cells. The surface of these lymphoid follicles is covered by a nonciliated epithelium. Ig^+ B cells are found within the NALT structures and distributed throughout the epithelium, similar to the $CD8^+$ cells. The dominant Ig isotype in both locations is IgY, whereas IgM^+ cells are less frequent and IgA^+ cells are relatively rare [43].

Lymphoid structures, which can be found in the lateral nasal glands and in their secretory duct in the chicken and quail [8], display a similar lymphocyte subset distribution pattern as the vestibular epithelium of the nasal cavity [43]. Little information is available regarding the response of these structures to antigenic challenge. Interestingly, birds raised under germ-free conditions develop the same lymphoid structures, with undistinguishable kinetics, as those kept in a specific-pathogen-free (SPF) environment or under commercial conditions [8]. Even though the available literature consists only of a few publications, these consistently report the presence of T and B cells

and a mucosa-associated lymphoid tissue (MALT) in the nasal cavity of birds.

Particle uptake by nasal tissue has been studied using trypan blue, ovalbumin, and fluorescence-labeled beads [42,45]. Both, the mucosal epithelial cells and the FAE actively take up particles as small as trypan blue and as large as 1 μm beads. Interestingly, beads coated with inactivated NDV antigen were predominantly found in cells expressing macrophage and dendritic cell markers but not in epithelial cells as the uncoated beads. Intra-nasally applied fluorescent beads become distributed throughout the respiratory tract and the paraocular lymphoid tissues, indicating that immunization by the nasal route may also induce mucosal immune responses in distant sites. Nasal vaccination in chickens has been investigated using different nanoparticles in combination with different adjuvants. Most studies focused on NDV, avian influenza virus (AIV), and infectious laryngotracheitis virus (ILTV) vaccination with few reports on avian pathogenic *Escherichia coli* (APEC) and *Salmonella* vaccination and demonstrated the induction of local IgA and systemic IgY responses [46]. Intranasal application of a nanoparticle-based NDV-DNA vaccine induced higher antigen-specific IgA antibody titers in mucosal tissues than intramuscular application but similar titers in serum [47].

11.2.5 The contribution of the trachea to respiratory tract immune responses

Constitutive lymphoid tissue has not been described in the avian trachea but scattered lymphoid cells, especially mononuclear phagocytes, are observed throughout the tracheal mucosa and their numbers increase posthatch. Visualization of the development of the lymphoid structures can nowadays be assessed *ex vivo* using whole-mount imaging of the entire tissue of colony-stimulating factor 1 receptor (CSF1R)-reporter transgenic chickens [48], without the need for tissue preparation and histology lowering the risk of missing smaller focal structures. In these birds, the CSF1R promoter region and Fms intronic regulatory element were used to drive expression of a fluorescent protein (eGFP or mApple) reporter and birds that carry a copy of this transgene show high levels of expression of the CSF1R reporter in the macrophage, monocyte, and dendritic cells lineages and at a much lower level in granulocytes [48]. Whole-mount imaging of the trachea showed small aggregates of CSF1R⁺ cells throughout the trachea at 1-week of age. From 4 weeks onward, areas with multiple lymphoid follicles were observed that contained tightly packed CSF1R⁺ cells and a clear halo, indicative for GC (Fig. 11.2.6), which become larger and more numerous as the bird ages [49].

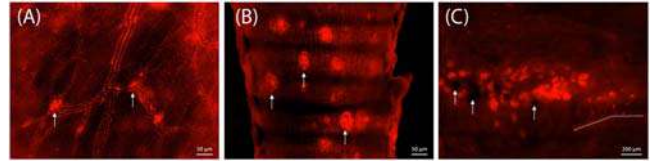


FIGURE 11.2.6 Visualization of organized lymphoid structures in the respiratory tract of a 4-week-old CSF1R-mApple transgenic chicken using whole mount analysis. (A) Air sac with CSF1R-mApple⁺ cells scattered and organized distribution that consists of mononuclear phagocytes lining the vessels and aggregate formations at bifurcations of the capillaries (arrows). (B) Trachea with scattered and tightly packed CSF1R-mApple⁺ cells forming a germinal center surrounded by a halo of transgene⁺ cells. (C) BALT region in the intrapulmonary bronchi; opening to secondary bronchi are marked by an arrow, primary bronchus by a dashed line. Modified from Dr. T. Costa [Sutton K, Costa T, Alber A, Bryson K, Borowska D, Balic A, et al. Visualisation and characterisation of mononuclear phagocytes in the chicken respiratory tract using CSF1R-transgenic chickens. *Vet Res* 2018;49(1):104].

Infection models with *Mycoplasma gallisepticum* have shown that the tracheal mucosa is highly responsive to infections and reacts with extensive lymphocyte infiltration followed by lymphoproliferation [50,51], a response pattern also shown for other infections [52]. CD8⁺ cells are found in clusters or lymphoid follicle-like structures within the tracheal mucosa, while CD4⁺ cells are spread randomly. Tracheal lesions characteristic for *Mycoplasma* infections predominantly consist of proliferating B cells [51]. Similar responses in the tracheal mucosa have been observed after infection with infectious bronchitis virus (IBV). Production of IBV-induced lesions is associated with massive lymphocyte infiltration in the tracheal lamina propria, the generation of numerous lymphoid follicles, and the immigration of plasma cells [53,54]. Interesting findings have been provided in a study by Javed and colleagues [55], who compared immune responses to *M. gallisepticum* infection in the trachea between vaccinated and unvaccinated chickens. While unvaccinated chickens had infiltrations of large numbers of B and T cells and some plasma cells, vaccinated birds developed secondary lymphoid follicle-like aggregates with far fewer lesions. In addition, in tracheal tissue of vaccinated birds manifold more mycoplasma-specific ASC could be identified than in unvaccinated animals. This indicates that in the tracheal mucosa lymphoid tissue can be induced by different infections.

11.2.6 The bronchus-associated lymphoid tissue

The avian lung exhibits both highly organized lymphoid structures and diffusely distributed lymphoid and myeloid cells. This was first described by Bienenstock et al. [56], who compared the lung immune systems of birds with those of several mammals and found lymphoid nodules in

the primary bronchus that had much similarity with Peyer's patches and other gut-associated lymphoid tissues. Hence, these structures were designated BALT. It was observed that BALT structures were constitutively present and far more frequent in the chicken than in any other species. However, this work was not followed up until growing interest in poultry respiratory diseases led to more detailed studies utilizing newly developed chicken leukocyte-specific monoclonal antibodies [57,58].

BALT structures develop at the junctions of the primary bronchus and the caudal secondary bronchi (Fig. 11.2.2) [13,57] as well as at the ostia of the air sacs [59]. While they are completely absent from the junctions with the cranial secondary bronchi in chickens [13], they are found regularly in the cranial part of the primary bronchus of turkeys [60]. At hatching no, or very few, lymphocytes are present at these locations [13,60,61], and although organized BALT nodules had not been observed prior to the third to fourth week after hatching [60,61], the use of reporter transgenic chickens facilitated visualization of these lymphoid structures. Small aggregations of CSF1R⁺ cells are first observed at 1 week of age, primarily at the vicinity of openings of the caudal secondary bronchi. With increasing age, these aggregates become larger and more numerous, and from 4 weeks of age onward, GC with tightly packed CSF1R⁺ cells were observed (Fig. 11.2.6) [49]. A fully developed BALT is found in birds a few weeks after hatch. It surrounds the entire openings of the secondary bronchi [60] and displays distinct B and T cell areas similar to other MALT [58]. Although not examined in detail, the number of follicles may decrease in the older bird but this could be attributed to the increase in lung size and follicles being dispersed over a larger surface area [49].

The mature BALT is covered by a distinct layer of epithelial cells called the FAE that harbors numerous lymphocytes [61,62]. Differentiation between FAE and interfollicular epithelium (IFE) can be visualized with the α -D-galactose binding lectin, jacalin. Jacalin does not bind to the FAE but binds to IFE cells [49]. FAE is made up of ciliated and nonciliated cells in variable numbers depending on the developmental stage and is devoid of goblet cells (Fig. 11.2.7). Some FAE cells have irregular microvilli on their luminal surface and are in close contact with lymphocytes and myeloid cells suggesting that they may be homologous to the M cells of Peyer's patches. More recently CSF1R-expressing M-like cells in the FAE covering the BALT have been described [49,63], however, particle uptake by these cells has not yet been demonstrated and it remains to be seen how lymphocytes in the chicken BALT encounter antigen [13]. A recent study demonstrated the appearance of lipopolysaccharide (LPS)-coated, fluorescently labeled microparticles in the BALT structures after intratracheal application and

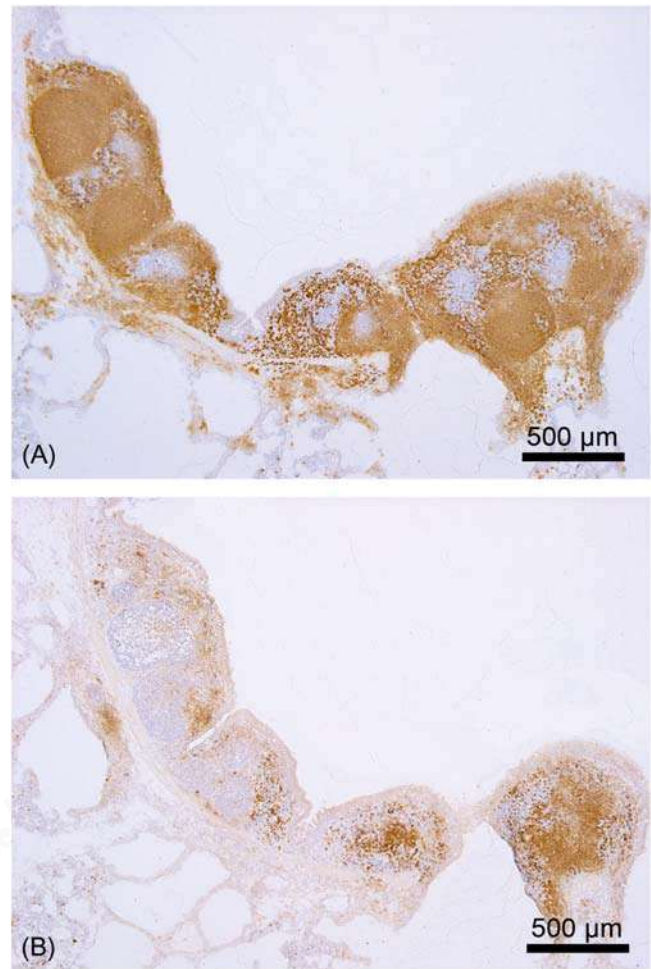


FIGURE 11.2.7 Fully developed BALT structure in a 12-week-old chicken. Immunohistological preparation is stained with (A) anti-chB6 (AV20) and (B) anti-CD4 (CT4) monoclonal antibody.

suggested that antigen-presenting cells (APC) located in the parenchyma might take up and transport antigen to the BALT structures to prime the immune response [64]. Evidence in favor of this hypothesis comes from work in mice where migration of antigen-loaded dendritic cells (DC) to BALT structures was shown to be crucially required for effective T cell priming and sustainment of the BALT [65]. Monocytes/macrophages and DC-type cells are abundantly present in the BALT, and are CVI-ChNL-68.1 and CVI-ChNL-74.2-positive, and express MHC II [58,66]. More recently, large populations of CSF1R⁺/putative CD11⁺/lysosomal-associated membrane glycoprotein 1 (LAMP1)⁺ cells were found to be distributed throughout the BALT area, some of which express MHC II [49].

In fully developed BALT nodules large B cell follicles are found (Fig. 1-2.8A), which are primarily made up of IgM⁺ cells while IgY⁺ and IgA⁺ B cells are present in much smaller numbers [61]. Both in chickens and turkeys,

GC are developed in most BALT nodules and are covered by a cap of $CD4^+$ T cells (Fig. 11.2.8B). In contrast, $CD8^+$ T cells are diffusely distributed between the lymphoid nodules and, as $CD4^+$ T cells, infrequent in the GC. Plasma cells are confined to the edge of the lymphoid tissue and found under the FAE [61].

BALT development is influenced by age and by environmental stimuli [58]. Single T lymphocytes are already present at predetermined locations in 1-day-old chicks and turkey poults while B cells are not evident until the second week. During the following weeks increasing numbers of $CD45^+$ cells accumulate and develop into organized structures with T cells in the center and B cells at the periphery [13,58]. As discussed, this pattern changes in older birds where B cell areas are found in the center of the lymphoid nodules, which are covered by $CD4^+$ T cells. GC formation is not observed until the end of the second week in conventionally reared birds and takes one more week in SPF chickens [61]. GC are composed of blast-like cells, which stain positive with a pan-B cell marker [recognized by monoclonal antibody (mAb) HIS-C1 or CB3] and with anti-Ig antibodies, as well as tightly packed IgM- or IgY-bearing cells with follicular dendritic cell (FDC) morphology [61,66]. FDCs express CVI-ChNL-74.3, CSF1R, CD11 but not MHC II and are characterized by trapping immune complexes via Fc receptors. In addition, GC contain a cell population with remnants of nuclei in their cytoplasm, also known as starry-sky or tingible body macrophages. In the chicken, these cells express TIM4, a receptor for phosphatidylserine and engulf apoptotic cells [49,66].

It is largely unknown if environmental stimuli are essential for BALT development. Early studies with germ-free chickens described lymphocytic infiltrates in the HG and the trachea but did not investigate the lung [8]. BALT development was shown to follow a similar time course in SPF and conventional chickens, even though slight differences were observed with regards to

GC development [61]. However, infections with pathogenic microorganisms increase the number of BALT nodules significantly [57]. Whether this response is an indication of local antigen-specific T and B cell responses or due to the immigration of lymphocytes remains to be demonstrated.

11.2.7 The immune system in the parabronchi

Surprisingly little is known about the structure and functional relevance of the immune system in the lung parenchyma [67]. Immunohistological staining for a pan-leukocyte marker (using mAb HIS-C7) revealed the presence of diffusely distributed leukocytes in the interstitial tissue of the lung [66] see also (Fig. 11.2.4). The majority of these cells were identified as belonging to the monocyte/macrophage (Fig. 11.2.8) and dendritic cell type because of their expression of major histocompatibility complex (MHC) class II molecules and their reactivity with the myeloid cell mAbs CVI-ChNL-68.1 and -74.2 [68]. Upon further analysis, the MHC II⁺ cells in the atrial septa and infundibulum also expressed CSF1R, putative CD11, and LAMP1 [49]. Thus the lung parenchyma is well equipped with resident phagocytes and APC, which are found in the interstitial tissue and in the parabronchial wall as early as 5 days of age. Lymphocytes are also widely distributed in this tissue. IgM-expressing cells appear in 1-week-old chicks, while IgA⁺ and IgY⁺ cells are not found at that time. At later stages, chB6⁺ (sometimes referred to as Bu1⁺) B cells and $CD3^+$ T cells are frequently observed. Most of these T cells show the classical cytotoxic T-lymphocyte phenotype with the expression of CD8 and α/β TCR molecules. $CD4^+$ cells and γ/δ T cells are less frequent [58]. While these reports clearly document the presence of an extensive system of immune cells throughout the gas exchange tissue, only a few studies have been published addressing its function and responsiveness to antigen challenge. In this context, it is interesting to note that inhaled particles smaller than 0.1 μ m in diameter reach the deep lung tissue and are

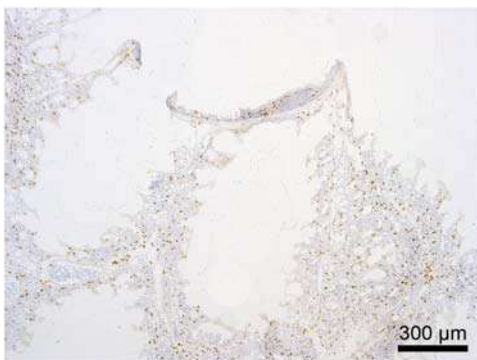


FIGURE 11.2.8 Diffusely distributed cells of the myeloid lineage in the lung tissue of an 8-week-old chicken stained with the monoclonal antibody KUL01.

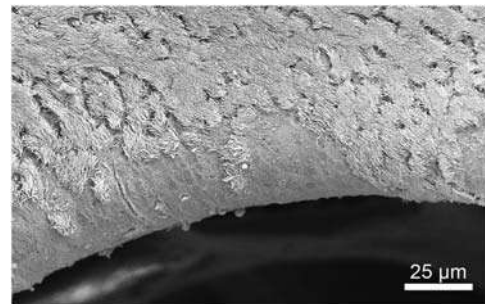


FIGURE 11.2.9 Ciliated and nonciliated epithelium covering the BALT structure at the opening of the primary bronchus to a secondary bronchus (SEM preparation).

taken up by the epithelial cells of the atria and infundibula and by interstitial macrophages [69]. Furthermore, intratracheally applied antigen-coated fluorescent beads with 1 μm diameter were actively taken up by phagocytic cells belonging to both the macrophage and the DC subsets, which showed an activated phenotype as indicated by MHC class II, CD40 and CD80 upregulation [64]. Thus the APC and lymphocytes of the parenchyma come into contact with inhaled antigens and may initially mount a local response but subsequently help to induce a systemic antigen-specific immune response. Support for this hypothesis comes from the frequent observation of lymphoid follicles with distinct T and B cell areas in the interstitium between the parabronchi (Fig. 11.2.4).

Type II pneumocytes secrete surfactant, which plays a role in the control of lung infection. In chickens, these cells cover the surface of the atria, show a cuboidal morphology with intracellular granules, and are specifically stained by the mAb 74.3 [70,71]. In addition, these cells express LAMP1 indicating phagocytic activity [49]. The content of the granules is secreted onto the atrial surface as well as at the basolateral side and forms a distinct structure called the trilaminar substance composed of surfactant [72]. Two surfactant proteins were identified in the chicken named surfactant protein A (SP-A) and chicken lung lectin (cLL) (see Chapter 8.5). Expression of SP-A in atrial epithelial cells and its secretion on the surface of the atria and infundibula was demonstrated by immunohistochemistry [73]. Likewise, cLL is primarily expressed in the chicken lung but its cellular origin is still unknown. Thus far, orthologues of mammalian SP-B, -C, and -D have not been described in chickens.

11.2.8 The phagocytic system of the respiratory tract

In the alveolar lung of mammals, macrophages are not only found in the tissue but also in the epithelial lining of the alveoli as a constitutively present cell population. They provide a first line of defense at the gas–blood barrier [74,75] and play a role in immunopathological events. Mammalian alveolar macrophages can easily be obtained for functional studies using lung lavage. Application of this method to chickens or turkeys yields comparatively few cells in the lavage fluid, as reported by several groups [76–79]. Phagocytic cells were estimated to be 20-fold less frequent in avian lavage samples than in those obtained from mice or rats [80] or were even considered to be entirely absent [81,82]. This discrepancy can be explained by differences in the lavage procedure, the immune status of the chicks, and the strain used for the study [83]. In addition, phagocytic cells may be derived not only from the lung but also, in variable amounts, from

the air sacs, as reported for chickens and ducks [79]. Phagocytic cells obtained by lavage have either been named “avian respiratory macrophages or phagocytes” (ARP) [83,84] or “free avian respiratory macrophages” (FARM) [6,76].

The origin and the distribution of FARM in the avian lung have been addressed using microscopic techniques. These studies have shown that macrophages are absent from the surface of the air capillaries but present on the epithelial lining of the atria and infundibula [6,78]. Interestingly, macrophages are also abundant in the connective tissue below this epithelium on the floor of the atria [49,78,82] and in the inter-atrial septa [64,67], indicating that phagocytic cells are strategically located at the start of the gas-exchange area to clear the air of inhaled particles before it reaches the thin and vulnerable air capillaries.

Leukocytes are present on the surface of the air sacs. Heterophils make up the majority of these cells followed by macrophages, while lymphocytes are relatively rare [14]. As in the lung parenchyma, mononuclear phagocytes can be detected in the connective tissue of the air sacs [82]. Ample scattered mononuclear phagocytes are already detected before hatch in CSFIR-reporter transgenic birds and from 1 week of age small aggregates, primarily located at the vicinity and bifurcation of capillaries, are detected. These cellular aggregates increase in size and number with age [49].

This distribution pattern of macrophages in the avian lung suggests that, even under noninflammatory conditions, phagocytes can migrate from the connective tissue to the surface of the gas-exchange area [76,77], even though some authors have argued against this happening [82]. Repeated lung lavages lead to an increase in macrophage numbers in the lavage fluid, indicating that macrophages can transmigrate into the air space either from the connective tissue or from the vascular system [79].

Under inflammatory conditions, heterophils and macrophages are rapidly attracted to the respiratory surface. A variety of stimuli have been used to characterize this process. Inoculation of incomplete Freund's adjuvant into the abdominal air sac induces large numbers of activated FARM into the lung and air sacs. These cells are highly adhesive onto glass surfaces, actively phagocytic and cytotoxic for *E. coli* as analyzed by in vitro assays [77]. Likewise, phagocytic cells from chickens elicited by intratracheal introduction of live bacteria show increased phagocytic activity compared with control cells [85,86]. This response was already evident within 2 hours of stimulation and showed peak activity about 6 hours later [87]. Furthermore, injection of *Pasteurella multocida* into the caudal thoracic air sac led to the rapid influx of large numbers of highly phagocytic heterophils with a minor population of macrophages [88]. The same rapid influx of heterophils and CSFIR+ macrophages was observed within hours post-inoculation when avian

pathogenic *E. coli* strains of serotype O1 and O2 were injected. Both bacterial strains induced strong inflammatory responses dominated by heterophils and not surprisingly given the huge genetic diversity of APEC, the immune responses between the bacterial strains differed. The bacteria were especially detected in heterophils and to a lower extent in CSF1R⁺ macrophages. Interestingly, phagocytosis assays using live and killed bacteria suggested that bacterial invasion instead of phagocytosis by the heterophils and macrophages contributed mostly to the uptake in vitro. However, the authors showed that bacterial invasion and survival in phagocyte populations in vitro is not predictive of events in the chicken lung [89]. The intratracheal introduction of heat-killed *Propionibacterium acnes* [80] or *Salmonella typhimurium* [90] also rapidly elicited FARM. By contrast, *P. acnes*, *E. coli* LPS, *Saccharomyces cerevisiae* glucan or incomplete Freund's adjuvant given intravenously induced only a weak, or no, macrophage efflux from the lung [80]. This was also demonstrated using a *P. multocida* vaccine that, after intratracheal but not after oral application, elicited phagocytic cells in the lung [91].

11.2.9 Handling of particles in the respiratory tract

In conventional poultry production, birds are exposed to high loads of aerosolized particles. Removal of these particles takes place in all parts of the respiratory tract with larger particles controlled in the upper respiratory tract and smaller ones reaching the more distal respiratory tissue. Several independent mechanisms complement each other to prevent tissue damage and control infections. The site of deposition of inhaled particles primarily depends on particle size. As shown by Hayter and Besch [92], larger particles with diameters between 3.7 and 7 μm are trapped and removed in the nasal cavities and trachea. Smaller particles ($\sim 1.1 \mu\text{m}$) are transported into the lung and the cranial air sacs while particles smaller than 0.1 μm pass through the lung and are deposited in the caudal air sacs. Clearance mechanisms in the upper respiratory tract and in the trachea primarily rely on the particles being trapped in mucus, its oral mucociliary transport, and expectoration or swallowing of the trapped material. A ciliated epithelium is present in the trachea, the primary bronchus, and the proximal parts of the secondary bronchi [93] while ciliated cells are rare in the air sacs [5,14]. Thus particles transported beyond the ciliated mucosa must be removed by cellular mechanisms. Using aerosolized inert iron particles with a diameter of 0.18 μm , Stearns et al. showed that particles were phagocytosed by FARM in the parabronchi and endocytosed by epithelial cells lining the atria and the proximal parts of the infundibula [69]. Iron particles were also found inside the tissue

macrophages underneath the atrial epithelium. This observation with ducks was subsequently confirmed by others for chickens and pigeons and demonstrated using different foreign particular material, as well as red blood cells [78,79]. It appears that birds have developed several lines of defense to protect the thin and vulnerable gas-exchange tissue if particles that are too small to be removed in the upper respiratory tract are transported into the deeper lung. Obviously, FARM and the epithelial cells of the atria and infundibula are the first to encounter and remove particles from the luminal surface of the avian lung [6,78]. Even though FARM are rare on the mucosal surface, these cells are potent phagocytes that take up microparticles more rapidly and at higher numbers per cell than rat pulmonary surface macrophages, thereby partially compensating for their paucity [94].

In the tissue, subepithelial phagocytes, interstitial macrophages [58,95,96], dendritic cells [64], and a poorly defined population of resident pulmonary intravascular macrophages [78] remove foreign particles which have crossed the epithelial barrier. The subsequent fate of this material is still not known. Theoretically, it could be transported from the lung to secondary lymphoid organs, as demonstrated in mammals. Alternatively, birds may present processed antigen locally to mount an antigen-specific immune response either in the constitutively present BALT or in organized lymphoid nodules which are inducible in many avian tissues by antigen inoculation. Such lymphoid aggregates have been observed in the avian lung parenchyma, but their function is still unclear. Interestingly, phagocytic cells which have taken up antigen-coated beads upregulate the cell surface molecules MHC class II, CD40 and CD80 which play a critical role in antigen presentation and T cell priming [64].

The handling of inhaled particles in the air sacs is even less well understood. Although a ciliated epithelium was described in the most proximal parts of the air sacs [14], transport back into the bronchi has not been demonstrated experimentally. Therefore phagocytic cells of myeloid origin are primarily responsible for particle clearance, even though this process is significantly slower than in the lung tissue [77,97].

11.2.10 The secretory IgA system in the respiratory tract

Antigen-specific protection of mucosae in the upper airway is achieved mainly using the humoral immune system [98,99] through the production and secretion of polymeric IgA and IgM [44]. These Ig are transported through the epithelial cells by a basolaterally expressed receptor protein, the poly-Ig receptor [100]. The avian homolog of this receptor has been recently cloned [101]

but its expression pattern in epithelial cells of the respiratory tract is so far not known.

Secretory Ig perform immune exclusion by inhibiting the uptake of soluble antigens and by blocking adhesion and invasion of epithelia by microorganisms [100,102]. As discussed previously, numerous B cells and ASC are present in the head-associated lymphoid tissues and throughout the respiratory tract of avian species, and Igs have been detected in the tears and lachrymal fluid [31,37], nasal [103], tracheal [37,50], and lung washings [55,102,104].

Secreted Igs in the respiratory tract are produced by bursa-derived cells [105], primarily of the IgA isotype, although IgY and IgM antibodies are also found [106]. Using a hemolytic plaque assay, Lawrence et al. demonstrated that the respiratory tissue is relatively enriched in IgA-secreting cells which could be the main source of mucosal IgA [107]. The mucosal IgA response is T cell dependent, since in vivo depletion of α/β T cells almost completely abolishes IgA production [108]. The protective role of secretory (s)IgA has been investigated in several infection and vaccination studies with economically important respiratory pathogens. Intranasal or eye drop vaccination with NDV induces antigen-specific IgA antibodies that can be detected in tears and tracheal [37,109,110] as well as lung washings [111]. Application of a live virus vaccine does not seem to be essential since inactivated NDV given by the intranasal route elicits a protective sIgA and sIgM response, which can be further enhanced by the addition of the mucosal adjuvant, cholera toxin B subunit [103]. Comparable results were obtained in *M. gallisepticum*-infected birds, which developed an antigen-specific IgA, IgM, and IgY response in washings of the upper and lower respiratory tract [112]. Functional in vitro assays demonstrated that these antibodies were protective but that protection did not correlate with the IgA titers in the washing, indicating that secreted IgM and IgY are also of relevance in mucosal defense [13]. Intratracheal infection with *M. gallisepticum* induces the accumulation of IgY⁺ and IgA⁺ B cells and plasma cells in the lamina propria. In contrast, vaccination prior to infection induced the formation of lymphoid follicles and of strongly elevated numbers of antigen-specific ASC as measured by an enzyme-linked immunospot (ELISPOT) technique. It also leads to a significantly higher proportion of *M. gallisepticum*-specific IgA and IgY antibodies in tracheal washings [55]. While IgA is most probably produced locally and secreted as a polymeric antibody, IgY antibodies may be locally secreted or transudated from the serum as suggested by some studies [55,113,114]. Induction of antigen-specific IgA and IgY antibodies in tear fluid and lower respiratory tract lavage samples was demonstrated in IBV-vaccinated birds [115,116] and was induced equally well by antigen

delivery through ocular instillation, spray, or drinking water [117]. Lung infections with a low pathogenic AIV (H7N1) induced antigen-specific IgM and IgY ASC within 1 week after primary infection and an increase of IgY and IgA ASC in response to secondary infection as measured by ELISPOT. This study further showed that the humoral immune response of the lung was induced locally and not in the spleen or other inductive sites [118].

11.2.11 Gene expression analysis as a tool to investigate host–pathogen interaction

Most of our knowledge of the respiratory tract immune system is based on morphological studies. Functional studies have largely been limited to the demonstration of antigen-specific antibody responses. Advances in avian genomics and the availability of new technologies such as the quantitative RT-PCR, transcriptomics, and transgene technology now enable more detailed investigations about the function of this mucosal defense system.

Innate protective factors are known to be important in the defense of the mammalian respiratory tract. Part of the innate immune system, type I interferons are potent antiviral cytokines that exert their activity by modulating the induction or suppression of interferon regulated genes (IRG). A recent transcriptomic study analyzed the time course of IRG expression in the lung after IFN α application and found IL-6 and avidin among the IRGs with the strongest upregulation [119].

By screening of expressed sequence tags and genome sequence databases, 14 different chicken β -defensins, designated gallinacins, have been identified (see Chapter 8.5). Gene expression in the trachea and in the lung was restricted to Gal-1 Gal-2, Gal -3, Gal-5, and Gal-7 [120], and tracheal expression of Gal-3 was significantly upregulated in response to *Haemophilus paragallinarum* infection [121]. With a similar approach, the family of chicken collectins was identified and their expression, together with the expression of SP-A, was demonstrated in the lung and tracheal tissue [122,123]. The gene expression of cSP-A and cLL is already high at embryonation day (ED)16, whereas chicken Collectin Liver 1 increased after ED16 and is upregulated post-hatch. Gene expression of chicken Collectin Kidney 1 in the lung remains low pre- and posthatch. cSP-A protein was mainly found in its secreted form in the epithelial lining of the parabronchi, primarily in the atria, and in some small segments in the infundibulum and in the lumen of the capillaries. Intracellular staining was occasionally seen in epithelial cells which likely reflect avian epithelial type II cell homologs or type II pneumocytes [73].

Mannose-binding lectin mRNA expression in the lung is barely detected during embryonic development and post-hatch [73] but can be upregulated in the lung and larynx after infection with IBV [124]. Progress in the identification of these antimicrobial peptides will help us to better understand the host response to pathogens, to identify host resistance factors, and to develop new therapeutic or preventative strategies for poultry farming, based on natural antimicrobial components [125].

Gene expression analysis using microarray and next generation sequencing (NGS) technology is now increasingly used to study host–pathogen interaction in the respiratory tract in more detail; the first results were published for infections with IBV in chickens [126] and AIV in chickens [127,128] and ducks [129]. Recently, HG transcriptome response to NDV in two inbred chicken lines has been characterized by RNAseq, revealing surprisingly little differences between challenged and nonchallenged birds of the same line [38]. Some studies used qRT-PCR and bio-assays to investigate gene expression of pattern recognition receptors (TLRs, RLRs), interferons (IFN- α , IFN- γ), and inflammatory cytokines (IL-1 β , IL-6, IL-8) in the context of AIV [130–133] and Marek's disease virus infection [134–138].

As discussed, the immune system of the avian lung is compartmentalized into organized BALT structures and diffusely distributed lymphocytes and myeloid cells in the gas exchange tissue. These structures will most likely respond to infections differently, thus necessitating focused tissue sampling for expression and functional studies in order to avoid misinterpretation of collected data. Isolation of parenchymal leukocytes has been described [139] and laser dissection microscope-guided probe sampling should enable detailed studies on BALT tissue. Likewise, it is now well appreciated that the respiratory epithelium plays a critical role in the early response to pathogens and closely interacts with the immune system [140]. Techniques to culture chicken lung epithelial cells have been described and used to investigate their response to AIV [141] and ILTV [142] infections. A recently established chicken lung epithelial cell line termed CLEC213 should further improve our understanding of the biology of these cells in infectious disease [143].

Posthatch immunization of poultry is frequently achieved by aerosol, spray, or eye drop vaccination and activates both the local and the systemic adaptive immune systems. Improvement of vaccines by rational design requires a comprehensive understanding of the way in which antigen is taken up, processed, and presented to the head-associated and respiratory immune system of birds, a process that is not understood. Therefore future research will have to focus on the characterization of the phenotype and function of professional APC in the respiratory tract and on methods that will allow direct targeting of antigen to APC and their activation by new adjuvants as was discussed by de Geus and colleagues [64,144].

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The avian reproductive immune system

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11.3.1 Introduction

The avian reproductive tract differs greatly from that of mammals in both its structure and function. Infection of the reproductive tract and particularly infection of developing eggs has consequences in the vertical transmission of disease to progeny. In the case of *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*), infection of table eggs for human consumption remains a major public health issue. Our knowledge of the reproductive tract-associated immune system, its response to infection and its regulation have advanced somewhat in recent years. In particular, the innate immune system and the production of antimicrobial peptides within the tract have begun to be defined including in the response to challenge. Furthermore, changes in the structure of the immune system related to the onset of sexual maturity in hens have also been described. This chapter reviews the current knowledge of this area and its relevance to infections of the reproductive tract and eggs by bacterial and viral pathogens.

11.3.2 The structure and function of the avian reproductive tract

The reproductive tract of female chickens consists of a functional left ovary and oviduct with vestigial organs on the right side [1]. The ovary reaches maturity from around 17–25 weeks of age, depending on the breed or strain of chicken [2]. The immature ovary consists of a central medulla surrounded by a cortex, which contains the developing oocytes, which is in turn surrounded by cuboidal epithelial cells. As the bird matures a distinct hierarchical follicular structure develops, with thousands of developing follicles present within the ovary. Toward sexual maturity a number of follicles begin to mature rapidly,

growing up to 40 mm in diameter. The mature follicles consist of a number of distinct layers surrounding the oocyte or yolk. Immediately surrounding the yolk is the vitelline membrane. This is surrounded by a perivitelline layer and the ovarian granulosa, which in turn is surrounded by a basement membrane, layers of theca and connective tissue and finally an epithelial layer [3].

Following ovulation, the mature oocyte or yolk is deposited into the oviduct [4]. The oviduct is a convoluted tubular structure that stretches from the ovary to the cloaca. In mature hens it is around 60–80 cm long when fully extended. During passage through the oviduct, the albumen, membranes, and shell of the egg are deposited around the yolk. The first section of the oviduct is the infundibulum, which receives the developing egg from the ovary. This is a funnel-like structure that has considerable secretory activity with goblet cells secreting mucous and ciliated cells facilitating movement down the oviduct. The developing egg passes into the magnum, the longest part of the oviduct, which secretes albumen around the yolk. Like the infundibulum, the magnum contains numerous ciliated and secretory cells [3]. The mucosal structure of the magnum is highly folded with underlying pyramidal proapical gland cells that secrete much of the albumen protein. The egg passes into the isthmus which produces the shell membranes. Following the deposition of the membrane, the egg passes into the uterus. It is retained within a pouch-like structure, in which calcification of the egg to form the shell occurs over a period of around 20 hours. The shelled egg then passes into the vagina and is eventually laid through the cloaca. The vagina also acts as a depository for spermatozooids after mating, which are released gradually to fertilize eggs in the ovary.

The male reproductive tract is, perhaps, less of an issue in terms of infectious disease and vertical

transmission of pathogens. Birds have two internalized testes which are located near the kidneys and are 5–6 cm in length in mature birds. As in mammals, spermatozoa develop within the testes. Unlike mammals, there are no accessory genital organs and seminal fluid is also produced by the testes. Semen is secreted via ducts to the epididymis and to the ductus deferens which connect to the phallus. Birds may have a protruding phallus, such as in ratites or many waterfowl, or non-protruding, as in galliforms and most passerines.

11.3.3 Structure and development of the reproductive tract-associated immune system in the chicken

11.3.3.1 Organization of lymphocytes in the reproductive tract

There are a number of descriptions of lymphocyte organization within the ovaries and oviduct. Histology and immunohistology have been largely utilized to determine these, though there are very few functional studies. Early histological examination indicated the presence of scattered lymphocytes and lymphocyte aggregates in the oviduct [5]. The distribution of immunoglobulin (Ig)-secreting cells within the reproductive tract was described in the 1970s, including the distribution of cells secreting IgA [6,7]. These studies indicated the presence of large numbers of IgA-secreting cells below the oviduct epithelium. The localization of B lymphocytes and their associated Ig subclasses in laying hens was described by Kimijima et al. [8]. This study indicated the presence of IgY⁺ B lymphocytes associated with epithelial and glandular tissue throughout the oviduct, with highest numbers located in the epithelium of the infundibulum, the glandular regions of the magnum, and the uterus. IgM⁺ and IgA⁺ B lymphocytes are found more frequently in glandular tissue of the magnum, though scattered cells are found throughout most areas of the oviduct [8]. Withanage et al. [9] investigated the distribution of B lymphocytes in the reproductive tract of mature hens and indicated that scattered cells are present throughout the oviduct, although they are found infrequently in the ovary and those present are IgM⁺ and IgA⁺ cells rather than IgY⁺ B lymphocytes. IgA⁺ B cells are found primarily under the epithelium, IgY⁺ B cells with tubular glands, and IgM⁺ B lymphocytes with the magnum and isthmus [9]. In situ hybridization of IgY γ -chain mRNA showed the presence of IgY⁺ B lymphocytes throughout the oviduct with greater numbers of γ -chain expressing cells associated with the stroma than the mucosa [10]. The oviduct is also considered to be an important site of maternal transfer of antibody to the egg, with IgM and IgA present

in the albumen, and IgY in the yolk which is transferred in the ovaries (for more details see Chapters 5 and 13).

T lymphocytes are present in both the ovary and the oviduct [9]. T lymphocytes are also associated with the loose lymphoid aggregates or nodes present in the oviduct. CD4⁺ lymphocytes are found most frequently in the lamina propria and are most numerous in the vagina. CD8⁺ cells are frequently associated with the epithelium and appear to form a major proportion of intraepithelial lymphocytes. CD8⁺ T lymphocytes are most numerous in the vagina and the infundibulum of the oviduct. Unlike B lymphocytes, both CD4⁺ and CD8⁺ T lymphocytes are found in significant numbers, associated with the ovaries [9]. The aggregates in the developing oviduct consist primarily of CD4⁺ cells, though smaller aggregates dominated by CD8⁺ cells are also present [11]. Large granular lymphocytes have also been described in the oviduct [12]. These are associated with glandular cells and the epithelium and are most numerous in the magnum and vagina. TCR $\gamma\delta$ lymphocytes are also found associated with the reproductive tract epithelium in significant numbers.

11.3.3.2 Distribution of macrophages and other cells

Immunocytochemical staining has indicated the presence of macrophages or macrophage-like cells in the ovarian follicles of the chicken [13–16]. MHC class II positive cells have been found in the thecal layer of preovulatory follicles and in the theca and granulosa of postovulatory follicles [13]. Macrophages are also present throughout the length of the oviduct, most frequently in the infundibulum and vagina [12,16,17]. The distribution of heterophils within the reproductive tract is less clear, although early reports indicate the presence of eosinophilic cells in the ovarian medulla [3].

In addition to a role in immunity to infection, ovarian macrophages and/or MHC class II positive cells are believed to be involved in the development of ovarian follicles and particularly in the regression of postovulatory follicles [13,16,18,19].

11.3.4 Local and systemic changes to the immune system at the onset of sexual maturity in hens

The onset of sexual maturity has pronounced effects both on the cellular population of reproductive tract-associated immune system and on the immune system in general. Studies in commercial laying hens that commence egg-laying at 18–20 weeks of age show that splenic populations of CD4⁺, CD8⁺, and $\gamma\delta$ T cells decline between 16 and 20 weeks of age leading to generalized suppression of

cellular responses. The populations of these cells also decline in the ovary, infundibulum, and magnum [11]. Furthermore, the organized structure of lymphocyte aggregates disappears. There is little change in the B lymphocyte populations. By 24 weeks of age, with the exception of $\gamma\delta$ T cells, the populations of T lymphocytes both in the spleen and reproductive tract begin to recover. These changes have an effect on susceptibility to infection and on vaccination, discussed later in this chapter.

Several groups have shown that, unlike lymphocytes, macrophage numbers increase in the reproductive tract at point-of-lay. Macrophages were found in higher numbers in the ovary of young laying hens compared with immature or older birds [19]. Similarly, macrophage numbers increase to a peak in the oviduct following the onset of sexual maturity [20], a finding recently confirmed [11]. Any functional change associated with the increase in immune cell numbers in the reproductive tract of hens following the onset of sexual maturity is not yet known. It has been proposed that these changes prevent infection or “clean” the oviduct at the commencement of egg laying, but this is pure speculation in the absence of any functional studies.

Attempts have been made to simulate the onset of sexual maturity through administration of sex steroids and in particular the estrogen diethylstilboestrol (DES) [12,16]. However, the changes observed are somewhat contradictory to more recent studies that follow the natural development of hens. The earlier studies suggest that raised levels of estrogens lead to increases in both T cells and Ig-secreting cells [16,18]. Large granular lymphocytes are first detected in the oviduct at 9 weeks of age and increase to a peak between 21 and 24 weeks of age following sexual maturity [12]. Administration of DES also increases lymphocyte numbers in the oviduct. In contrast, lymphocyte numbers dropped in both ovary and oviduct at point-of-lay in naturally developing hens [11]. It may be argued that the levels of hormones administered have little significance to real physiological levels and their use is evidently detrimental to the health of experimental hens. However, given the difficulty in maintaining experimental animals for long periods experimentally, such approaches that mimic the hormonal changes at sexual maturity have some merit but clearly need considerable refinement.

11.3.5 The innate immune system and the reproductive tract

In recent years, there has been considerable progress in understanding the role of the innate immune system in the reproductive tract. Our understanding of the importance of secreted antimicrobial peptides, in particular β -defensins, and the

distribution of toll-like receptors (TLR) and other pattern-recognition receptors in the avian reproductive tract has developed considerably since the earlier editions of this book.

TLRs 1–2, 2–1, 2–2, 3, 4, 5, 7, 15, and 21 are expressed in the ovary and vagina [21,22]. TLRs 1–2, 2–1, 2–2, 3, 4, 5, and 7 were found throughout the oviduct, though levels of expression were highest in the uterus and vagina [23]. Stimulation with lipopolysaccharide (LPS) leads to increased expression of TLR4 in the oviduct and vagina and increased expression of both TLR4 and TLR15 in the ovary [22,23]. Stimulation with LPS also leads to the expression of the proinflammatory cytokines interleukin (IL)-6 and IL-1 β and the chemokine CXCLi2 in the ovarian follicles and stroma [24]. There is also a recruitment of heterophils and CD4⁺ cells to the theca layer of the ovarian follicles, though both expression of cytokines and cell recruitment appear fairly transient. The administration of a range of TLR ligands to the oviduct has shown a functional role in recognition of a wide range of bacterial and viral expressed molecular patterns that lead to expression of IL-6 and IL-1 β along with the expression of some β -defensins [25]. Inactivated infectious bronchitis virus (IBV) stimulates the expression of TLR-3, TLR-7, and MDA-5 which recognize viral RNA and lead to cathelicidin and defensin production [26].

Antimicrobial peptides are expressed in the reproductive tract epithelium and may play a key effector role in protection. The antimicrobial histone proteins H1 and H2B are expressed in the ovary and oviduct [27] but the role of β -defensins has provoked more interest. As many as 11 avian β -defensins or gallinacins are expressed in the reproductive tract of the chicken [22]. Gallinacins 4, 7, and 9, all of which are active against *Salmonella*, are expressed throughout the ovary and oviduct [28]. Gallinacins 3, 11, and 12 [29,30], which are also expressed throughout the reproductive tract, show increased expression after administration of LPS. Immunolocalization of the defensin proteins, however, indicates that little protein is secreted in the upper oviduct and that the presence of the egg in the uterus also leads to lower levels of defensin secretion, but that defensins may be incorporated into the egg membrane and shell thereby offering some protection to the egg. In addition to the gallinacins, a novel defensin family termed ovodefensins, related to β -defensins has been described [31]. Gallin, a member of the ovodefensin family, was identified in egg white through proteomic approaches [32]. Recombinant gallin has antimicrobial activity against bacteria and is thought to contribute with other peptides to protection of the developing embryo. Gallin is produced in the magnum and shell gland areas of the oviduct where it may be incorporated into developing eggs [31] (for more details see Chapter 13).

It is clear that the reproductive tract of the chicken has mechanisms to recognize the threat from pathogens and to respond through signaling via pattern recognition receptors such as TLRs and production of chemokines

and cytokines. There also appears to be a formidable arsenal of defensins and cathelicidins produced in the reproductive tract. It is perhaps worth noting that the greatest levels of both defensins and TLR expression are in the lower parts of the tract. We speculate that this structure affords significant protection to ascending cloacal infections, though may be less effective in dealing with transovarian infections. There is also recent evidence of expression of TLRs and defensins in the testes that increase in response to *Salmonella* infection [33].

11.3.6 The reproductive tract immune system in infection

11.3.6.1 Bacterial infections of the reproductive tract

A number of bacteria may infect the reproductive tract of chickens, including some strains of *Escherichia coli* that lead to egg peritonitis and salpingitis [34]. However, the most studied and best-described bacterial infection of the reproductive tract is *S. enterica*. Two serovars have a particular affinity with reproductive tract infection. These are *S. enterica* serovar Enteritidis, the serovar most associated with human food-borne salmonellosis, and serovar Pullorum, the cause of the systemic pullorum disease. Both serovars may be vertically transmitted by egg infection.

Much work on avian salmonellosis has concentrated on egg infection by *S. Enteritidis*, primarily as a consequence of its importance as a major food-borne zoonotic infection. A number of bacterial virulence factors have been implicated in this process [35,36]. The bacterium infects both developing and newly formed eggs in the ovary and oviduct [36,37]. The rate of egg infection with *S. Enteritidis* is low after experimental and natural infection [36,37]. The exact nature of the infection is poorly understood, as it is not completely clear whether *S. Enteritidis* infects at a young age, persists, and infects eggs, or if infection occurs during the laying period. Although some studies suggest birds infected at a young age develop a carrier state that leads to egg infection [36], others suggest that it is almost impossible to reproduce this experimentally [38]. Given the low frequency of infection, it seems likely that a carrier state could develop in a small number of animals and is greatly influenced by the genetics of the host. Infection of the reproductive tract and transmission to eggs is considerably more frequent with *S. Pullorum*, where about half of experimentally infected hens develop a carrier state with the reproductive tract becoming infected [39]. In this case, the site of infection is predominately within splenic macrophages, with reproductive tract infection becoming prevalent only at sexual maturity. The ability of *S. Pullorum* to persist within macrophages is dependent on the *Salmonella* pathogenicity island 2 (SPI2) type III secretion system, a bacterial apparatus that translocates bacterial effector proteins or toxins to the host

cell. The primary function of SPI2 is to interfere with intracellular trafficking within macrophages, thereby inhibiting phagolysosome fusion [40]. In mammalian species the *S. Typhimurium* SPI2 system modulates the immune response, in particular inducing IL-10-mediated regulatory responses in maintaining persistent infection [41]. It is possible that the *S. Pullorum* system plays a similar role. Type III secretion systems are also important in *S. Enteritidis*, with both SPI2 and pathogenicity island 1 effectors involved in invasion and survival in macrophages and oviduct epithelial cells in vitro [42] and the SPI2 system playing a key role both in intestinal colonization and systemic spread in vivo [43].

11.3.6.2 The immune response to *Salmonella* infection of the reproductive tract

The immune response to *Salmonella* infection can be divided into responses that occur systemically during infection or vaccination and local responses that occur within the reproductive tract. We will deal first with the systemic response. Studies with killed and live *S. Enteritidis* vaccines indicate the generation of both humoral and cellular responses [44] [45–47]. Oral infection with wild-type *S. Enteritidis* has indicated that a strong humoral response is generated [38]. In common with *S. Typhimurium* infection of chickens, clearance of bacteria from systemic sites occurs two to three weeks postinfection (pi), a time that coincides with peak expression of interferon (IFN)- γ and antigen-specific T cell proliferation [48,49]. There is a peak of IFN- γ , IL-12 and IL-18 expression in the spleen at 2 weeks postoral infection with *S. Enteritidis* [50] and, as is the case with *S. Typhimurium*, there is a peak in antigen-specific T cell proliferation at 3 weeks pi [51]. In sexually immature birds, the biology of infections with *S. Enteritidis* and *S. Typhimurium* and the immune responses against both pathogens are very similar. As yet, it is not clear which elements of the response are protective against subsequent infection. Although it appears that cell-mediated responses are important, the initial success of killed bacterins as vaccines in the control of *S. Enteritidis* in layer flocks in the United Kingdom suggests that antibodies also play an important role in protection to reinfection.

Persistent *S. Pullorum* infection also induces antigen-specific T cell responses and particularly strong humoral responses with specific IgY titers of 1 in 60,000 or greater [39,52]. The T cell response to mitogenic stimulation is halved in infected birds compared with controls, suggesting an overall suppression of the cellular response [52]. Early in *S. Pullorum* infection the increase in expression of Th1-associated cytokines (IFN- γ , IL-18) seen in the spleen during infection with serovars Typhimurium [48,49], Gallinarum [53], and Enteritidis is absent, though

interestingly there was a significant increase in IL-4 at the same time-point [50]. This suggests that *S. Pullorum* generates a Th2-dominated response, which is in keeping with its infection biology in establishing persistent intracellular infection accompanied by a strong antibody response, though it appears that IFN- γ -mediated responses are generated later in the infection. We have previously hypothesized that *S. Pullorum* persists in macrophages whilst suppressing Th1 responses, requiring the SPI2 type III secretion system for persistence, whilst the host produces a Th1-mediated response in an attempt to clear the infection. The balance of host response and pathogen immunomodulation results in the carrier state. Such a system has also been shown in mice persistently infected with *S. Typhimurium*, where administration of anti-IFN- γ antibodies pushes the equilibrium in favor of the pathogen, resulting in severe systemic infection [54]. In the *S. Pullorum* carrier state the equilibrium is broken by the onset of sexual maturity. In hens the onset of lay is accompanied by a dramatic decrease in splenic T lymphocyte numbers [11] and in antigen-specific and mitogen-stimulated T cell proliferation [52]. This has the effect of allowing replication of the *Salmonella* in the spleen and liver, along with the spread of infection to the ovaries and oviduct. Moreover, the fall in numbers of lymphocytes, and in particular $\gamma\delta$ T lymphocytes, in the reproductive tract may mean that infection is more readily established within the ovary and upper oviduct. Although there is as yet no function assigned to the $\gamma\delta$ T cells in the reproductive tract, they play a key role in the initiation of protective responses to systemic and gastrointestinal *S. Enteritidis* infection [55]. From a public health viewpoint, hens show increased susceptibility to *S. Enteritidis* challenge at point-of-lay, and the efficacy of a live attenuated vaccine is reduced to the extent that egg infection may be produced by systemic challenge with *Salmonella*. Immunosuppression at the point of lay offers a clear “window of opportunity” for pathogens to infect or reemerge from latency. Indeed there is considerable anecdotal evidence of increased susceptibility to helminth, protozoal, and viral infections at this time-point as well as direct evidence of recrudescence of both *Salmonella* and IBV associated with the onset of lay [56]. In carrier state infections, the mechanisms by which *Salmonella* is transported to the reproductive tract from the spleen are unclear, though it is conceivable that, during the influx of macrophages to the reproductive tract at the onset of lay [17], cells infected with *Salmonella* are also directed to this site.

Our knowledge of the local immune response in the reproductive tract to *Salmonella* infection is even less clear, though there have been advances in understanding the innate response that is described earlier in this chapter. A particular limitation of the studies that have been

reported is the use of intravenous or intraabdominal challenge of old laying hens with a high inoculum of *S. Enteritidis* [17,57,58]. This infection model does lead to reproductive tract infection, though only as part of a transient general systemic infection with considerable pathology and it is very unlike the situation found in egg infection associated with younger birds. Nevertheless, these studies have shown that there is a surge of both CD4⁺ and CD8⁺ T cells into the ovaries and oviduct 7 days pi, peaking at 10 days pi, whereas B lymphocytes peak at 14 days pi [17,57]. Both return to preinfection levels by 21 days pi. In contrast, macrophage numbers decrease initially, followed by an increase. Other recent studies suggest that the influx of T lymphocytes to the ovaries is very rapid, occurring within 12–24 hours pi [58]. *Salmonella*-specific IgY and IgM antibodies are also secreted into the oviduct following infection, determined by enzyme-linked immunosorbent assay of oviductal washings, with a peak at 14 days pi although, perhaps unexpectedly, there was only a transient change in levels of IgA [59]. In these studies, the increases in both antibodies and lymphocyte numbers coincided with bacterial clearance. Due to the nature of the experimental model, it is not clear whether this is a reflection of a specific reproductive tract-associated response or merely an extension of the systemic response to *Salmonella*. Antibodies were also been detected in serum and secreted into the reproductive tract in vaccinated and unvaccinated chickens challenged intravaginally [60].

Little is known about the nature of infection and immunity in the male reproductive tract. While it is clear that *S. Pullorum* does not infect the male reproductive tract in the same way as in hens [52], the behavior of *S. Enteritidis* is less clear, although there is expression of TLRs and defensins in the testis following *Salmonella* challenge [61]. There are two reports suggesting that *S. Enteritidis* may be transmitted sexually via semen and that this route enhances the ability of *S. Enteritidis* to persist in the female reproductive tract [62,63]. Intriguingly, a recent study indicated that seven TLRs and nine defensins are expressed by chicken sperm cells and that the expression of four of the defensins increased following LPS challenge [64]. The authors postulate that the defensins may offer sperm cells protection to pathogen challenge within the female reproductive tract.

11.3.6.2.1 Viral infections of the reproductive tract

Thus far, no viruses have been identified that exclusively replicate in the reproductive tract of birds, although many viruses causing systemic infections may also replicate in the female and male reproductive tracts during the period of active viral replication. Transient transmission of the virus through the

embryonated egg may also occur during the viral replication period. Normally, the development of general antiviral immune responses results in the elimination of the agent from the reproductive organs so these infections will not be discussed in this section. However, in some instances virus may become established in the reproductive organs as a latent and/or persistent infection, even in the presence of virus-neutralizing (VN) antibodies.

The following viruses are of particular interest for the establishment of latent and/or persistent infection in the reproductive tract: avian leukosis virus (ALV), reticuloendotheliosis virus (REV), chicken infectious anemia virus (CIAV), and two genera of adenovirus (Aviadenovirus and Atadenovirus). The mechanism for the establishment of persistent infection is reasonably well understood for ALV and is probably similar for REV [65,66]. Congenital infection with ALV or REV results in the development of immunological tolerance to these pathogens. These birds become persistently viremic and are unable to produce VN antibodies (referred to as V^+A^-) or to clear the virus from the reproductive tract. In V^+A^- chickens, virus is transmitted from the albumen-secreting glands in the oviduct to the developing egg. Infection at hatching from congenitally infected chickens results mostly in V^-A^+ chickens. Some of these birds may become intermittent shedders of virus into the eggs. The reasons why the VN antibodies are unable to completely clear the infection in the uterus are not known.

Avian adenoviruses, belonging to the genera Aviadenovirus and Atadenovirus, are reported to establish latent infections, which may result in subsequent reactivation and vertical transmission. Fadly et al. [67] reported that aviadenovirus could remain latent in a specified-pathogen-free flock for at least one generation while the birds were positive for VN antibodies. McFerran and Adair [68] suggested that egg drop syndrome (EDS) virus, belonging to the genus Atadenovirus, can also establish a latent infection. The concept of latent adenoviruses has been confirmed by studies with human adenoviruses [69,70].

The location of latent Aviadenovirus or Atadenovirus in the reproductive tract has not been studied, though Smyth et al. [71] found viral replication of EDS virus in the infundibulum and especially the pouch shell gland after experimental infection, as well as limited infection in lymphoid tissues. The latter location may be important for latent infections in view of the report that human adenovirus can maintain itself in some dividing lymphocyte cell lines for at least 150 days pi. The mechanisms controlling virus replication and vertical transmission have not been elucidated for avian and mammalian adenovirus, although increased production of E3 protein in absence of E1A products may be important in human adenovirus latency [69]. For avian adenoviruses it has also been suggested that steroid hormones could be involved, based on

reactivation of Aviadenovirus and Atadenovirus when VN antibody-negative hens reach sexual maturity [68].

CIAV, the only member of the genus Gyrovirus of the Circoviridae, can be detected in the reproductive tract of hens in the absence or presence of VN antibodies, suggesting the presence of latent virus. The reasons for the lack of virus clearance are not understood but, based on serological data, it is unlikely to be caused by the induction of tolerance. Latent virus or viral DNA can be transmitted to the embryo with little or no consequences for the embryo and this latent transmission may occur over several generations [72–74]. In contrast, vertical virus transmission during active infection in the hen results in clinical disease in young chickens [75]. Viral reactivation is likely to be under control of steroid hormones, such as estrogen, interacting with hormone response elements, which have been demonstrated in the promoter region of CIAV [76]. Activation is also controlled by strong repressor elements in this region [77]. The only evidence for the presence of latent infection is the occurrence of seroconversion after sexual maturity, which can occur in a few birds or up to 100% of the flock before the end of the first laying cycle.

IBV, particularly the highly virulent QX variant of this coronavirus, can cause an active infection in the reproductive tract leading to a severe drop in egg production (reviewed by [78]). Infectious bronchitis is mostly controlled by vaccination and numerous studies have addressed systemic immune responses, but very few have investigated local responses in the reproductive tract. Sevoian and Levine [79] reported significant increases of plasma cells, lymphocytes, and heterophils into the lamina propria of the oviduct between 6 and 76 days pi suggesting the local production of antibodies in response to infection. Almost 30 years later Raj and Jones [80] found anti-IBV IgY and IgA in oviduct washings and demonstrated that these antibodies could be produced in the oviduct. It is likely that vaccine-induced IBV-specific (memory) CTL [81] and defensins and cathelicidins play an important role in protection. Recently, expression of key pattern recognition receptors, β -defensins, cathelicidins, and IFN- β , α , β , and γ in the uterine mucosa of hens following an intra-oviductal challenge with IBV indicating the capacity for a strong innate and potentially adaptive response [26].

The immune responses in the male reproductive system to viral infection are less well understood than the responses in the female reproductive tract. Transmission through semen has been described for these viruses but their importance for virus spread is not known.

11.3.6.3 Responses to vaccination in the reproductive tract

In previous editions of this book we discussed the limited knowledge we have of vaccine responses in the

reproductive tract, particularly to *Salmonella* given the extensive use of vaccines to control egg infection, although as we discuss below there are many fundamental gaps in our basic understanding of the response, there are a few studies that have begun to address this. *Salmonella*-specific IgA [82] and IgY antibodies [83] have been detected in the oviduct following the use of killed vaccines. Live attenuated and inactivated Newcastle disease virus vaccines have been shown to elicit IgY responses throughout the reproductive tract and higher levels of IgA and IgM in the magnum and infundibulum of the oviduct [84]. Although limited, these studies show there is a localized adaptive mucosal response in the reproductive tract following vaccines.

11.3.6.4 The chicken as a model-understanding immunity in ovarian cancer

The laying hen has a prolific rate of ovulation and along with humans is the only animal to develop spontaneous ovarian cancer. As such in the last decade, the hen has been widely adopted as a model for ovarian cancer as reviewed by Johnson and Giles [85]. As part of this research immunological parameters associated with tumor formation have been investigated. Some of the earliest research found hens with ovarian tumors produce both antitumor antibodies and autoantibodies to the ovary and oviduct which as thought to be associated with pathology [86,87]. More recently it has been shown these tumors are associated with chronic inflammation and high expression of IL-1 β and IL18 [88].

11.3.6.5 What do we need to know—directions for future research?

In the earlier editions of this book we described the reproductive tract-associated immune system as something of a “black box” given our poor understanding of its function. Since then, research has begun to address the role of the innate immune system in the female tract and we have a clearer understanding of the changes that underlie point-of-lay immunosuppression. Nevertheless, there are still considerable gaps in our knowledge, particularly in relation to function and regulation. In particular, the following questions still need addressing:

11.3.6.6 What are the functions and phenotypes of the cells in the reproductive tract?

Although our understanding of the structure and composition of the reproductive tract immune system has developed greatly in recent years, we have still only scratched the surface in terms of defining the function of these cells.

We now know that there are TLRs in the reproductive tract and stimulation with LPS, killed virus and a range of ligands or challenge of oviductal cells with *Salmonella* elicits expression of a range of cytokines and chemokines and elicits the production of antimicrobial peptides. However, we know little of the phenotypes of CD4⁺, CD8⁺, and $\gamma\delta$ T cells throughout the reproductive tract, other than their distribution and relative numbers. Furthermore, we have no understanding of the regulation of immunity in the reproductive tract. For example, while we know there are inflammatory responses to stimulation with LPS, these are short-lived. Could these be as tightly regulated as inflammatory responses in the gastrointestinal tract [49]? As the toolkit of avian immunology expands, our understanding of the immune system within the avian reproductive tract should be further unlocked.

11.3.6.7 How does the immune tissue of the reproductive tract integrate with the rest of the immune system?

The extent to which the reproductive tract integrates with the immune system as a whole or functions as a local system is not known. It is not clear whether localized infection in the reproductive tract induces substantial systemic responses, or whether systemic infections induce a substantial adaptive response in the reproductive tract, including responses induced by vaccination. Our current lack of knowledge of the reproductive tract immune system makes it impossible to predict if a local infection of the reproductive tract induces a cytokine response that leads to a more extensive systemic response.

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Impact of the gut microbiota on the immune system

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12.1 Introduction to the microbiota and avian immune system

Chickens, like all production animals, are *meta*-organisms that maintain a diverse population of microorganisms on their barrier surfaces, collectively named the microbiota. The chicken gastrointestinal tract is rich in microbial biodiversity, playing home to hundreds of ≥ 500 bacterial phylotypes or ~ 1 million bacterial genes, which equates to 40–50 times the number in the chicken genome [1–3]. Over 1000 different bacterial species inhabiting the healthy chicken large and small intestine have been identified [4,5]. Furthermore, also within the chicken gut, although largely ignored until recently, reside other diverse microorganisms, such as viruses and fungi, that coexist with the host in a mutually advantageous relationship [6–10]. Most studies seeking to elucidate the relationship between the chicken microbiota and health and disease have been fundamentally observational; the gut microbiota compositions of diseased and healthy birds compared and subsequently associated with clinical signs and/or performance (weight gain, feed intake, feed conversion ratio).

Our understanding of the interface between the microbiome and avian host immunity is almost exclusively based on correlational studies which do not establish causality. For example, there are dozens of reviews describing the effects of feeding pre- and probiotics to chickens and the immune response [11–13]. Clearly, the need to identify the causal relationships and mechanisms by which the gut microbiome influences the avian host immune system, both locally and systemically, is vital and remains an area for research in the future.

Evidence from humans and murine models has clearly established the causal role of the gut microbiota in shaping, educating, and developing the immune system [14,15]. To

date, virtually none of these microbes have been shown to modulate specific immune parameters in chickens. Therefore, large segments of this review will be correlative based on data generated in mammals and not yet confirmed in chickens.

The host microbiota embodies the microbial communities present on the skin and the mucosal surfaces of the body. The healthy lung and reproductive tract of chickens and turkeys have historically been considered sterile, but recent culture-independent analyses have identified respiratory and productive tracts microbiota [16–19]. The interaction between these colonizing microbes and the avian host may be considered mutualistic since various physiological host functions are positively influenced by the microbiota. To date, there have been no reports characterizing the skin microbiota of poultry. Interestingly, the gastrointestinal (GI), respiratory, and reproductive tracts are connected by a common mucosal system suggesting that modulation of the gut microbiota may not only modulate the immune responses of the GI tract but may also impact the immunity of the lung and reproductive organs, thus affecting the lung and reproductive health and diseases. Fecal microbial transplants (FMTs) of swine have demonstrated protective effects on primary respiratory or urogenital infections [20–24].

With the advent of next-generation sequencing technologies, our understanding of the complex relationship between the host and its resident microbes has been fundamentally changed. The microbes that inhabit the intestinal barrier surface are not the result of random colonization but are shaped by interactions with the host and are therefore unique to each tissue site [25]. Chickens have evolved in the context of microbial colonization; therefore, there are multiple host functions dependent upon cues derived from the microbiota [25,26]. Not least of these is the

immune system, which can sense the microbiota directly via its expression of microbe-associated molecular patterns, enzymatically derived metabolites, and foreign antigens [14,25]. Thus, the immune system relies upon the microbiota for protection against invading pathogens, but this close relationship comes with a significant risk in that if it becomes perturbed, responses against the microbiota can contribute to disease.

12.2 Microbiota, metagenome, and microbiome

The collective genes that an individual's intestinal microbiota encompasses are known as the microbiome. The terms microbiota and microbiome are often incorrectly used interchangeably in the literature. Microbiota refers to the microorganisms that live in an environment such as the intestine and includes viruses, bacteria, fungi, and protozoa in the environment [27]. In the literature, one also sees other phrases such as commensal bacteria and gut microbiota correctly used in place of microbiota. The microbiome refers not only to the microbiota but also the collective genes, genomes, and gene products (microbiota metabolites and proteins) found in the local environment [27]. So, discussing the microbiome is far more complex than the microbiota. Lastly, the metagenome defines the genetic potential of the microbiota that incorporates the genomes of all microbe populations and the genes within the microbial populations, including the plasmids within the different bacterial populations of the microbiota [27].

As mentioned previously poultry are naturally adapted to hosting a complex GI microbial community. The upper small intestine contains bacterial cell densities of approximately 10^5 CFU per gram of gut contents with cell densities increasing through the small intestine with $>10^9$ per gram of contents in the lower small intestine, particularly in the ileum [1]. Bacterial cell densities can reach $>10^{11}$ CFU per gram of contents in the ceca [3].

The GI tract, or "gut," regulates homeostasis of the microbiological, physiological, and physical functions that allow the host to endure infectious and noninfectious stressors that it encounters [28–32]. Because the gut has the greatest surface area separating the environmentally exposed lumen and the internal subepithelial tissue, it is constantly exposed to infectious and noninfectious stressors making it an active immune organ containing more resident immune cells than any other organ in the host. The gut mucosal immune system, a highly regulated network of innate and acquired elements, provides a remarkable ability to respond and modify to these extremely diverse encounters [33,34]. The development of the different divisions of the immune response has corresponded with the acquisition and maintenance of a symbiotic

microbiota. The microbiota trains, stimulates, and functionally adjusts the different features of the immune system [35,36].

12.3 GI tract and immune system of poultry

The GI tract lumen can be considered as being part of the external environment. One of the most important roles of the gut is providing a barrier between the internal and external environment, as the GI tract is the site of colonization and invasion of many pathogenic organisms. Through this and several other functions, the GI tract has an important role in the immune function of a bird [37,38]. Posthatching is a vital time for the development of the gut immune system of the bird. The immune system must differentiate between food, beneficial bacteria, and harmful pathogens, so there is a fine balance between tolerance and response to antigens. This is fundamental to gut health as a prolonged immune response can cause problems such as villus atrophy. The sequential development of various phases of intestinal immunity have been characterized in broiler chickens during the first weeks of posthatch: (1) innate development and influx of immune cells; (2) immune differentiation and specialization; and (3) maturation and immune regulation [39–41].

12.3.1 Intestinal barrier system

The description and function of intestinal innate defenses have been detailed in mammals but have not been well characterized in chickens. The intestinal innate defenses are characterized by a "mucosal firewall," a system of barriers that separates the luminal side of the intestine from the subepithelial tissues [14,42]. The reliability of the mucosal firewall is vital for the interactions between the immune system components and the intestinal contents. The first component of the mucosal firewall is the microbiological barrier where the microbiota live in or at the upper mucus layer. These commensal bacteria function to provide colonization resistance against pathogen colonization, produce metabolites/components that modulate immune signaling, and promote immune homeostasis [14,43,44]. The second firewall is the chemical barrier consisting of the mucus overlaying the gut epithelium. The mucus regulates contact between the commensal bacteria and the epithelial cells. This division between the epithelium and commensals is achieved by the activity of the mucus produced by goblet cells in the epithelium, antimicrobial peptides released by the epithelial cells, and mucosal IgA produced by B cells in the intestine [44,45]. The third component of the firewall is the physical barrier provided by the single-cell epithelial cell layer. The intestinal epithelium is a single enterocyte cell layer that

assists the absorption of nutrients while providing a physical barrier that prevents both pathogen invasion and extra-intestinal translocation of commensal microbes. Besides being the primary barrier preventing a microbial breach of the intestine, the epithelial cells should also be considered part of the cellular component of the innate immune response possessing pattern recognition receptors (PRRs) for sensing microbial-associated molecular patterns (MAMPs), but also capable of producing cytokines and chemokines to drive an inflammatory response against pathogen infection. The final component of the mucosal firewall is the immunological barrier where the professional immune cells (macrophages, dendritic cells, lymphocytes) reside in the lamina propria [46]. Further innate sensing of microbes is conducted by the macrophages and dendritic cells which can present antigens to T cells resulting in the differentiation and activation of various T cell subsets (Th1, Th2, Th17, or Treg) [46]. Specialized epithelial cells (goblet cells, Paneth cells, and M cells) of the GI tract function together with lymphoid, myeloid, and stromal cells to secrete mucus, antimicrobial peptides, IgA, and chemokines that limit direct contact between the epithelium and infectious agents and activate target cells that mediate innate defenses [47–51]. The importance of these epithelial defense mechanisms is highlighted by the ability of enteric pathogens to target these mechanisms to achieve invasion and dissemination [52–56].

Newly hatched chickens have some degree of immune resistance via the innate immune mechanisms that involves macrophages, dendritic cells, heterophils, and natural killer cells, which recognize and control pathogens [57,58]. The innate immune system is relatively nonspecific and has recently been found to be able to mount memory response, known as trained immunity, which increases host resistance to reinfection [59]. Conversely, the acquired immune system is specific and has memory and is mediated by B and T lymphocytes. Macrophages are part of the innate and adaptive immune system of the broiler and can destroy pathogens directly [60] as well as secrete cytokines and chemokines that act as mediators of the inflammatory process. Lymphoid cells, such as natural killer cells, T cells and B cells, can be found in key immune tissues in the GI tract called the gut-associated lymphoid tissue (GALT). GALT is crucial in developing the immune system of the GI tract and is the largest component of the birds' entire immune system [61,62]. The Bursa of Fabricius, Peyer's Patches, and cecal tonsils are all parts of the GALT. The Bursa of Fabricius is a sac-like structure located between the cloaca and sacrum. The Bursa of Fabricius is the key lymphoid organ of the bird, and is responsible for the development and maintenance of the B-cell [63,64]. Its removal during embryonic life can prevent the development of B cells and the production

of antibodies [65]. Peyer's Patches are made up of lymphoid tissues situated at numerous sites along the small intestine [66]. One Peyer's Patch is always situated in the ileum, 5–10 cm from the ileum–cecal junction [67]. Peyer's Patches are involved in local protection in the GI tract as regulators of the gut immune response against intestinal pathogens and contain T and B cells and are the main source of precursors of IgA-producing cells [68–70]. Cecal tonsils contain T and B lymphocytes and are located in each cecum where they join the ileum.

Studies have described how altering the microbiome affects GALT, while others have focused on how GALT regulate the microbiome and prevent invasion [71]. The relationship between host immune tissues and GI microbes is multifactorial and sensitive to several genetic and environmental factors.

Many of the interactions between host and microbial cells are mediated by host receptors that recognize a broad array of microbial cell components (MAMPs), such as flagellin, glycoproteins, and lectins [72]. These PRRs are expressed on a variety of cell types, particularly antigen-presenting immune cells like dendritic cells and macrophages, and some enterocytes; they include groups of receptors such as toll-like receptors (TLR), nod-like receptors (NLR), and C-type lectin receptors [72,73]. The signaling pathways activated by interactions between MAMPs and PRRs can be activating or inhibitory, and lead to a variety of different immune responses [74].

12.4 Influence of the microbiota in immunity

12.4.1 Germ-free chickens

Relatively few studies have evaluated the immune development in germ-free and gnotobiotic chicks. The development of GALT is restricted in the absence of microbes [75,76]. In germ-free chickens, there was a dramatic delay in the production and presence of serum and intestinal IgA [77]. Likewise, there was a striking lack of B cell development in the bursa of Fabricius, lamina propria, and cecal tonsils of germ-free chickens when compared to conventional chickens [77,78]. Goblet cell density and MUC2 expression were decreased and protective ability of mucin in the ileum of germ-free chicks was different in the germ-free birds [79–81]. Lastly, both CD4 + (T helper) and CD8 + (T cytotoxic) cells were not found in the intestinal tissues of germ-free chickens [77]. Most recently, Volf and colleagues [82] found a total lack of immunoglobulins in the cecum of germ-free chickens when compared to conventional birds. Furthermore, there were reduced protein expression involved in redox potential, extracellular matrices, and arginine metabolism in the ceca of the germ-free birds [82].

12.4.2 Antibiotic-treated chickens

Until 2006 when the European Union banned the use of antibiotics as growth promoters (AGPs) in animal feed, subtherapeutic doses of antibiotics were used in poultry production worldwide for growth promotion [83–85]. Although the exact mechanism of action of the growth-promoting ability of AGPs is still unclear, there are three theories that have been proposed: (1) AGPs remodel the microbial diversity and relative abundance which then imparts an optimal microbiota for bird growth [86], (2) AGPs act as antiinflammatory agents that induce alterations in the microbiota that diminish the effects of inflammation [87], and (3) AGPs alter the microbiota composition such that prevent the induction of inflammatory responses in the gut [88–92].

Other studies have shown that the effects of AGPs on the microbiota composition and immune development occurred during the starter phase of the broiler: 1–21 days posthatch [93,94] when the immune system is rapidly developing and is profoundly affected by the early microbial colonization of the gut [95]. For example, a short (24 hours) exposure of day-old chicks to therapeutic levels of amoxicillin had a limited effect on the microbial composition after day 5 posthatch but induced a reduction in immune gene expression and decreased macrophages numbers in the jejunum for 2 weeks [93]. Interestingly, this antibiotic treatment resulted in increased intestinal barrier function perhaps implying a developmental priority of the gut from immune development to strengthening barrier function since the barrier function is the first “line of defense” against pathogens [14]. Curiously, therapeutic levels of antibiotics given to broilers at the end of the starter phase-beginning of the grower phase (15–20 days posthatch) also only induced a temporary (2–3 weeks) effect on microbial diversity and composition while decreasing the expression of immune genes (IL-2, IL-8, IFN- γ , IL-1 β) [96].

12.4.3 Fecal microbial transplants

FMT supports complex microbe–microbe interactions which are essential for successful bacterial colonization, such as nutrient competition and cross-feeding of metabolites but are not represented when using single- or multiple-strain applications. FMT is not a new concept for the poultry industry as evidenced by competitive exclusion where newly hatched chickens could be protected against colonization by *Salmonella enteritidis* by dosing a suspension of gut contents derived from healthy adult chickens [97]. This colonization resistance concept of adding beneficial bacteria to the intestine has led to the development of probiotics and prebiotics and has been recently reviewed extensively and so will not be reviewed here [27,98–102].

More recently, it was shown that early exposure (day-of-hatch) to adult-derived microbiota shaped and accelerates the maturation of cecal microbiota, which is paralleled by an increase in NK cells and enhanced NK cell activation in the intestine [103]. FMT of day-of-hatch chicks with adult microbiota resulted in increased natural antibody titers in layer chickens [104] as well as increased mRNA levels of proinflammatory cytokines in the intestine of broiler chickens [105]. To take FMT a step further and a more practical approach with poultry would be spraying or brushing of eggs and or newly hatched chicks with the gut microbiota from healthy adult birds [106]. The topical spray treatment of eggs with adult cecal contents altered broiler chicken microbiota and accelerated the normal microbiota development [107]; whereas, swabbing of the egg surface once during incubation with diluted adult cecal content did not lead to significant differences in alpha diversity nor in the pattern of bacterial colonization between treated and control birds [108]. Although the inoculation technique may have led to the discrepancies in the gut colonization results, neither trial evaluated the effects of the FMT treatment of the pre-hatch eggs on the immune development or response of the newly hatched birds.

12.4.4 Layer-type chickens versus broiler chickens

The fecal and cecal microbiota of laying hens is generally more complex than the fecal and cecal microbiota of broilers [109,110] which may explain major functional difference [111] and suggests the basis for differences in performance between the types of chickens. Furthermore, both the innate and acquired cellular response of layer-type chickens is much higher than the response of broilers. Broilers appear to specialize in the production of a strong short-term humoral response and layer-type chickens in a long-term humoral response in combination with a more robust cell-mediated response which may correspond to their longer life expectancy [112,113]. However, there have been no studies reported demonstrating a correlation between the gut microbiota genotype, diversity and abundance, and immune development and function.

12.5 Gut microbiota–immune system communication

The crosstalk that occurs between the host and its gut microbiota affects host metabolism, immunity, and health mediated by dietary nutrients, host and microbiota metabolites, microbial structural components, as well as antimicrobial compounds. Microbiota growth and anatomical location are regulated by the host through the production

of nonspecific antimicrobial peptides such as defensins [114,115], sIgA [116,117], and miRNAs that regulate bacterial transcripts and bacterial growth [118].

The commensal microbes in the intestinal tract sense the local environment to induce biochemical pathways to activate bacterial metabolism that allows them to avoid, alter, and/or survive host innate immune killing. Moreover, some microbial-based molecules can promote specific commensal processes that are beneficial to both host and microbe. Similarly, the host detects the microbes through their production of specific molecules or components with unique molecular patterns that lead to activation of innate and acquired immune responses. Thus, the adaptation of the commensal bacteria (as well as viruses and fungi) living in the intestine of a host has resulted in a mutually beneficial coexistence for both microbiota and host during homeostasis [25,119,120]. The interdependent relationship between host and microbiota pointedly influences the host immune response to induce an immune tolerance to commensal microbes while also maintaining responsiveness to invading pathogens [121–123]. Altering the intestinal microbial communities in chickens disturbs this immune balance and leads to immune dysregulation which facilitates the establishment of infectious diseases such as: (1) acute viral infections including avian influenza, Marek's, Infectious Bursal Disease, and Newcastle Disease [124–129], (2) bacterial infections including *Campylobacter* [130–132] and *Salmonella* [133], and (3) coccidial infections [134,135].

Lastly, in poultry, there is a clear lack of information on the role of commensal microbiota in vaccine efficacy. As of now, there has been a single study that has described shifts in the composition of gut microbiota of chickens that resulted in changes in cell- and antibody-mediated immune responses to vaccination against influenza viruses [136].

12.5.1 Components of the microbiota

The microbiota is directly engaged in maintaining the functional innate immunity of the host. The host immune system consistently senses the intestinal microenvironment to determine the metabolic state and colonization status [137]. In the steady-state, the metabolites and/or components of the commensal microbiota are recognized by various PRRs, including TLRs and NLRs, to regulate intestinal epithelial barrier function, cellular lifespan of phagocytes, and induce secretion of antimicrobial peptides and IgA [137,138]. Furthermore, beneficial bacteria ferment dietary fibers to produce small chain fatty acids (SCFA) which stimulate the production of antiinflammatory cytokines [137,138] that drive the production of regulatory T cells (Tregs). In addition, the microbiota influences the priming signal of the inflammasome

activation that leads to the transcription of IL-6, as well as pro-IL-1 β and pro-IL-18.

In mammals, there is growing evidence that some commensal and symbiotic bacteria have MAMPs that promote a tolerant host phenotype. *Bacteroides fragilis* polysaccharide A, for example, interacts with TLR2 on regulatory T-cells (Tregs) to promote tolerance and inhibit TH₁₇ responses [71,139]. Other bacteria, particularly *Clostridia* species, are able to stimulate the development of Tregs as well [71,139,140], while attachment of segmented filamentous bacteria, notably *Candidatus Sagvella*, in the ileum has been shown to stimulate differentiation of proinflammatory Th₁₇ type cells [140].

12.5.2 Microbial metabolites

It has also become apparent that the intestinal immune system can also detect the metabolic state of the microbiota by recognition of microbial metabolites via their PRRs [137,138]. The microbiota, using several biochemical pathways, metabolizes both diet- and host-derived metabolites that then influence various components of the intestinal immune system. For example, the microbiota converts nondigestible fibers to SCFA (acetate, propionate, butyrate) that have several antiinflammatory activities on chicken immune cells both in vitro and in vivo [141–143]. Butyrate is best known for its beneficial effects on intestinal barrier function, antiinflammatory activity, and as the primary source of energy to intestinal epithelial cells of the large intestine [144–147]. In chickens, butyrate has a minimal direct effect on the expression of proinflammatory cytokine transcription (IL-1 β , IL-6, IFN- γ) or nitric oxide production by macrophages but suppresses the induction of these cytokines by LPS in vitro and in vivo [141,148,149]. Furthermore, dietary supplementation with sodium butyrate increases the expression of multiple host defense peptides throughout the intestinal tract of chickens and the clearance of *S. enteritidis* following an experimental infection [148,150].

12.5.3 Microbial epigenetic modifications

Epigenetics involves genomic modifications through post-translational and posttranscriptional modifications induced by environmental factors but without modifying the nucleotide sequence of the host cell [151]. Epigenetic mechanisms regulate transcriptional control by external environmental including host–microbe crosstalk [152,153]. Since epigenetic events do not alter the DNA, the epigenomic effects are associated with the attachment of different chemical groups to DNA, histones, and chromatin posttranslationally and the epigenetic alterations can persist for several generations [151,154]. These epigenetic alterations affect both the chromatin structure and serve as recognition elements for

proteins with motifs dedicated to binding particular modifications.

Since the gut microbiota plays such a pivotal role in poultry metabolism, microbiota-induced epigenetic alterations by dietary nutrients could be a significant environmental factor affecting poultry performance and health. Based on studies in mammals, microbiota-generated metabolites of dietary components can be epigenetic activators of gene expression that modify or inhibit enzymes involved in epigenetic pathways [152,155–157]. This can best be exemplified by the production of SCFA produced by intestinal microbiota by bacterial fermentation of non-digestible carbohydrates as described previously.

12.6 Gut microbiota: immune homeostasis

Based on a new concept brought forward by Baumler and colleagues, homeostasis of the gut microbiota–immune interactome is a “state of immune competence” [158,159] that involves two complimentary elements: the traditional protective antiinfective immunity and a distinct type of immune response called microbiota-nourishing immunity [158,159]. During homeostasis, the microbiota can effectively inhibit colonization and overgrowth by invading microbes such as pathogens. This phenomenon is called “colonization resistance” and is associated with a stable and diverse microbiota in tandem with a controlled lack of inflammation, and involves specific interactions between the mucosal immune system and the microbiota [160–163]. According to Byndloss and colleagues [158], the first step in intestinal immune protection against pathogens is microbiota-nourishing immunity which functions in maintaining a balanced intestinal microbiota so that colonization resistance may be initiated. Microbiota-nourishing immunity involves the activation of two components of colonization resistance: direct (microbiota origin) and indirect (host origin) activities which function to protect the “microbial organ” within the host intestinal organ. The second component of the intestinal immune response is the activation of the conventional antiinfective immunity which prevents colonization and growth of pathogens through antimicrobial peptide production, inflammasome activation, and the expression and production of IL-17, IL-10, and IL-22 [14,71,163–167]. As described above, in a healthy microbiota, the commensal microbiota provides colonization resistance against potential pathogens in the gut. However, when perturbations (antibiotics, dietary changes) disrupt the commensals, colonization resistance is compromised. Pathogens like *Salmonella typhimurium* and *Clostridium perfringens* can exploit the ecological opportunity by the availability of resources to expand. Thus, the pathogens change the playing field by inducing inflammation and causing other

physiological changes (diarrhea) that influence the intestinal environment to promote their own growth [168].

12.7 Gut microbiota: immune dysfunction: dysbiosis and inflammation

The microbiota in the GI is impacted, and potentially altered, when exposed to antibiotics, infection of the host by pathogens, diet, and several other host- and environmental-dependent influences. As described previously, during homeostasis, the bacteria work together to produce beneficial metabolites that are used by the host. Dysbiosis (dysbacteriosis) occurs when there is a perturbation in the microbiota composition and/or function that leads to an imbalance between beneficial and harmful bacteria resulting in an unwanted immune response against commensal bacteria. Dysbiosis leads to a decrease in bacterial diversity and essential functions directly affecting metabolic activity which, can then deprive the host of valuable end products leading to poor gut health and bird performance [99]. Based on the Baumler concept [158], dysbiosis is weakening in microbiota-nourishing immunity that disrupts colonization resistance, thus, allowing pathogens a physiological “opening” to exploit for colonization. As described above, in a healthy microbiota, the commensal microbiota provides colonization resistance against potential pathogens in the gut. However, when perturbations (antibiotics, dietary changes) disrupt the commensals, colonization resistance is compromised. Pathogens like *S. typhimurium* and *C. perfringens* can expand by exploiting the ecological opportunity of increased availability of nutritional and physiological resources. Thus, the pathogens change the playing field by inducing inflammation and causing other physiological changes (diarrhea) that influence the intestinal environment to promote their own growth [168–170].

Intestinal inflammation is involved in dysbiosis in poultry [171,172], but it is not clear if the inflammation is the cause or the effect of the disruption in gut homeostasis [173–176]. However, both pathogenic (microbial infection or tissue injury) and metabolic (excess dietary nutrients) inflammation cause dysbiosis [172]. Targeted use of dietary interventions, direct-fed microbials (DFM) including pro/prebiotics, and other bioactive compounds may constitute an effective therapeutic strategy to rebalance gut dysbiosis and restore colonization resistance. However, existing microbe-targeted therapies, including prebiotics, probiotics, enzymes, and DFM have been developed for enhancing performance (increase the nutritional value of feed, improved feed conversion, breast meat yield, egg production) not for normalizing immune cell function. Nevertheless, there are a few examples of the use of commercial feed additives improving immunity or decreasing gut inflammation including β -mannanase [177,178], amino acids [179,180].

12.8 Managing the microbiome for immune modulation

It is beyond the scope of this chapter to discuss and review the use of preprobiotics and DFM as means of manipulating the microbiota. A recent book has collectively discussed all aspects of gut health [181]. However, it should be noted that the interest in identifying DFM and probiotics that exhibit immunomodulatory activity has grown over the past decade, our understanding of the regulation of host immunity by the microbiota is still largely based on correlational studies. Such correlational studies do not demonstrate causality. Undoubtedly, the need to identify the mechanisms by which the microbiota influences the avian intestinal immunity remains overriding and an area where much research is required in the future.

Manipulation of the intestinal microbiota to enhance the development, maturation, and education of the intestinal and systemic immune response signifies a promising therapeutic strategy for the future in poultry production. The intestinal microbiota has a collective metabolic activity equal to a virtual organ within an organ, and the mechanisms underlying the conditioning influence of the bacteria on mucosal homeostasis and immune responses are only beginning to be resolved in poultry. The trillions of microbes that reside in the avian GI tract are jointly producing thousands of small molecules and metabolites that have local and systemic effects on host physiology. Identifying effector microbes that causally affect host phenotype and deciphering the underlying mechanisms should be the ultimate goal of avian microbiome research in order to ultimately achieve the development of new microbiota-based therapeutics. In mammals, two complementary approaches have been used: the first is the identification of a desired immune phenotype and narrowing down the microbiota to identify responsible effector bacteria, while the second identifies bacteria-derived molecules and metabolites and characterizes their effects on the host immune system [71,138,164,182]. Together, these strategies should also provide the basis for the rational design of microbial and metabolite-based therapeutics for poultry health and welfare. More importantly, identifying these individual effector strains and understanding the mechanisms of host/microbial interactions are becoming increasingly possible.

The gut microbiota produces a myriad of unique small metabolites which interact with the host locally or systemically upon accumulation in the serum. “Postbiotics” are defined as microbe-derived bioactive compounds which may confer a health benefit [183]. They represent a potential “short-cut” to achieve a therapeutic effect while circumventing the technical challenge to seed live microbes which produce them. As engraftment of probiotic strains or successful utilization of prebiotics may depend on the

individual gut microbiome composition [184], these inter-individual differences may render the health effects of pre- and probiotics unpredictable. In this light, bacterial metabolites may represent a more controlled, reproducible, and reliable therapeutic strategy. Moreover, they may ease storage, delivery, and application [184]. A recent study described the mechanism of action of a postbiotic in the context of a *C. perfringens* challenge model. The postbiotic improved lesion scores, reduced intestinal *C. perfringens* counts and mortality, and improved weight gain compared to challenge groups without the postbiotic. The postbiotic predominantly affected the innate immune response and appears immunomodulatory where it reduced the proinflammatory responses and generated a homeostatic-like response [185].

The shaping and development of the immune response strongly suggest that the microbiota has a considerable influence on the response to vaccination. In commercial broiler production, the immune response and the microbial community develop in concert during the first few weeks posthatch when the majority of vaccinations are given. Thus, the composition of the early microbiota could play an adjuvant-like role in the bird’s responses to vaccines. For example, the administration of immune-dedicated probiotics or immune-based postbiotic formulations could prompt the intestinal commensals to contribute to the microbiome-innate immunity-acquired immunity axis interaction with a vaccine-directed immune response. Involving the commensal microbiota as a vaccine adjuvant, not only provides increased protection against infections but also maintains immunological tolerance in the intestine. Such an adjuvant-like activity of the chicken intestinal microbiota has recently been shown to modulate avian influenza vaccination in chickens [136]. Obviously, further research will be forthcoming in this area.

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Innate defenses of the avian egg

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13.1 Introduction

The avian egg has been shaped through >120 million years of evolution, and is a successful reproductive adaptation to the terrestrial environment to resist physical and pathogen challenges from the external environment, while satisfying the metabolic and nutritional needs of the developing embryo. During evolution, the structures and contents of avian eggs have been adjusted and optimized to combat microbes from a potentially contaminated hen oviduct/cloaca during egg formation (vertical transmission), and to impede potential contamination by pathogens from the environment, after laying (horizontal transmission). To achieve this vital function, the egg of avian species has several complementary and balanced levels of protective systems. Several of these structures are deposited during the process of egg formation in the ovary (the yolk including the inner perivitelline layer) and oviduct (sequentially, the outer perivitelline layer, the egg white or albumen, and the eggshell membranes together with the calcified eggshell). These primary structures are a source of nutrients and energy at precise time points during embryonic development, but also exhibit physical, structural, and molecular specificities that collaborate to protect the embryo [1] (Fig. 13.1). During incubation, these initial structures are progressively modified following protein and nutrient assimilation by the embryo, water exchange, physicochemical changes in egg compartments, and the development of complex vascularized extraembryonic structures including the amniotic, the allantoic, and the yolk sacs (Fig. 13.1). The extraembryonic membranes are epithelium-like structures that ensure vital functions such as digestion, gas exchange and add several strata of new physical, molecular, and cellular protection. Altogether, these nonspecific mechanisms, of both maternal and embryonic origins, constitute the sole line of defense of the fertilized egg (innate immunity).

Although organs of the immune system, including bursa of Fabricius and thymus (primary lymphoid organs) and spleen, cecal tonsils, Peyer patches, gut-associated lymphoid tissue (secondary lymphoid organs), initiate their development during the second half of embryonic development, the resulting components of adaptive-specific immunity (antibodies, immune cells) are fully functional only after hatching. This chapter aims to integrate data currently available on the various levels of egg defense that function during incubation, considering the *Gallus gallus* species as a well-studied model of avian species.

13.2 Egg basic structures and their role in innate defense

Avian eggs possess very efficient and orchestrated systems to protect the embryo during incubation, until hatch. These include the microbiota covering the outermost layer of the eggshell (the cuticle), physical barriers (the eggshell, the eggshell membranes, and the perivitelline layer), physicochemical barriers (egg white, alkaline pH and viscosity), and proteins and peptides that exhibit a diversity of antimicrobial activities (Fig. 13.2) [2]. The efficiency of all these protective systems and levels is attested by the remarkable capacity of the avian egg to resist bacterial contamination. Indeed, only a few pathogenic bacteria, including *Salmonella enterica* Enteritidis, are able to pass through all these mechanisms, to survive in the unfavorable environment of the egg white, while “waiting” for a loophole/weakening of one of these protections to proliferate and disseminate within the egg.

Of all these systems, and despite the presence of maternal immunoglobulin Y (IgY), the yolk remains the most susceptible to microbial contamination as it contains essential nutrients (initially to assist embryo growth) that

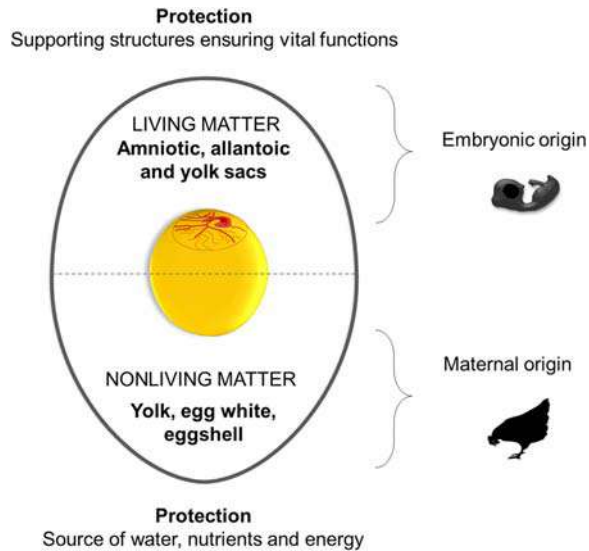


FIGURE 13.1 Origin and general functions of egg macrocomponents (original figure).

support proliferation and dissemination of opportunistic invading pathogens. The yolk is mechanically maintained in the center of the egg by two chalazae (translucent twisted filaments made of proteins attached at the two opposite extremities of the egg yolk) in order to avoid contact with the internal side of the eggshell membranes where pathogens can potentially accumulate, and to protect the yolk from shocks.

13.2.1 Physicochemical barriers

The egg possesses two major distinct physical barriers, the eggshell as a mineralized barrier essentially composed of calcium carbonate, and two types of acellular membranes, the eggshell membranes and the perivitelline layer (or vitelline membrane) that are each composed of two layers (internal and external) of specific protein fibers. The plasticity and the molecular composition of the eggshell membranes are essential, as these structures constitute the matrix for the deposition of mammillary knobs, which will be the seeding sites for the growth of calcite crystals that will form the eggshell. The eggshell membranes also prevent excessive loss of internal water through eggshell pores. The perivitelline layer and associated chalazae protect the yolk content from leaking toward the egg white while maintaining its contents at a distance from potential bacterial contamination arriving through the eggshell. The perivitelline layer covers the embryo and exerts mechanical constraints to shape the embryo and to drive morphogenesis [3]. The integrity of the eggshell membranes and perivitelline layer is pivotal to ensure the reproductive success of the avian embryo and may be partly altered by a long storage duration and

by high temperatures prior to incubation. Such alterations are characterized by an increase in the volume of the air chamber (water loss) forming between the two layers of the eggshell membranes and by altering the structural integrity of the perivitelline layer, thus compromising the development of the embryo and the extraembryonic yolk sac. The viscosity and the alkaline pH of egg white also constitute two major contributing factors that affect the survival capacity and migration of bacteria toward the yolk.

The eggshell

The eggshell is a critical physical barrier against mechanical stress and microbial entry. The avian eggshell is made of calcium carbonate (95%, calcite polymorph) and 3.5% of proteins, polysaccharides, and proteoglycans. The chicken eggshell is a natural porous bioceramic resulting from the sequential and temporally regulated deposition of its different layers within specific segments of the hen oviduct. It is a highly ordered structure exhibiting a polycrystalline organization [4–6]. The mechanical properties of the shell are crucial determining factors to keep the egg interior free of pathogens. A major factor of the eggshell solidity is the organization, orientation, and morphology of the calcite crystals, which determine the shell texture and consequently its mechanical properties. From the outside to the inside, the eggshell consists of the cuticle and its associated microbiota, the palisade and mammillary calcified layers, and finally the eggshell membranes (Fig. 13.2).

The cuticle is a proteinaceous biofilm, which acquires its microbial features after laying (microbiome barrier); it constitutes the first barrier against trans-shell penetration by pathogenic microbes. Microbes on the surface of the eggshell are likely to influence colonization by pathogenic bacteria through direct inhibition and/or competitive exclusion. The composition of the eggshell microbiome is a heritage from both maternal microbiota (cecum/feces) where the egg meets cecal secretions in the cloacal segment during oviposition, and from the nesting environment (contaminated litter/feathers and air environment including dust). At laying time, the egg surface is still moisturized, but will progressively dehydrate during incubation. The surface characteristics of the egg, the loss of the moisture layer, and the presence of antimicrobial molecules composing the cuticle [7,8] are likely to dictate the bacterial communities that will survive on the surface of the eggshell.

The literature on the composition of the eggshell microbiome of eggs originating from current commercial broilers and layers is quite sporadic, as hatching eggs are systematically disinfected, usually by chlorine/formaldehyde fumigation, to avoid trans-shell contamination that

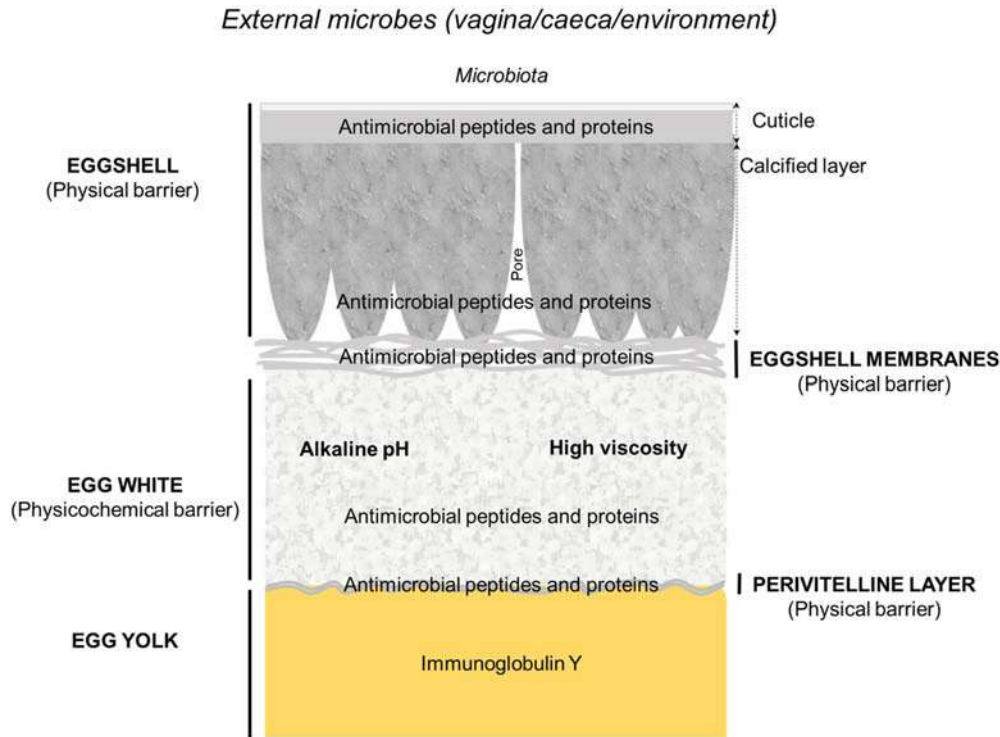


FIGURE 13.2 Primary contribution of egg components in the protection of the embryo. Original figure inspired from [2].

could compromise embryo viability. Moreover, some authors have reported that the bacterial load on the surface of the egg varies according to hen age and also strongly depends on the interaction between rearing systems and practices in each country, and the methods used for detection and data quantification/validation [9]. In a recent study performed on eggs from a broiler breeder strain, the most abundant bacterial communities present on the eggshell surface were shown to be essentially from the *Firmicutes*, *Proteobacteria*, and *Actinobacteria* phyla, with *Clostridium*, *Heliobacterium*, and *Turicibacter* as the dominant genus [10]. These results are in accordance with the core microbes identified in the gut of laying hens, but also in chicks from broiler breeds [11]. In this study, authors also showed that the disinfection process reduced the bacterial load from an average of 10^4 colony-forming units corresponding to a reduction of *Actinobacteria* group and an increase of bacteria from the *Bacteroidetes* and *Proteobacteria* phyla [10]. Although a moderately high variability between samples was observed, this study highlighted that eggshell disinfection is rarely complete and may destabilize the natural microbial biofilm (cuticle) of the eggshell by favoring some bacterial communities at the expense of others. The predominance of Gram-positive bacteria in the microflora on the shell exterior is possibly explained by their higher tolerance to dry

conditions. However, Gram-negative bacteria are abundantly present in the interior of decaying eggs [12]. The shell and contents of eggs can be contaminated by a variety of pathogens such as *Campylobacter jejuni*, *Listeria monocytogenes*, *Escherichia coli*, *Yersinia enterocolitica*, and especially *S. enterica* serovar Enteritidis [12]. *Staphylococcus* spp. are among the Gram-positive bacteria that are mainly found in the eggshells of contaminated eggs [13].

Elucidating the origins, the contributing factors, and the transmission processes that govern eggshell microbiomes may be of particular interest as there is increasing evidence that eggs may function as transgenerational carriers of the maternal microbiome to the egg and the resulting embryo [11]. Indeed, some components of the maternal oviduct microbiota appear to be transferred to the embryo through the egg white and are assumed to constitute most of the embryo gut bacterial population [14]. Such a process may be considered as an additional level of immune defense to prepare the embryo and the chick for environmental microbes after hatching. Understanding which factors drive microbial communities on eggshells throughout incubation may lead to a better comprehension of evolutionary strategies that prevent colonization of the egg surface by pathogenic bacteria [15].

The completeness of the cuticle itself, as the most external layer of the egg, is thus essential to guarantee that the egg remains free of pathogens. Eggs with the best cuticle coverage are rarely penetrated by microbes [16]. It constitutes a protective layer, which is impermeable to microbes and is under strong natural selection. Birds living in damp or microbiologically challenged environments (flamingos, pelicans) have thicker cuticles [16,17], suggesting some evolutionary pressure.

This layer is composed of organic constituents that are deposited in the uterus in the last 2 hours of the egg formation process. At oviposition, the surface of the eggshell is moist and since the cuticle is still soluble, it is not fully protective. Therefore the bacterial risk is high at this stage. However, the cuticle will dry in less than 3 minutes [18] and plugs the eggshell pores, thereby limiting bacterial trans-shell penetration [19]. The cuticle also contains antibacterial compounds (bacteriostatic and bactericidal), which exert an antimicrobial protection until the cuticle is dry and are assumed to participate in defining the protective microbiota that will colonize the eggshell surface. However, the literature on this specific assumption remains scarce.

The cuticle and outer eggshell protein extracts from three species of domestic birds (ducks, goose, and domestic chickens) [20,21] and other anseriformes (wood duck, hooded merganser, Canada goose, mute swan) [22] have all been shown to exhibit antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. In addition, other extracts also possess activities against *E. coli* [21]. It is strongly believed that the composition of the cuticle has evolved according to environmental challenges and thus depends on bird species and habitats. Remarkably, the egg cuticle of the cavity-nesting hooded merganser is composed of potent antimicrobial molecules compared with other species [21]. Moreover, nanostructuring of the cuticle layer seems to be specifically observed in bird taxa that nest in humid infection-prone environments, which suggests that these features play a critical role in antimicrobial egg defense [23]. Indeed, these nanospheres are highly effective in decreasing the attachment of bacteria to the shell surface, thus limiting bacterial penetration [24]. The area where eggshell infection occurs might also be of particular importance as the deposition of cuticle onto the eggshell is not homogenous at the blunt and sharp ends [25] and may differ in quality depending on hen age and egg freshness [26].

When considering protein composition of the cuticle, 47 proteins were identified using proteomic methods [8,16]. Several of these proteins are known to have antimicrobial activity (lysozyme C, ovotransferrin, myeloperoxidase, ovocalyxin-36, cystatin, ovoinhibitor, and other members of the serine protease inhibitor family) and

some of them have been reported to be more concentrated in specific areas where cuticle plugs the shell pores [7]. Finally, a significant association was observed between ovocleidin-116 and estrogen receptor alleles, and cuticle deposition [16,27], suggesting that genetic selection might be possible to increase cuticle deposition in chicken species.

Underneath the cuticle, we find several constitutive calcified layers, which consist of crystals of calcium carbonate that contain 2%–3% of an occluded organic matrix. During eggshell formation, calcification is initiated in the red isthmus segment of the oviduct. The initial step is the deposit of organic cores on the eggshell membranes (mammillary knobs), which are seeding sites for initiation of shell calcification. Eggshell calcification progresses in the uterus with a massive accumulation of the transient amorphous calcium carbonate (growth phase, palisade layer) [28]. The eggshell crystal texture results from a physicochemical process that is mediated and controlled by the proteins of the organic matrix secreted in the uterine fluid [29]. Matrix proteins play key roles to stabilize the transient form of calcium carbonate, influencing the selection of the calcite polymorph, controlling the orientation of crystal growth [28,30]. These interactions define the eggshell ultrastructure that is responsible for its mechanical properties. This structure is traversed by pores, which allow gaseous exchange across the eggshell to support embryonic development. On the surface of the palisade layer, a thin layer of small vertical crystals (vertical crystal layer) is observed, which constitutes the last calcified layer (terminal phase). Finally, the eggshell surface acquires its cuticle layer (see paragraph above), which is of variable thickness and may be incomplete.

The various phases of eggshell formation are associated with proteins that are secreted into the uterine fluid, whose profile and abundance are differentially regulated at each stage of eggshell formation (initiation of mammillary knobs, growth, and terminal phase). Proteins of the organic matrix of the eggshell have been widely studied, including a proteomic survey that identified novel components and quantified about 300 eggshell proteins at the key steps of shell calcification, including antimicrobial proteins [30]. By combining data from this and other published eggshell proteomes [7,8,31–37], about 900 unique proteins could be identified with various abundances [38].

The eggshell membranes are considered the innermost layer of the shell and are deposited during 1.5 hours as the forming egg transits the white isthmus [5,39]. They are composed of highly crossed-linked networks of extracellular fibers that run parallel to the eggshell surface [40]. The inner membrane envelops the albumen and is not calcified, while the fibers of the outer shell membrane penetrate the tips of the mammillary cones of the calcified shell where they initially provide the nucleation (seeding)

sites for mammillary cone mineralization [5]. Moreover, the inner membrane is lined by a lysozyme-rich limiting membrane that is in contact with the albumen [40]. Globally, these layers are semipermeable and allow the exchange of gases and water while acting as a physical and chemical barrier to bacteria. The fibers consist of 10% collagen (mainly type X) and 70%–75% of other proteins and glycoproteins containing lysine-derived cross-links [31,41,42]. The major structural protein is stabilized by disulfide-crosslinks and has been termed CREMP (cysteine-rich eggshell membrane protein) [31,43]. Close to 300 distinct eggshell membrane proteins have been identified so far [31]. The respective contribution of all these proteins to the structure and the integrity of eggshell membranes is under active investigation.

Egg white

Mainly composed of water (~88%) and proteins (~9.7%–10.6%), egg white is a biological fluid that globally resists microbial contamination. These properties are multifactorial, as they rely on the presence of a number of antimicrobial molecules (see paragraph below), combined with particular physicochemical properties, such as alkaline pH and viscosity [44]. Egg white is a heterogeneous fluid, composed of three distinct superposed layers, each exhibiting distinct viscosities, namely, the outer and inner thin egg white layers (in contact with the eggshell and the egg yolk, respectively) separated by a thick egg white layer. The chalaziferous layer surrounds the perivitelline layer and forms twisted filament structures (chalazae) that connect the yolk to each pole of the egg. In fresh eggs, at 20°C and a shear rate of 34.2/s, the apparent viscosity of thick egg white is approximately 40-fold higher than that observed for the thin egg white [45]. It forms a capsule around the egg yolk and contributes, together with the chalazae, to maintain the egg yolk in a centered position in the freshly laid egg. Egg white is generally bactericidal toward Gram-positive bacteria, and bacteriostatic or bactericidal toward Gram-negative bacteria [46]. Therefore egg white constitutes, together with the eggshell and the perivitelline layer, a unique model of passive innate defense, as only a few bacteria, including *S. Enteritidis*, are able to naturally enter eggs via vertical or horizontal contamination, and survive in the egg white. Among *S. enterica* subspecies enterica, the serotype Enteritidis has a higher capacity to survive in egg white compared with other *Salmonella* serotypes [47]. The antibacterial nature of egg white strongly depends not only on its physicochemical and chemical properties (alkaline pH, viscosity, antimicrobial molecules) but also on the environmental temperature [44]. While bacteriostatic effects against *S. Enteritidis* are observed at moderate temperatures, egg white exhibits a

bactericidal anti-*Salmonella* activity against the same bacteria at 42°C (hen body temperature) or above [48]. Major changes in the *S. Enteritidis* transcriptome related to nutrient deprivation (in particular iron and biotin restriction), cell damage/stress, and a shift in energy metabolism and catabolism, are induced by egg white environmental exposure at 45°C [49]. Some global transcriptomic and proteomic approaches were conducted to investigate the gene response of *S. Enteritidis* to the egg white. At bactericidal temperature (45°C), major changes in the *S. Enteritidis* transcriptome related to nutrient deprivation (in particular iron and biotin restriction), cell damage/stress, and a shift in energy metabolism and catabolism, were found to be induced by egg white environmental exposure [49]. At 37°C, egg white upregulates a number of *S. Enteritidis* genes associated to DNA damage repair, alkaline pH adaptation, osmotic stress adaptation, envelope damage repair, *Salmonella* pathogenicity island 2, iron absorption, and biotin synthesis [50]. At the protein level, proteins associated with iron acquisition, cofactor and amino acid biosynthesis, transporter, regulation, and stress responses were upregulated, while proteins associated with energy metabolism, virulence and motility, and chemotaxis were in contrast downregulated [51]. Besides, a genome-wide microarray-based transposon insertion library screening revealed that lipopolysaccharide (LPS) biosynthetic pathways and the serine protease/heat shock protein (HtrA) were crucial factors that contribute to bacterial survival in egg white at hen body temperature (42°C) [52]. Interestingly, a recent study reported that the bacterial outer membrane channel TolC (which is part of the multidrug efflux pump systems) is activated by egg white at 42°C and protects *S. Enteritidis* against ovotransferrin-mediated growth inhibition [53]. All these observations underline the detrimental properties of egg white against bacteria and the survival strategies developed by *S. Enteritidis*.

The egg white pH at lay is about 7.6, but it increases at a temperature-dependent rate to 9 or above within few days of storage in air. This rise in pH results from the loss of CO₂, present in the egg white as a dissolved gas or in ionic form, through eggshell pores. The pH of the egg albumen indeed depends on the equilibrium between dissolved CO₂, HCO₃⁻, CO₃²⁻, and proteins [54]. Concentrations of HCO₃⁻ and CO₃²⁻ are governed by the partial pressure of CO₂ in the egg environment. In fertilized eggs, egg white pH is alkaline during the first 2 days of incubation. However, this is followed by a drop in pH resulting from the secretion of hydrogen ions by the blastoderm and the production of CO₂ by the developing embryonic tissues [55], leading to a microenvironment that is optimal for embryonic development [56]. The alkaline environment present in the egg albumen after storage, or in early incubation, is generally unfavorable for

nonextremophilic bacteria, which usually grow at pH 5.5–9.0. Indeed, most bacteria must maintain an intracellular pH within the narrow range of 7.4–7.8, compatible with optimal functional and structural integrity of cytoplasmic proteins. The alkaline pH mainly impacts the respiratory status of bacterial cells that must maintain the proton motive force and the electrochemical potential across their membranes. Numerous strategies are exhibited by bacteria to cope with alkaline environments, such as the upregulation in proton intake and retention (e.g., via modulation of the ATP synthase and monovalent cation/proton antiporters), metabolic changes leading to an increase in acid production, and changes in the cell surface layers contributing to cytoplasmic proton retention [57]. In addition to accelerating proton import, high pH also represses the energy-expensive systems of flagellar biosynthesis and chemotaxis [58]. In this regard, *S. Enteritidis* lacks flagella at alkaline pH [59], which may limit its motility in the egg white and its access to yolk nutrients.

The egg white viscosity also affects the motility of flagellated bacteria [60,61]. The gel-like structure of the thick egg white inhibits the motility of flagellated bacteria and their migration toward the egg yolk. Indeed, the swimming speed of *S. Typhimurium* and its flagellar rotation rate are significantly altered in the thick albumen, compared to the thin albumen [62]. The thin and the thick egg albumen are both repellent for *S. Typhimurium* and cause reversals of the flagellar rotation [62]. The gel-like structure of thick egg white is due to the presence of a sulfated glycosylated protein of very high molecular weight, namely, ovomucin, whose levels in the thick egg white layer were estimated to be about four times higher than that in thin egg white layer [54]. This protein is composed of two subunits: an α subunit with a low carbohydrate content (MUC5B), and a carbohydrate-rich β subunit (MUC6). The viscosity of egg white is highest at lay, and it progressively decreases over

time as the pH rises, with a rate depending on the temperature of storage. This liquefaction event, called egg white thinning, may promote bacterial motility and migration toward the nutrient-rich egg yolk. Interestingly, ovomucin can interact in solution with the antibacterial lysozyme via electrostatic interactions to form a water-insoluble complex. Although the molecular mechanisms of egg white thinning are not precisely characterized, it is hypothesized that it might result from the dissociation of ovomucin-lysozyme complexes under alkaline conditions. Thus the liquefaction process might be associated with the solubilization of some antimicrobial proteins initially entrapped in the gel structure, resulting in the release of their intrinsic antimicrobial activities.

Perivitelline layer (vitelline membrane)

The perivitelline layer, also known as the vitelline membrane, is a thin protein layer covering and encapsulating the egg yolk nutrients destined for the developing embryo. In addition to its role in fertilization and in early embryonic development, the perivitelline layer also participates in the protection of both the embryo and the yolk nutrients during the early incubation. Indeed, this biological barrier protects embryonic cells against the alkalinity of egg white, and it limits the diffusion of egg yolk nutrients to the egg white, and the penetration of bacteria from the egg white to the egg yolk.

In chicken eggs, the perivitelline layer is almost entirely composed of proteins. About 140 different proteins were identified by mass spectrometry [63]. The perivitelline layer consists of two superposed layers, the inner and the outer perivitelline layers, in contact with the egg yolk contents and the egg white, respectively, which are separated by a very thin granular “continuous membrane” [64]. The chicken internal perivitelline layer is initially produced in

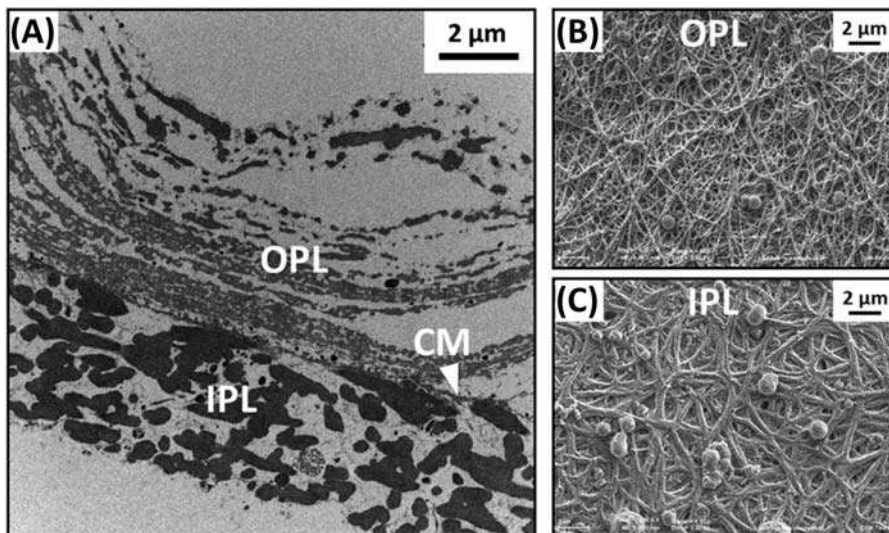


FIGURE 13.3 Micrographs of chicken egg perivitelline layer in cross-section by transmission electron microscopy (A), and of outer and inner surfaces by scanning electron microscopy (B and C, respectively). OPL, outer perivitelline membrane; IPL, inner perivitelline membrane; CM, continuous membrane. ©Plateforme IBiSA de Microscopie Electronique, University and CHRU of Tours, France, S. Georgeault.

the ovarian follicle before ovulation and consists of a three-dimensional network of fibers [64,65] (Fig. 13.3). At the molecular level, the filamentous structures are mainly composed of Zona Pellucida proteins ZP1–ZP3 complexes assembled into helical fibrils, which further assemble into bundles to form the ZP1–ZP3 matrix. Besides, Zona Pellucida D protein (ZPD) aggregates and binds to ZP1–ZP3 matrix to form the inner perivitelline layer matrix [66]. ZP proteins are presumably involved in sperm binding and the acrosome reaction. No direct innate immune functions were reported for chicken ZP proteins to date, although ZPD might have some immune roles by homology with the human protein uromodulin, which is involved in antibacterial protection and immune regulation within the human urinary tract [66]. The outer perivitelline layer is synthesized in the proximal part of oviduct (infundibulum) following ovulation and covers the inner perivitelline layer. It consists of a variable number of sublayers superposed on each other and containing long and straight fibrils [64] (Fig. 13.3). The major proteins composing chicken outer perivitelline layer are ovomucin, lysozyme, vitelline membrane outer layer proteins 1 and 2 (VMO1 and VMO2, now referred to as avian β -defensin-11, AvBD11) [67,68]. Although the perivitelline layer protein pattern may vary depending on avian species [69], the innate immune properties of these major compounds (see Section 13.2.2) strongly support the antimicrobial function of outer perivitelline layer. It has been hypothesized that lysozyme contributes to perivitelline layer structure via electrostatic interactions with ovomucin [67,70]. As demonstrated in quail, VMO2/AvBD11 might have a structural role in outer perivitelline layer, in mediating the binding of outer perivitelline layer with inner perivitelline layer, possibly via an association with ZP1 and ZP3 proteins [71]. The maintenance of outer perivitelline layer integrity is crucial to prevent or limit bacterial penetration from the egg white into the yolk. However, outer perivitelline layer integrity can be challenged by several parameters, especially the conditions of egg storage prior to incubation (e.g., high temperature, long duration).

Yolk

The yolk is the main source of nutrients for the embryo, and consequently, when bacteria reach this egg compartment, they take advantage of this nutrient-rich milieu to proliferate, disseminate, and invade all egg compartments including the embryo. Although the egg yolk contains high levels of maternal immunoglobulins, these are ineffective against bacterial invasion as they cannot counterbalance the exponential growth of bacteria when they reach the yolk. Thus the contribution of the yolk to innate immunity of the egg and protection of the developing embryo appears to be quite limited at least during the first half of incubation. The transfer of yolk IgY to the embryo seems to

occur essentially during the second half of incubation with the contribution of IgY receptors of high affinity that are expressed by the yolk sac during the later stages of development [72]. These yolk maternal immunoglobulins are particularly important at hatching, as they constitute the first line of defense and compensate for the immaturity of the chick gut, posthatch (refer to Section 13.5.2).

13.2.2 Antimicrobial molecules

The avian egg contains a myriad of antimicrobials distributed within all egg structures (eggshell, egg white, perivitelline layer, egg yolk) [73,74]. Their diversity and levels, however, greatly differ depending on the egg structure considered. A list of the most abundant egg antimicrobials is presented in Table 13.1.

In the egg yolk, as mentioned in a previous section (See Section 13.2.1), the antimicrobials are not abundant (with the exception of immunoglobulins) in contrast to the high levels of nutrients that make this compartment particularly sensitive to microbial contamination.

In the eggshell, the bioavailability and activity of these compounds as antimicrobial molecules may be limited due to their integration within the mineral phase and to the fact that the organic matrix (proteins) represents only 3.5% of the eggshell, which is essentially composed of calcite. However, some of these constituents might be released and solubilized during the demineralization process occurring in incubated fertilized eggs (as a calcium source for the embryo), and provide additional protection locally between the inner eggshell and the chorioallantoic membrane (CAM). The first evidence of the presence of antimicrobial proteins in the shell was derived from investigations into mechanisms of eggshell mineralization. Lysozyme and ovotransferrin are well-known antimicrobial proteins that were identified as eggshell matrix proteins [40,75–77]. Such a role was also ascribed to ovocalyxin-32 (OCX-32) and ovocalyxin-36 (OCX-36), which are enriched in the eggshell [54,76,77]. Proteomics exploration of eggshell matrix proteins during the calcification process allowed the identification of 35 antimicrobial candidates [30]. Recombinant OCX-32 protein was reported to be antimicrobial against *B. subtilis* [78]. OCX-36 is related to the family of bactericidal permeability-increasing (BPI)/LBP [LPS (lipopolysaccharide)-binding proteins]/PLUNC (palate, lung, and nasal epithelium clone) proteins, which are well-known in mammals for their involvement in defense against bacteria. They belong to the superfamily proteins known to be key components of the innate immune system, which act as the first line of host defense. LBP binds the lipid A component of the LPS layer of Gram-negative bacteria [79]. BPI also binds LPS, leading to permeabilization of the cytoplasmic membrane and to a decrease in the

TABLE 13.1 Major egg antimicrobial proteins and their localization in the egg.

Protein name	Gene ID/gene symbol	Antimicrobial properties	Localization
Avian beta-defensin 3	395363/AvBD3	Antibacterial	ES
Avian beta-defensin 6	407776/AvBD6	Antibacterial	ES
Avian beta-defensin 9	414343/AvBD9	Antibacterial	ES, EY
Avian beta-defensin 11	414876/AvBD11	Antibacterial, antiviral, antiparasitic	EW, PL, ES
Avidin	396260/AVD	Antibacterial	EW, PL, EY
Beta-microseminoprotein-like	101750704/MSMB3	Antibacterial	EW
Cystatin	396497/CST3	Antibacterial, antiviral, antifungal, antiparasitic	EW, PL, EY, ES
Gallin/Ovodefensin A1	422030/OvoDA1	Antibacterial	EW, ES, PL
Immunoglobulin A and M			EW
Immunoglobulin Y	—		EY
Lysozyme	396218/LYZ	Antibacterial, antiviral, antifungal	EW, PL, EY, ES
Ovalbumin-related protein X	420898/OVALX	Antibacterial	EW, PL, EY, ES
Ovocalyxin-32	395209/RARRES1	Antibacterial	ES
Ovocalyxin-36	419289/BPIFB3	Antibacterial	ES, EW
Ovodefensin B1	1067780806/OVODB1	Antibacterial	EW
OvoglobulinG2/TENP	395882/BPIFB2	Antibacterial	EW, PL, EY, ES
Ovoinhibitor	416235/SPIK5	Antibacterial	EW, EY, ES
Ovomucin (alpha and beta subunits)	395381/LOC395381 (alpha)	Antibacterial, antiviral	EW, PL, ES
	414878/MUC6 (beta)		
Ovotransferrin	396241/TF	Antibacterial, antiviral	EW, PL, EY, ES
Phosvitin	424547/VTG1	Antibacterial	EY
Pleiotrophin	418125/PTN	Antibacterial	EW, ES
Vitelline membrane outer layer protein 1	418974/VMO1	Antibacterial	EW, PL, EY

Note: ES, eggshell (including eggshell membranes); EW, egg white; EY, egg yolk; PL, perivitelline layer.

electrochemical gradient of the bacterial cell leading to death. Purified OCX-36 showed significant binding activity for LPS derived from *E. coli* and could modestly inhibit the bacterial growth of *B. subtilis*, *E. coli*, and *P. aeruginosa* [80,81]. Furthermore, OCX-36 and enzymatically digested OCX-36 can differentially modulate immune responses in vitro and in vivo [81]. Interestingly, OCX-36 protein is unique to birds, as its gene counterpart cannot be detected in either mammalian or bony fish genomes [82]. Ovocleidin-17 and ansocalcin are C-type lectins that are abundant proteins of the chicken and goose eggshell. They were found to be bactericidal against *P. aeruginosa*, *B. subtilis*, and *S. aureus*, with an enhanced activity in the presence of calcium. These authors also demonstrated that these two proteins bind to bacterial polysaccharides [83].

OCX-36 was shown to be also abundant in the eggshell membranes [80] along with avian β -defensins (AvBDs,

Chapter 8.5, Soluble Components, acute phase proteins and fever). AvBDs are cationic peptides with biocidal activity against a wide array of pathogenic bacteria, viruses, and fungi [84,85]. A large number of AvBDs are expressed in oviductal segments of chicken [86–88]; of these, AvBD3, AvBD9, and AvBD10 proteins have been detected in the chicken eggshell membrane by proteomics [31,88,89]. Similarly, gallin (ovodefensin A1) with anti-*E. coli* activity [90,91] is present in chicken eggshell membranes [89].

The highest concentration of active antimicrobial molecules is found in the egg white and in the perivitelline layer. Depending on the protein considered, these antimicrobials may exhibit antibacterial, antiviral, antifungal, or antiparasitic activities (Table 13.1). The egg antimicrobials display bactericidal or bacteriostatic activities via different but complementary mechanisms: (1) via interaction with bacterial cell walls that further triggers permeabilization and bacterial death (e.g., lysozyme,

avian beta-defensins, etc.); (2) by decreasing the bioavailability of nutrients required for microbial growth (ovotransferrin and avidin strongly bind to iron and biotin, respectively), and (3) by inhibiting microbial proteases that are virulent factors of infection (e.g., ovoinhibitor, cystatin, etc.). Interestingly, some of them have no orthologue in mammals (AvBD11, OVALX, AVD, OvoDA1) or in reptiles (AvBD11, OVALX, OvoDA1) [92,93], suggesting that they have a specific role in the innate protection of the avian egg, or other biological functions related to avian reproduction.

Lysozyme (LYZ) and ovotransferrin (TF) are the most abundant antimicrobial proteins in the chicken egg. LYZ is a 14 kDa cationic protein representing 3.4% of total egg white proteins. Its antibacterial effect is mediated by different mechanisms. The enzymatic-dependent mechanism relies on the muramidase activity, which catalyzes the degradation of the glycosidic [1–4] beta-linkage between the N-acetylglucosamine and the N-acetylmuramic acid of the peptidoglycan layer in the bacterial cell walls. This enzymatic-dependent lytic action is mainly observed in Gram-positive bacteria, although some of them can develop resistance mechanisms. The muramidase activity may be reduced in stored eggs due to alkaline conditions; however, it is noteworthy that LYZ can still be substantially bactericidal at pH 9 [94]. Interestingly, the enzymatically inactive LYZ mutant D52S and heat-inactivated LYZ also possess bactericidal properties against Gram-positive bacteria [95], suggesting that LYZ can use alternative mechanisms such as membrane disruption or induction of autolysins. LYZ can also exhibit antibacterial properties against Gram-negative bacteria via a nonenzymatic mechanism, by increasing the permeability of the outer and inner layers, as previously shown in *E. coli* [96]. The antibacterial effect against *E. coli* and Gram-negative bacteria likely involves the cationic features and the numerous positive charges of the molecule that are assumed to destabilize the bacterial wall similarly to many cationic peptides including defensins.

TF is a 77.7 kDa glycoprotein representing 12% of the egg white protein. This iron-chelating protein creates an iron-deficient environment, resulting in the inhibition of bacterial growth (bacteriostatic effect). Of note, iron-chelating activity of TF is increased under alkaline conditions. Besides its bacteriostatic effect, TF was also reported to have bactericidal activities against *S. aureus* and *B. cereus* [97,98].

The outer perivitelline layer contains high amounts of LYZ and avian beta-defensin 11 (AvBD11). AvBD11 is a 9.3 kDa protein consisting of two beta-defensin domains and has been recently defined as the archetype of a new structural family named double beta-defensins. This small protein exhibits a broad spectrum of activities against both Gram-positive and Gram-negative bacteria while it also bears activities against *Eimeria tenella* parasite and

H1N1 virus, in vitro [99]. However, the potential of this peptide against such viral and parasitic challenges during embryonic development remains unknown.

13.3 Modification of egg structures during embryonic development

Chicken embryonic development takes 21 days and the innate defenses initially present within the egg disappear gradually during incubation. The integrity of the perivitelline layer is severely modified during incubation as it serves as a substratum for the growth and expansion of the yolk sac during incubation, and progressively disappears. The physicochemical properties of egg white are also strongly impacted by the development of an embryo. Starting from 9.5, the pH of the fertilized egg white gradually decreases to 7.5 by embryonic incubation day (EID) 12 [100] due to the respiratory metabolism of the embryo. This decrease in pH possibly modifies the antibacterial properties of egg white proteins; (e.g., increased hydrolase activity and decreased iron chelation activity). Moreover, during embryonic development, the water contained in the egg white is transferred to the yolk and to developing extraembryonic fluids, which will increase the viscosity of egg white and may reduce the mobility of potential pathogens. As a result of water transfer, the egg white volume decreases and the protein concentration rises (threefold increase at EID8). The antibacterial properties of egg white proteins progressively decline during the first half of incubation, even though it might be partly counterbalanced by the increase in protein concentration [100]. The eggshell also undergoes major changes during incubation. From the second half of incubation (EID10/EID11) to hatching (EID21), the eggshell is degraded/solubilized to provide calcium for the embryo [101,102], which subsequently facilitates the emergence of the hatchling from the egg. However, this weakened eggshell could also more easily result in contamination of the egg and embryo in the later stages of development. Eggshell degradation and subsequent oral absorption of egg white by the embryo is concomitant to the development of the immune system in the embryo, first observed at EID10. By EID18, the chicken embryo is capable of producing both an innate and an adaptive response to pathogens [103–105]. On the other hand, chick embryonic tissues express TLRs from EID3 onward, recognizing viral ligands and responding to them, and thereby exhibiting an innate preparedness [105–107] (See Section 13.4.1). However, the immune system of the embryo is immature during incubation suggesting that other structures, such as the extraembryonic sacs that develop concomitantly to the alteration of initial defenses may also contribute to protecting the embryo. A comprehensive assessment of the

role of the extraembryonic structures in the defense of the embryo is not yet available. Their strategic position around the embryo and their role in the assimilation of the compounds from the Eggshell, yolk, and egg white, however, give these compartments priority for the relocation of the molecular defenses initially present in the egg and the implementation of new defense compartments. The appearance of extraembryonic membranes (chorioallantoic membrane or CAM, amniotic membrane, and yolk sac membrane) during incubation is thus believed to add an additional level in the defense of the egg and the embryo. When these extraembryonic structures start to degenerate from EID18, the embryo is “virtually” immunocompetent although immature.

13.4 Embryonic immunity

The major components of the vertebrate's immune system are mucosal barriers, soluble serum proteins (e.g., complement system), and leukocytes, including phagocytes, lymphocytes, and natural killer (NK) cells [108–110]. Phagocytic cells such as granulocytes and mononuclear phagocytes [macrophages and dendritic cells (DCs)] are derived from myeloid lineages, whereas lymphocytes and NK cells are derived from lymphoid lineages [111,112] (see Chapter 8: Introduction to the avian innate immune system; properties, effects and integration with other parts of the immune system). In mammals, these cells arise from single pluripotent hematopoietic stem cells from the yolk sac, indicating an extraembryonic origin of hematopoiesis [113] (see Chapter 3: Development of the avian hematopoietic and immune systems). For all vertebrates, molecular pathways involved in the development and differentiation of hematopoietic cells into lineage-specific cells are conserved [114]. In the chicken embryo, large numbers of CD2+ cells in the peripheral blood, as well as CD45+ cells in the yolk sac of EID2 embryos (prior to the development of liver and thymus), strongly suggest a dominant yolk sac origin for most immune cells [115]. Moreover, despite not being fully characterized, embryonic tissues express TLRs and are able to recognize and respond to pathogen-associated molecular patterns (PAMPs), thus possessing a certain degree of immune preparedness. However, data for other pathogen recognition receptors (PRRs) are scarce or lacking. Below we provide details on the ontogeny and function of certain cells and molecules, notably those pertaining to the innate immune system.

13.4.1 Toll-like receptors

Toll-like receptors (TLRs) (see Chapter 8: Introduction to the avian innate immune system; properties, effects and integration with other parts of the immune system) recognize microbes by binding to PAMPs such as LPS,

lipoteichoic acid, lipoproteins, and glycoposphatidylinositol. Most TLRs, including TLR2A, TLR3, TLR4, TLR5, TLR7, TLR15, and TLR21 are expressed as early as EID3 in whole-embryo preparations [116]. High expression of TLRs in late embryonic stages may suggest the ability of the embryo to produce effector molecules after PAMPs recognition. For example, at EID12, TLR4 is highly expressed in the liver and brain, while its expression is higher in the intestine by EID18 [116]. Therefore the dynamics of TLRs expression could explain the acquisition of immunocompetence prior to hatching. Although not fully immunocompetent, EID18 the chicken embryo is capable of mounting an innate immune response that closely resembles that of hatchlings [117]. In a recent study, the effect of TLR2, TLR4, and TLR21 ligands on the expression of genes encoding different classes of cytokines (e.g., IFN- α , IFN- β , IFN- γ , IL-1 β , iNOS, and OAS) in the CAM was investigated. However, treatment with Pam3CSK4, CpG ODN, and LPS did not modulate the expression of type-1 interferons (IFN- α and IFN- β) in the CAM of embryonated eggs while the expression of IFN- γ was indeed stimulated, thus highlighting the complex regulation of embryonic TLRs [118]. In addition, a possible role for TLRs in regulating the development of chicken embryos is yet to be further confirmed [116].

13.4.2 Macrophages

Many tissue-resident macrophage populations arise from embryonic precursors in the yolk sac of vertebrates, take residence in these tissues prior to birth and maintain themselves locally throughout adulthood, independent of a major contribution from bone marrow-derived precursors [119] (See Chapter 3: Development of the avian hematopoietic and immune systems and Chapter 8: Introduction to the avian innate immune system; properties, effects and integration with other parts of the immune system). Similarly, avian macrophages are identified in the yolk sac and those from relatively mature embryos are functionally competent, which may suggest a role in embryonic development [120]. Moreover, myeloid progenitors of yolk sac origin colonize the organ primordia of the chicken embryo to differentiate into multiple types of cells, including tissue-resident macrophages [121]. Avian embryonic macrophages have shown to possess different physiological roles, including dead-cell phagocytosis [122], directing axonal and central nervous system vascular growth [123], and development of a network of lymphoid tissue [124].

13.4.3 Heterophils

Heterophils are detected in the blood of newly hatched chicks [125] but data on heterophil ontogeny and function

in the chicken embryo are relatively scarce. They are predominant cells in the blood and gastrointestinal tract of chicken hatchlings compared to mature birds [126]. Phagocytosis, degranulation, and oxidative burst are reduced in heterophils from newly hatched chicks compared to those from mature birds [127,128]. Heterophils isolated from 2- and 10-day-old chicks previously inoculated in ovo with CpG-ODN (an agonist of TLR21) at EID18, showed increased degranulation when stimulated by killed or live *S. enterica* serovar Enteritidis [129]. These data suggest that the innate immune response of heterophils in late-stage embryos may be boosted to adult levels through TLR stimuli as already observed for other vertebrates [130], thus paving the way to in ovo immune stimulation approaches to improve animal robustness in early life.

13.4.4 Dendritic cells

Chicken DCs (see [Chapter 8](#): Introduction to the avian innate immune system; properties, effects and integration with other parts of the immune system) are a relatively understudied cell population compared to their mammalian counterparts. They are phenotypically characterized by the expression of a combination of markers such as putative CD11d, DEC205, CD83, and MHC class II surface molecules [131–135]. Nevertheless, their ontogeny, phenotype (i.e., subsets), and function are yet to be characterized, notably during embryonic development. It has already been shown that DCs derived from the bone marrow of embryos at EID12–18 express several classes of PRRs, including TLRs, and respond to stimulation with TLR ligands and viral infections [134]. In terms of ontogeny, Dóra and colleagues showed that CD45⁺ MHC-II⁺ ramified cells arising from the yolk sac expand in the para-aortic region before colonizing the bursa of Fabricius at EID10. These cells are likely to give rise to precursors of bursal DCs [121,136].

13.4.5 T lymphocytes

Avian T cell development is remarkably similar to that of mammals, although it has some unique features. Using quail–chick chimeras, it was shown that the thymus becomes populated with cells originating in the three germ layers (i.e., ectoderm, mesoderm, and endoderm). The hematopoietic component (which differentiates in the thymus into T lymphocytes, macrophages, and medullary DCs) is derived from the mesoderm. All lymphocytes developing in the thymus are of extrinsic origin. This was demonstrated by experiments in which the thymic rudiment was removed at regular intervals from EID3 to EID7 quail or chick embryos and grafted into the somatopleure of the other species. The thymic epithelium becomes attractive to

blood-borne hematopoietic cells at precise stages in the development. T cell progenitors colonize the epithelial thymus in three successive waves: the first beginning at EID6, the second at EID12, and the third occurring around EID18 until just after hatching [137]. Although the mechanisms controlling the internal clock that governs thymic influx in the embryo remain unknown, each wave of colonization is correlated with a peak in the number of progenitor T cells present in the blood circulation [138]. Progenitors in each wave give rise to $\gamma\delta$ T cells about 3 days earlier than the appearance of $\alpha\beta$ T cells. At EID12, about 5 days after the initial influx of thymocyte precursors, a subpopulation of thymocytes begins to express the $\gamma\delta$ TCR-CD3 complex on the surface [139]. $\alpha\beta$ TCR-bearing T cells that express the V β 1 variable domain are first detected in the thymocyte population on EID15, and these become the predominant type of thymocytes by EID17–18 [140]. $\alpha\beta$ TCR-bearing cells expressing the V β 2 variable domain emerge around EID18. After expansion in the cortex, these CD4CD8 double-positive cells undergo clonal selection and maturation to become either single positive CD4 or CD8T cells [141].

In the spleen, $\gamma\delta$ T cells appear at EID15, whereas the TCR $\alpha\beta$ V β 1 cells do not reach the spleen until EID19. TCR $\alpha\beta$ V β 2 cells are not found in the spleen until the second day after hatching [142]. These three T cell populations display distinct homing patterns in the spleen: $\gamma\delta$ T cells preferentially home to the sinusoidal areas of the splenic red pulp, whereas both the $\alpha\beta$ T cells (V β 1 and V β 2) are located in the periarteriolar sheaths [143].

$\gamma\delta$ T lymphocytes belong somewhere “in-between” the innate and adaptive immune systems. Chicken $\gamma\delta$ T cells represent around 30%–50% of peripheral T lymphocytes depending on the age and breed [144]. During ontogeny, $\gamma\delta$ T cells are the first T lymphocytes to appear in the thymus. They are believed to be involved in immunosurveillance, as the functional responses of conventional $\alpha\beta$ T cells (which require clonal expansion) in human neonates are impaired [145]. Thymic $\gamma\delta$ T cells represent around 30% of all thymocytes between EID12–15 [146]. From EID15, they are found not only in the spleen but also in the intestinal epithelium, suggesting that the chicken embryonic intestinal epithelia are able to support T cells development and/or homing [143]. Chicken $\gamma\delta$ T cells express all TLRs, except type 1 TLR2 [147,148]. In mammals, $\gamma\delta$ T cells respond to stimulation with PAMPs and produce cytokines [149]. Although information on chicken $\gamma\delta$ T cell responses to TLR stimulation is lacking, based on their TLR expression profile, we surmise that chicken $\gamma\delta$ T cells are also able to recognize PAMPs and are engaged in diverse innate immune responses [150] (see [Chapters 3](#): Development of the avian hematopoietic and immune systems, and [Chapter 6](#): Avian immunology, for more details).

13.4.6 Natural Killer cells

NK cells are the predominant innate lymphocyte subsets that mediate antiviral responses, mainly through the production of type I interferons (IFN- α and - β) and the release of proteases (e.g., granzymes) [151]. NK cells are present in the embryonic spleen and intestine of chicken embryos at relatively higher numbers [152]. NK cells characterized as CD8⁺ cells lacking both B cell and T cell receptors appear in embryonic spleen around EID14, before T cell migration from the thymus to peripheral lymphoid tissues. More recently, a novel blood lymphocyte bearing no markers for B or T cells except CD4 and CD5 were identified [153]. They express several NK cell markers including CD25, CD57, CD244, the vitronectin receptor, and other molecularly undefined NK cell markers, and they can be induced to produce IFN γ protein. In the intraepithelial lymphocyte compartment and also in the embryonic spleen, NK cells are found in abundance by EID19 [154,155]. The cytotoxic antiviral/antitumor properties of chicken NK cells differ among chicken strains and their cytotoxic potential is believed to be not fully developed until 6 weeks of age [156]. On the other hand, NK cells purified from chicken embryos at EID14 were found to be functionally similar to those isolated from the spleens of 4-week-old chickens [157] (see [Chapter 8.4: Natural killer](#)), suggesting that NK cell ontogeny and function in chickens merits to be further investigated.

13.4.7 Cytokines and chemokines

Cytokines regulate survival, proliferation, differentiation, and migration of primitive or mature innate immune system cells (see [Chapter 9: Avian cytokines and their receptors](#)). Although their ontogeny and function in the chicken embryo were not yet thoroughly investigated, some of them are relatively well studied. In chickens, macrophage colony-stimulating factor (CSF1) regulates the survival and proliferation of primitive macrophages during embryonic development by signaling via its receptor, CSF1R, and through the activation of different transcription factors [158]. Genes coding for several cytokines were shown to be differently regulated during embryonic immune system development. For example, baseline expression levels of IFN- γ , IL-4, IL-10, or IL-18 are identified in the spleen at EID12, with increased expression levels at later embryonic stages [159].

IFN was first described as a product of influenza virus-infected chick embryo cells, capable of inducing resistance to infection with homologous or heterologous viruses [160]. However, at present, there is a paucity of information around which organs have developed this capability, and further, which components of the IFN

system are regulated during embryogenesis. For types I and III IFNs, gene expression increases gradually from EID14 [161]. In the lung, the relative levels of type I and type III IFN increase across embryogenesis. The levels of IFN- α , IFN- β , and IFN- λ increase 100-fold, 55-fold, and 45-fold, respectively, at EID18 relative to EID10. In the spleen, IFN levels appeared to increase earlier, at EID14 [161]. Concerning type I IFN receptors, both IFN- α R1 and IFN- α R2 are poorly expressed during embryonic stages but are markedly increased at the time of hatch in both the lung and spleen [161]. Moreover, chicken embryonic cells can be stimulated in vitro to express type I IFN as early as EID15 and in an age-dependent manner, thus highlighting the functional relevance of this system during embryogenesis [162]. It may be that in the chicken IFN activity improves over time, despite the observation that relative levels of constitutive IFN decrease after hatch because of a greater access to receptors.

IL-7 is a master regulator of lymphopoiesis, acting primarily on cells of the lymphoid lineage. In chickens, the expression of IL-7 receptor, IL-7R α , is detected as early as EID7 in pooled primary and secondary lymphoid organs. Interestingly, IL-7R α expression levels are lower at EID20, suggesting that early expression of IL-7R α is correlated with cell differentiation and survival, as expected [163].

Chemokines are a large family of chemotactic cytokines involved in the migration of innate and adaptive immune system cells. There is very little information on the role of chemokines in chick embryos. As in other vertebrates, chemokine ontogeny in chickens is expected to occur in the very early stages of embryogenesis and may continue posthatching. The CXC chemokine CXCL14, which is involved in connective tissue development, appears in the earliest stages during chicken embryonic development [164]. Similarly, the stromal cell-derived factor-1 (SDF1), which also belongs to the CXC family of chemokines, appears as early as the primitive streak stage (a structure that forms in the blastula during the early stages of avian, reptilian, and mammalian embryonic development) [165]. Furthermore, the CXCR4-CXCL12 (SDF1) system is critically involved in the colonization of the bursa of Fabricius by prebursal B-cell precursors [166,167] and was shown to regulate egress of mature B-cells from the bursa around hatch [167] (see [Chapters 3 and 4](#) for more details).

13.5 Extraembryonic structures and innate immunity

The development of an embryo intimately depends on supporting vascularized appendages, namely, the extraembryonic sacs that persist until the transition from the

embryo to the autonomous hatched chick. In birds, they consist of four structures that are continuous with the embryonic body: the yolk sac, the amniotic sac, the chorion, and the allantois; the latter two fusing to form the allantoic sac [108,168,169]. As embryonic development proceeds, the membranes forming the lining of incipient extraembryonic sacs grow and spread within the egg (Fig. 16.4).

The cellular composition of these extraembryonic sacs initially originates during gastrulation, when the blastula reorganizes to form three germ layers, namely, the ectoderm, the mesoderm, and the endoderm. Thereafter, during neural tube formation (neurulation), the mesoderm splits into two sublayers, the somatopleural layer beneath the ectoderm and the splanchnic layer in contact with the endoderm. The amniotic sac is an extension of the ectoderm/somatopleural layer while the allantoic sac and the yolk sac arise from fusion of the splanchnic layer/endoderm.

The yolk sac encloses the yolk and is involved in the predigestion (partial proteolytic processing) and assimilation of yolk nutrients throughout development. The amniotic sac is composed of a thin membrane filled with an aqueous solution that surrounds and encloses the embryo to protect it from dehydration and mechanical shocks. The allantoic sac arises from an outgrowth of the ventral wall of the embryonic hindgut and consists of a complex cellular structure that grows in close contact with the eggshell internal surface to ensure gas exchange and mineral transport (Fig. 13.4). It becomes filled with embryonic

metabolic wastes discharged into the so-called allantoic fluid (AIF).

All three extraembryonic sacs develop concomitantly to ensure specific and complementary physiological functions that are crucial for the development of the embryo. The delimitation enforced by the eggshell confines the growing embryo in a fixed spatial volume and imposes a sequential equilibrium between all egg compartments, the water composing the yolk and the egg white being progressively transferred into the forming embryonic sacs. These structures, which are extensions of the embryonic body, are essential to trigger very rapid protective/defensive mechanisms to resist the negative impact of physical or microbial challenges (eggshell damage/microbial penetration through an altered/degraded eggshell). It is noteworthy that the amniotic and allantoic sacs both degenerate during the last days of incubation and are discarded at hatch, while the yolk sac is partly resorbed in the embryo abdominal cavity as a continuum of the immature digestive gut of the chick.

13.5.1 Amniotic sac

The amniotic sac arises after EID2 from the ectoderm layer that forms the inner layer of the amniotic sac, and from the somatopleural mesoderm as the outer layer. The inner ectodermal layer undergoes very little differentiation while the outer mesodermal layer is progressively differentiated into smooth muscle cells that are arranged

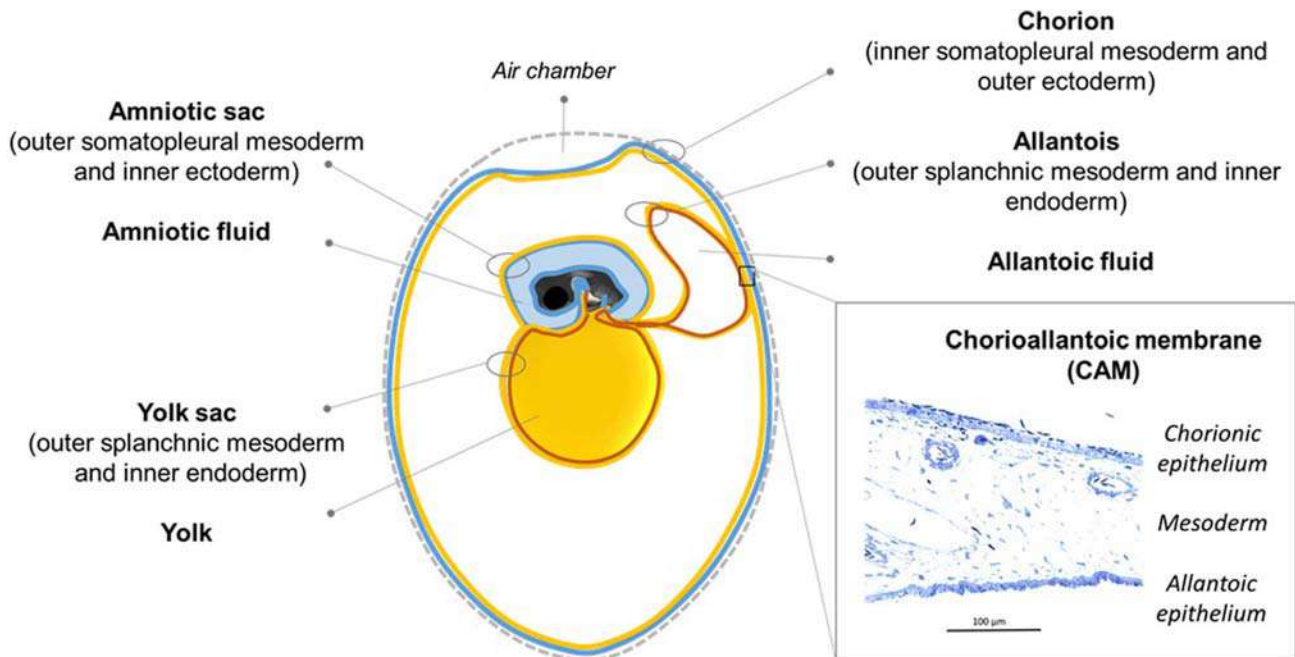


FIGURE 13.4 Overview of extraembryonic appendages. The ectoderm, mesoderm, and endoderm derived tissues are illustrated by blue, yellow, and orange lines, respectively. The eggshell is in grey (dashed line). *Inset: semithin micrograph showing the chorioallantoic membrane at EID17* ©Plateforme IBiSA de Microscopie Electronique, University and CHRU of Tours, France, S. Georgeault (original figure).

in muscle bundles to form peculiar double-layered structures resembling stars [169]. The amniotic membrane exhibits contractile activity as soon as EID5 [170] and is capable of responding to mechanical, thermal, electrical, or chemical stimuli/injuries. Thus the primary role of the amniotic sac in the protection of the embryo is physico-chemical, as it cocoons the embryo to prevent dehydration and adhesion to other egg structures; it also mitigates the negative impact of potential mechanical shocks or thermal changes while permitting embryonic movement and growth.

The elasticity of this very thin membrane is very high. It is continuously tense as it is progressively filled with a translucent aqueous fluid reaching up to 4.5 mL around EID15 after the massive influx of the albumen [171]. The ionic composition of amniotic fluid (AmF) (chloride, sodium, potassium, phosphorus, magnesium, calcium, iron, and sulfur) varies during developmental stages [171]. Its concentration in proteins is very low during the first half of incubation but strongly increases afterward as the concentrated albumen flows into the AmF [172]. The antimicrobial potential of the AmF essentially derives from the antimicrobial activity of egg white proteins that enter the amniotic cavity during the second half of incubation [92]. Since the embryo starts to swallow the AmF/egg white mixture from EID13 onward, we recently hypothesized that this fluid might contribute to the establishment of the primary innate immunity of the digestive tract [92]. In addition, since the embryo is immersed in this fluid, the embryonic feathers and skin are likely to be covered by a moisturized biofilm originating from the AmF, that would contribute to protecting the newly hatched chick from exogenous agents and pathogens [92], similar to the human vernix in neonates. It might also help in the adaption of the chick skin from a liquid to a gaseous milieu, by preventing superficial skin dehydration.

13.5.2 Yolk sac

The formation of the yolk sac is a highly coordinated sequence of events leading to a very peculiar and complex structure consisting of a medial vascularized region known as the area vasculosa and a peripheral nonvascularized region termed the area vitellina. It expands onto the perivitelline layer to enclose the totality of the yolk by EID15 [169] and thus physically protects the highly nutritive yolk. The yolk sac serves as a yolk/embryo interface that mediates the transfer of nutrients and bioactive components from the yolk to the embryonic circulation, via its very high vascularization. The yolk sac is continuous with the embryo intestine but the transfer of yolk nutrients is mediated by the engulfment of yolk spheres by the apical face of the yolk sac endodermal epithelial

cells. These cells acquire the typical characteristics of polarized epithelial cells with numerous villi and invaginations [173,174]. The weight of the yolk sac increases up to EID16 (4 g) then decreases by EID20 (2 g), as it is progressively retracted into the abdominal cavity of the embryo concomitant with the closure of the mid-gut [169]. This yolk-to-blood transfer of nutrients also requires an orchestrated expression of enzymes, transporters, receptors, and plasma carrier proteins at specific developmental stages [175]. The role of the yolk sac in innate immunity during embryonic development is thus physical, cellular (as its high vascularization allows the circulation of immune cells), but also molecular with the expression of antimicrobial molecules. It also mediates the translocation of maternal immunoglobulins from the yolk to the embryo blood. During the last week of incubation, the yolk sac remains attached to the embryonic intestine thanks to the yolk stalk (resembling a peduncle) that develops to form a structure similar to the intestine wall [169]. By EID19, 2 days before hatching, the yolk sac together with the remnant yolk starts to enter the body cavity simultaneously to the progressive closure of the mid-gut. This umbilicus closure is usually only partial at hatch and remains a major gateway for microbial infection of the chick. It is noteworthy that the Meckel's diverticulum, a unique avian lymphoid structure, will develop after hatching at the junction of the duodenum and jejunum, following the connection between the yolk stalk and the small intestine [176,177] (see Chapter 2: Structure of the avian lymphoid system).

It is now well known that one of the main contributions of the yolk sac to innate immunity of the older embryo and the newly hatched chick is its capacity to mediate transfer of maternal immunoglobulin Y (IgY) from the yolk to the embryo via its vascularized network. The transfer of maternal IgY is a two-step receptor-mediated process. It starts with the progressive accumulation of the maternal immunoglobulins in growing follicles during vitellogenesis [178]. At maturity, a follicle will contain 100–200 mg of maternal IgY [178]. The second step requires the endocytosis of yolk IgY by yolk sac receptors to reach the embryonic blood. The concentration of IgY stored in the yolk is variable among individuals (1–8 mg/mL of yolk [178,179]), between strains [179], and is dependent upon hen age [180]. It intimately depends on the IgY concentration in the maternal blood and yolk follicles [178]. Indeed, the total IgY concentration found in offspring serum is positively correlated with IgY concentration in hen serum and in yolk [181]. During incubation, IgY is uptaken from the yolk after binding to the chicken yolk sac receptor (FcRY) [182], endocytosed at acidic pH, and transcytosed. The ligand/receptor dissociation then occurs at the slightly basic pH of the embryo blood to release IgY [182,183]. IgY uptake

occurs predominantly in the last few days before hatching (EID17 to hatch) but low concentrations of IgY in the bloodstream can be observed as early as EID7 of incubation [178] (see **Chapter 5: Structure and evolution of avian immunoglobulins**).

IgY is the most prominent immunoglobulin isotype of the egg, being essentially concentrated in the yolk, but egg white also contains low concentrations of IgA and IgM (6–14 µg/mL) [179]. An increase of the egg white derived-IgA in the yolk sac is detectable from EID14 onward [184]. These data suggest that IgM is likely to increase similarly in the yolk sac [185]. Whether this IgA and IgM increase results from an accumulation of egg white components directly across the yolk sac membrane [184] or after oral absorption of egg white/AmF mixture [92] remains to be elucidated. These immunoglobulins are quite selective as they can “only” protect the chick from early infection by pathogens against which these maternal antibodies were initially produced. They reflect the rearing environment of laying hens. Thus the accumulation of IgY in the yolk may be considered as a maternal anticipation of microbes that the chick is likely to encounter after hatch.

In addition to this passive immunity, it is now well established that the yolk sac contributes to a transient wave of hematopoietic cells including macrophages [186,187]. Although there is a lack of evidence that these macrophages can be recruited at the vicinity of a potential physical wound, they are clearly capable of recognizing and engulfing microbes that are attached to the blood vessel wall [186] (see **Section 13.4**). The yolk sac serves as a support for the circulating immune cells (monocytes and macrophages, from EID10 and 12, respectively) [188]. Moreover, there is increasing evidence that the yolk sac epithelium steadily expresses basal levels of antimicrobial peptides including avian beta-defensins (AvBD1, 2 and 7), lysosomal proteases (cathepsin B, legumain), and the iron-sequestering ovotransferrin from EID13 onward [175]. In addition to AvBD1, 2, and 7, a recent report also demonstrated the expression of AvBD10 [189] with an increasing expression of all four avian beta-defensins up to EID13, followed by a decrease in expression until EID19. Using *in situ* hybridization, the authors also showed that AvBD10 mRNA was essentially expressed in the endodermal epithelial cells of the yolk sac, while acidophilic granulocytes expressed AvBD1, 2, and 7 and were present in the yolk sac between EID9 and EID17 [189]. In contrast, the expression of AvBD6, AvBD8, AvBD11, and AvBD13 was barely detectable, and that of AvBD13 tended to increase at EID15 [189]. To our knowledge, the expression of other antimicrobial proteins, cytokines, and pattern recognition receptors (including TLRs) by the yolk sac has not yet been explored.

Thus the complete repertoire of yolk sac molecules and cells that may participate in innate immunity is not

completely understood. It would be interesting to investigate how yolk sac immunity could be stimulated remotely by a cellular wound occurring in the CAM (following eggshell damage). Indeed, these two extraembryonic annexes are likely to communicate via vascularization as they are both continuous with the embryonic body.

13.5.3 The allantoic sac

Morphology and structure of the chorioallantoic membrane

In birds, the CAM is a placenta-like structure that progressively develops during incubation and ultimately lines the inner eggshell membranes [190,191]. It is formed of three layers, which include the chorionic epithelium, the mesoderm, and the allantoic epithelium (**Fig. 13.4**). During growth, blood capillaries and sinuses invade the chorionic layer in close proximity with ambient air arriving via eggshell pores (**Fig. 13.4**). The CAM is a nexus for many different physiological and metabolic processes including acid-base balance, breathing, and calcium solubilization from the eggshell that is reallocated to assist bone and tissue formation in the embryo [192]. The highly vascularized CAM occupies a strategic position, as it forms a lining under the eggshell and totally surrounds the embryo by the end of incubation, allowing the cells of innate immunity to act locally, in case of bacterial penetration through the eggshell. CAM vascularization starts from EID4 and continues until EID14 of embryonic development [193]. The highly vascularized CAM becomes the functional respiratory organ for the embryo from EID8 to EID19, when pulmonary respiration of the embryo is initiated [102,194]. By EID8, the CAM contains a developed capillary plexus, which becomes progressively associated with the outer surfaces of chorionic epithelial cells. The capillary plexus is localized at the surface of the ectoderm adjacent to the eggshell membranes at EID14 [195]. From the second half of incubation (EID10/EID11) to hatching (EID21), one major function of the CAM is to solubilize the inner part of eggshell to release the minerals that are required for the mineralization of the embryo skeleton [101,102,190]. By the end of incubation, the thinning of the eggshell facilitates the emergence of the hatchling from the egg [101]. But, this weakened eggshell could increase the susceptibility of the egg and embryo for contamination in the later stages of development [196]. Concomitantly, this eggshell solubilization may contribute to the release of occluded antimicrobial proteins from the eggshell organic matrix, which may trigger the formation of a local protective biofilm at the interface between the CAM and the inner part of the eggshell. Proteins associated with innate immunity and antimicrobial protection are abundant in the

vascularized CAM and associated eggshell membranes at EID19: OCX-36 (BPIFB3), TENP (BPIFB2), and AvBD11, which would also contribute to innate protection against pathogens as the shell is weakened before hatching [197]. Deposition of lipopolysaccharide directly on the CAM induces a significant inflammatory response, resulting in heterophils and monocytes recruitment to the stimulated site [198], confirming its immune responsiveness. In case of bacterial penetration through the shell, this strategic position allows immune cells to act locally. By EID10–14 [106], the CAM provides a functional response to TLR ligands. The CAM response to viral infection was pivotal in identifying the presence and action of IFN- γ [199]. Indeed, this molecule was shown to be released by EID10–EID11 chicken CAM after incubation with inactivated influenza virus [199]. Thus the elaboration of the CAM during embryonic development introduces an additional tissue layer/barrier, which is a rich source of antimicrobial peptides and proteins.

Composition and function of the allantoic fluid

The allantoic compartment is associated with the chorio-allantoic sac, which results from the fusion of the chorion and the allantois at EID5–EID6 [172] (Fig. 13.4). The AIF is a product of embryonic metabolism and primarily results from kidney filtration of plasma [200]. This fluid remains hypoosmotic to embryonic plasma throughout incubation; however, uric acid concentration increases approximately twofold during development. Uric acid is essential for avian embryonic water balance, since oxidation of protein to urate produces more metabolic water than oxidation of fat [201]. As the volume of AIF decreases from EID13, these compounds are concentrated and precipitate to form urate crystals [202]. From the second half of incubation, secretion of protons into the compartment induces a drop in pH of about 2 units by EID18/EID19 to a pH <6 [172,203]. This acidic pH would challenge bacterial survival in AIF, if it became contaminated, since a deviation of even one unit from pH optimal can reduce microbial growth rates by about 50% [204].

AIF proteolytic activities increase from EID8 to EID16, as assessed by in-gel and in-solution assays [172]. Characterization of proteolytic enzymes in embryonic fluids identified 12 proteases in the AIF, compared to five in the AmF [172]. However, there is no clear evidence that AIF plays a significant role in antibacterial defense during embryonic development.

13.6 Concluding remarks

The calcareous egg of birds (and reptiles) is a unique reproductive model that comprises at least four levels of

protective barriers to assist the embryo throughout its development: eggshell microbiome, physical barriers (mineralized, proteinaceous barriers, and epithelial barriers), physicochemical barrier, and antimicrobial molecules. The exact role of the eggshell in the establishment of the cuticle microbiota as a potential primary innate immunity of the chick remains to be characterized. This microbiota is regulated by the environment and by hatchery practices (disinfection and absence of any contact with hens). Indeed, the presence of adult hens during incubation in hatchery environment has been shown to impact the microbiota development in newly hatched chickens, as hens are efficient donors of *Bacteroidetes* and *Actinobacteria* [205]. The eggshell microbiome also depends on the habitats of birds and on the microbes present in the environment. It is noteworthy that within bird species, some subtle variations of egg structures or protein composition, likely resulting from evolutionary processes to adapt to the environmental constraints of the bird habitat, can be observed in perivitelline layer [69] and eggshell [16,17,206–208]. However, the biological significance of these modifications remains largely unexplored, especially in wild species. Moreover, although the function of the various basic components in chicken egg defense has been extensively reviewed, little is known on the ability of extraembryonic appendages to respond to microbial and physical aggression. The CAM is assumed to have a major role in egg defense with regards to its strategic position beneath the eggshell, which is directly exposed to bacterial and physical aggressions of the eggshell. The structural and cellular features of the CAM and its dense vascularization are believed to undergo major changes upon immune stimulation and depending on its developmental stage. Knowing that the CAM is capable of cellular and molecular responses when immunostimulated by bacterial LPS [198] or viruses [199], it will be of interest to explore whether such immune changes (cells and molecules) can function as warning signs for the embryo (via vascularization), to increase embryo/chick preparedness to challenged environments, after hatching.

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Avian immunosuppressive diseases and immune evasion

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14.1 Introduction

Control of infectious diseases is essential for the production of healthy poultry flocks and this is generally achieved by extensive vaccination programs in combination with good management practices, including biosecurity measures to reduce the risk of infection. The success of vaccination programs depends on the ability of the birds to mount a vigorous immune response after vaccination. In addition to the innate ability of a particular bird to mount an immune response to a vaccine or an infection, there are numerous external factors influencing the level of protective immunity. The purpose of this chapter is to review the mechanisms and impact of immunosuppression, which can have multiple causes, and the ability of pathogens to counteract immune responses by immunoevasion.

In this chapter immunosuppression is defined as “A state of temporary or permanent dysfunction of the immune response resulting from insults to the immune system and leading to increased susceptibility to disease” as originally proposed by Dohms and Saif [1] with the addition of “and often a suboptimal antibody response” as suggested by Lütticken [2] to which we add “and suboptimal innate and cell-mediated responses.” Such immunosuppression often results from infection of cells of the immune system, leading to their impaired function against the primary and subsequent infections, in a nonspecific manner. For this chapter, we define immunoevasion as “pathogen-initiated responses counteracting the immune responses to the specific pathogen.” The major difference is that immunosuppression is the consequence of the overall replicative strategy of the causative agent, resulting in increased susceptibility to other pathogens but not necessarily to the causative agent, while immunoevasion is

achieved by specific pathogen-encoded determinants, primarily favoring replication of the causative agent. In some instances infections can result in immunosuppression and immunoevasion, for example, infection with Marek’s disease virus (MDV).

14.2 Immunosuppression

14.2.1 Introduction

Immunosuppression is a major problem for the poultry industry, but actual figures indicating the scale of the problem are difficult to find. Infection with pathogens and/or environmental factors, including management errors, can result in immunosuppression, and interactions between the two usually exacerbate the problem. In an excellent review article, Hoerr [3] examined the many environmental and infectious causes of immunosuppression in chickens and turkeys, which are complex and can have a major impact on profitability of broiler and layer flocks (Fig. 14.1). Environmental stressors include the later period of incubation, hatching, and chick handling. Other stressors include suboptimal housing conditions and mycotoxins. Any infection causing clinical disease may result in immunosuppression, but the focus here is on pathogen-induced immunosuppression in the absence of clinical disease.

Viruses causing immunosuppression and/or clinical disease at any age include MDV, reticuloendotheliosis virus (REV), reovirus, and, although controversial, avian leukosis virus (ALV). Other pathogens—for example, chicken infectious anemia virus (CIAV)—may cause clinical disease in young chicks, but the major damage is caused by subclinical infection resulting in immunosuppression. Infectious bursal disease virus (IBDV) also

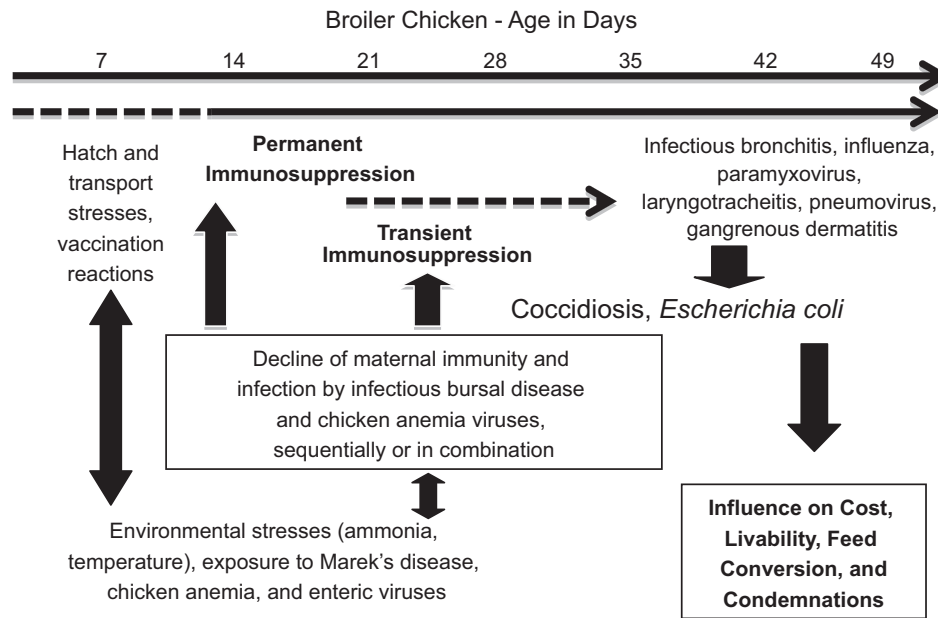


FIGURE 14.1 Immunosuppressive interaction in broiler chickens. A high-yield broiler may hatch in an energy-depleted state and be subjected to suboptimal handling and transportation systems, and experience delays in acquiring nutrition, all while processing attenuated live vaccines that produce cytolytic infections. The brooding environment may not meet thermoneutrality, and placement on reused litter creates the potential for exposure to ammonia and potentially immunosuppressive agents such as MD, chicken anemia, and enteric viruses. With the decline of maternal immunity, active infection by IBDV can be permanently immunosuppressive at or before 14 days of age and, at least transiently immunosuppressive when it occurs at an older age. Also, with declining maternal immunity, CIAV can produce a cytolytic infection in the thymus and contribute to the immunosuppression initiated by IBDV. Following this sequence, it is possible for immunocompetency to be compromised in a variable percentage of the flock. This can contribute to increased severity of infections by respiratory viruses and the ability to control secondary respiratory infections by *E. coli*. Coccidiosis and gangrenous dermatitis may likewise emerge as problems from 25 days of age and older. Collectively, this sequence of events occurs to variable degrees and influences livability, feed conversion, condemnation at processing, and total production cost. Each immunosuppressive influence represents a control point to prevent immunosuppression and improve health and welfare. [3] From Hoerr FJ. *Clinical aspects of immunosuppression in poultry*. Avian Dis 2010;54:2–15 with permission from the American Association of Avian Pathologists.

causes clinical disease in young chicks, but results in damage to immune tissue, particularly the bursa of Fabricius and bursa-derived lymphocytes, compromising the host's ability to mount effective responses upon subsequent infection by other pathogens. Caution is needed, however, in the interpretation of the immunosuppressive effects caused by reovirus, MDV, REV, and IBDV especially with older studies; because it is not always clear whether these viruses were free of CIAV. For example, Jakowski et al. [4] reported hematopoietic destruction in chickens with Marek's disease, but it was later learned that tumor material harvested from these birds was positive for CIAV (Wellenstein, personal communication, cited in reference [5]). In addition to viral infections, coccidial infections have been linked to immunosuppression.

14.2.2 Stress-induced immunosuppression

The term "stress" is often used but remains poorly defined and can affect many systems within a stressed animal [6]. The consequences of stress may depend on the duration of the stress period. In contrast to chronic stress, short-term stress can have a beneficial impact on the immune responses

(reviewed in [7]). In this chapter we will only briefly address the negative impact on the immune responses. Most environmental factors causing immunosuppression are related to management problems such as inadequate water or food supply, ammonia in the houses, temperature stress, "social interactions within a flock," and so on. Most of these stressors enhance the production of corticosterone (CORT). Selection for high versus low CORT concentration in blood plasma can influence the degree of stress-induced immunosuppression. In addition, the presence of fungal toxins in feed is an environmental stressor.

It has been recognized for a long time that social stress can exacerbate the disease. A classic example is the study by Gross [8] in which MDV-exposed chickens were kept in a social stressful environment by moving one chicken from one cage into another cage every day. These chickens developed a higher incidence of tumors than MDV-infected chickens kept in a low-stress environment. The effects of social stress on MD were especially enhanced in birds selected for high plasma CORT concentrations. Inoculation with chemicals blocking 11- β hydroxylase, which mediates the conversion of deoxycorticosterone to CORT in the adrenal glands, reduced the impact of social

stress on Marek's disease and other diseases [8,9]. Inoculation of chickens with CORT using pharmacological doses resulted in a rapid lymphoid depletion in thymus, bursa, and spleen [10]. These authors suggested that bacterial infections (e.g., *Escherichia coli*) may cause stress-type lesions in the bursa similar to CORT-induced lymphoid depletion. High levels of CORT in hens reduce egg production and decrease testosterone and progesterone in the yolk influencing the quality of the chicks [11]. In addition, embryonic exposure to increased levels of CORT can alter the juvenile stress responses [12].

Heat stress has a major negative impact affecting broilers and laying hens. The importance of heat stress is expected to increase in the near future as a consequence of global warming. In addition to immune responses, heat stress affects also the physiology of the chickens, increases feed consumption and reduces the growth rate and egg production. High temperatures impact the sympathetic–pituitary–adrenal axis and, as a consequence, the hypothalamic–pituitary–adrenal (HPA) axis resulting in increased levels of plasma CORT (reviewed in [13,14]). Chronic increased levels of CORT can reduce proinflammatory responses [15,16], suppress immune organs and immune cells (reviewed in [15]). The impact of heat stress has consequences when the effects of pathogen-induced immunosuppression are studied. These studies need to be done in climate-controlled facilities in order to reduce or eliminate heat stress as a confounding factor.

14.2.3 Mycotoxin-induced immunosuppression

There is an extensive literature on fungal toxins or mycotoxins and in this chapter we provide only a very limited overview of the importance of mycotoxins. Mycotoxins are often present in feed and can confound the analysis of pathogen-induced immunosuppression because mycotoxins can influence immune responses in addition to cause clinical disease. However, the impact of mycotoxins on immune responses is not always clear because low doses of certain mycotoxins may actually have beneficial effects by stimulating growth, while high doses may have a negative effect. This phenomenon, named hormesis, has been described: e.g., for aflatoxin B1 (AFB₁) affecting growth [17]. In addition, there are many different mycotoxins that may have different effects on the immune responses, gut physiology, and growth of especially broilers. AFB₁ is the best known fungal toxin for poultry, although chickens are rather resistant to the toxic effects compared to ducks and turkeys (reviewed in [18]). After ingestion, AFB₁ is bioactivated in the liver by cytochrome P450 into the highly toxic component aflatoxin-8,9-epoxide, which can bind to DNA, or after being hydrolyzed, bind to proteins, causing cytotoxicity [19]. The consequences are cell death in the primary lymphoid organs, damage to the

intestinal integrity and, especially in broilers, decreased performance. Antibody and CMI responses are in general decreased [3,18], although at low levels antibody responses may actually increase during initial exposure [18]. Chicks hatched from hens fed on diets containing aflatoxins also showed impaired immune functions when tested at 2–3 weeks of age, but it is not known how long after hatching the effects last [20]. AFB₁ does not seem to affect the barrier function of the intestinal tract or the intestinal innate immune responses, although the data are not conclusive [18].

The genus *Fusarium* is a source for several mycotoxins such as Fumonisin B1, deoxynivalenol (DON), other trichothecenes (e.g., T-2), zearalenone (ZEA), and ochratoxins (e.g., OTA) have also been implicated as immunotoxicants in chickens, turkeys, and ducks (reviewed in [21,22]). Low levels (15 ppm) of fumonisin B₁ (FB₁) given to broilers in the feed for 3 weeks reduced macrophage activity, reduced secondary antibody responses to Newcastle disease vaccines, and reduced gene expression of interleukin (IL)-1 β , IL-2, interferon (IFN)- α and IFN- γ . This level of FB₁ does not cause a decrease in growth performance and is considered an acceptable level in chicken feed [23]. Two toxins belonging to the trichothecenes group, T-2 and DON, have been linked to immunomodulation in chickens [24,25]. For example, administration of sublethal doses of T-2 to chickens between 1 and 7 days of age reduced herpesvirus of turkeys (HVT)-induced protection against challenge with MDV [26]. DON inhibits the synthesis of DNA, RNA, and proteins and therefore affects tissues with a high cell turnover such as epithelial cells in the intestinal tract and cells in immune organs [21]. The effects of DON on the integrity of the intestinal tract are complex and are different for the different parts of the intestinal tract. Claudin 1 (*CLDN1*) mRNA is upregulated strongly in the duodenum but less in the jejunum by 5 mg DON/kg feed [27]. Osselaere et al. [28] reported increased expression of Claudin 5, which was also reported by Chen et al. [29]. These authors noted also an increase in spleen size with increased cellular proliferation, apoptosis, and DNA damage at the end of the 16-week experiment in slow-growing chickens. Several groups have suggested that the changed permeability of the intestinal tract may cause increased bacterial challenge leading to higher IgA levels than in chickens without DON in the feed. Low doses of T-2 and DON have also been linked to increased IgA levels and titers against Newcastle disease virus (NDV), while high doses cause immunosuppression. Broilers fed a diet containing DON and challenged with very virulent (vv) IBDV developed more severe effects than broilers fed a diet lacking DON [30].

ZEA is a nonsteroidal estrogenic mycotoxin that can bind to estrogen receptors (reviewed in [22,31]). There is

little information on its immunotoxicity in chickens. However, it is possible that the presence of ZEA or its metabolites can interact with CIAV, because the promoter/enhancer of CIAV resembles an estrogen receptor [32]. Miller et al. [32] have shown that estrogen binds to the CIAV promoter/enhancer in studies using reporter genes driven by the CIAV promoter sequence. Thus far, there are no studies published on the potential of ZEA to increase transcription and thus viral replication of CIAV.

The interactions between trichothecenes and immune responses are complex [33]. Low doses can actually stimulate immune responses by upregulation of cytokines, and chemokines, while high doses activate caspases resulting in apoptosis. OTAs are highly toxic in many species, including chickens, and can be found in eggs from OTA-fed chickens. Innate and acquired immune responses are decreased and susceptibility to disease is increased in chickens fed OTA ≥ 1 pm and in their progeny [34–36]. For example, OTA at 2 ppm for 14 days significantly increased mortality caused by *Salmonella enterica* subspecies *enterica* serovar Gallinarum [37]. Combinations of the different mycotoxins are frequently present in feed and interactions between them often increase the immunosuppressive effects [38–40]. The presence of mycotoxins in feed and possible embryonal transmission has important implications and can confound the interpretation of immune dysfunctions in commercial flocks when virally induced immunosuppression occurs.

14.2.4 Coccidia-induced immunosuppression

Two groups of coccidian species have been linked to immunosuppression, *Cryptosporidium baileyi* and *Eimeria* species, although the evidence is rather limited in both cases. Infection with the latter has been linked to decreased mitogen stimulation responses [41], but antibody responses to T cell-dependent and -independent antigens were not affected [42]. *Cryptosporidium baileyi* replicates in the epithelial cells of the bursa of Fabricius and respiratory tract of chickens and is probably more prevalent than diagnostic cases indicate [43]. Oral inoculation of young chickens with high doses of *C. baileyi* caused histopathological lesions in the epithelium and interfollicular tissue of the bursa [44] and decreased antibody responses to T cell-dependent and -independent antigens [45,46]. Titers to infectious bronchitis virus (IBV), NDV, and avian influenza virus (AIV) vaccine were also decreased after inoculation of chicks with *C. baileyi* [47–49]. However, Abbassi et al. [50,51] found that infection with *C. baileyi* did not increase Marek's disease or affect the efficacy of the MDV vaccine strain CVI988. Interestingly, MDV infection before or after challenge with *C. baileyi* aggravated the latter. Likewise, MDV vaccination followed 4 days later with *C. baileyi* challenge

caused respiratory lesions within 6 days (see also section on MDV).

14.2.5 Virus-induced immunosuppression

As mentioned before, we will only discuss viruses that may cause immunosuppression independently of clinical symptoms, although the independence may be a temporal feature, as is the case for the tumor-inducing viruses and, depending on the age of challenge, CIAV and IBDV. The following viruses and their effects on the immune responses are discussed: IBDV; CIAV; reovirus; adenovirus, and the three tumor-inducing viruses ALV, REV, and MDV. Detailed information on virus replication, pathogenesis, pathology, and immune responses for these viruses can be found in several chapters in *Diseases of Poultry* 13th edition [52] and *Avian Virology: Current Research and Future Trends* [53].

Infectious bursal disease virus

IBDV, belonging to the Birnaviridae, has been divided into two serotypes, of which only serotype 1 causes immunosuppression and disease in chickens. Several pathotypes are recognized within serotype 1 varying from mild to vv. Strains within group 1 have been further subdivided into genetic groups based on sequencing. Based on the sequence information, Jackwood et al. [54] recently proposed a nomenclature based on serotype, country of origin, original name, year of isolation, and genotype (although the nature of the two genome segments of this bi-segmented virus may also need to be considered).

The genome of IBDV is characterized by a double-stranded (ds)RNA genome consisting of segments A and B. Segment B codes for the viral RNA-dependent RNA polymerase, which may be involved in pathogenicity [55]. Segment A codes for two structural proteins (VP2 and VP3), an autoprotease (VP4) and a small nonstructural peptide (VP5), which partly overlaps with the open reading frame (ORF) coding for VP234 [56]. VP3 is bound to the viral dsRNA and also interacts with VP1 [57]. The VP2–3–4 polypeptide, but not the mature VP2, VP3, or VP4, arrests B cell division possibly by interfering with the cell cycle, given that it does not affect cell viability [58].

It has long been recognized that serotype 1 strains replicate in B cells expressing chB6 (previously named Bu-1) and surface immunoglobulin (sIgM) [59,60] resulting in apoptosis and atrophy of the bursa of Fabricius. The depletion of B cells expressing sIgM affects mostly primary antibody responses. Infection, especially at 1 day of age, also results in a significant decrease of sIg expressing B cells in spleen and peripheral blood lymphocytes but does not affect circulating CD4⁺ and CD8⁺ cells [61,62]. IBDV also replicates in the B cells in the cecal tonsils resulting in

an influx of mostly CD8⁺ T cells [63]. The damage to the bursa is transient, follicles become repopulated with lymphocytes and tissue architecture is restored, but primary antibody responses remain depressed until at least 7 weeks postinfection (PI). Ultimately, however, they also recover. The duration of the recovery process depends on the age of infection and the virulence of the strain [64,65]. The consequence of apoptosis of the immature B cells is a severe immunosuppression with impaired antibody responses and increased susceptibility to other pathogens, especially when birds are infected before 3 weeks of age (reviewed in [56,59,66]). Recently, IBDV-induced immunosuppression has also been linked to significantly reduced efficacy of vaccine-induced protection against highly pathogenic AIV [67]. IBDV-induced immunosuppression also increases the bacterial load of *Campylobacter jejuni* [68], most likely by a reduction of B lymphocytes in the lamina propria of the caeca negatively affecting the antibody titers against *C. jejuni* [69]. Interestingly, infection with vvIBDV also alters the cecal microbiota, although the significance of this change is not yet clear and further studies will need to be conducted [69].

The mechanism(s) involved in the induction of apoptosis are complex and only partly elucidated. Using a reverse genetics approach, Yao et al. [70] showed that deletion of VP5 did not prevent virus replication in vivo but did prevent the development of bursal lesions, suggesting a role for VP5 in the pathogenesis. Interestingly, in vitro studies suggest that very early after infection VP5 is antiapoptotic [71], probably through binding to the p85 α regulatory subunit of phosphatidylinositol 3-kinase (PI3K) [72]. Activated PI3K phosphorylates Akt, which results in survival of infected cells early during infection. During the later stages of infection, VP5 accumulates in the plasma membrane [73] and causes apoptosis [74]. The induction of apoptosis was linked to activation of the Jun NH₂-terminal kinase (JNK) [75], although it was not resolved if VP5 or VP2 was responsible for the activation, which has also been linked to apoptosis in infected B cells [76].

Type I IFNs (IFN- α and IFN- β) can be triggered by binding of dsRNA to chicken melanoma differentiation-associated protein 5 (MDA5) and TLR3. However, early during IBDV infection there is no or little induction of IFNs [77], for which in vitro studies offer possible explanations. Thus VP3 blocks the binding of the dsRNA to MDA5, specifically interfering with the production of IFN- β [78]. In addition, the chicken staufen protein 1 (chSTAU1) binds dsRNA preventing access of MDA5 to IBDV dsRNA [79]. VP4 also inhibits the production of type I IFNs by interfering with the K48-linked ubiquitylation of the glucocorticoid (GC)-induced leucine zipper [80]. The finding that VP3, 4, and 5 interfere with the production of type I IFNs suggests the importance of interference with early production of IFNs in order to

allow the completion of the viral replication cycle. The addition of IFN- α to cultures early after infection resulted in massive apoptosis [81] confirming the importance of inhibiting type I IFNs.

In addition to the apoptosis of IBDV-infected B cells, noninfected B cells can also undergo apoptosis [82], perhaps caused by the rapid increase of IFN- γ shortly after infection [83]. In summary, the molecular basis for IBDV-induced apoptosis of B cells is the result of a complex set of interactions between the different viral proteins and infected B cells. The recent demonstration by Dulwich et al. [84] that vvIBDV will replicate in ex vivo bursal B cells offers additional routes for investigating the phenomenon.

Withers et al. [85,86] described two types of follicles emerging after recovery from IBDV infection in neonatal chicks: small follicles lacking a distinct cortex and medulla and large follicles with rapidly proliferating B cells and a normal structure. B cells in the large follicles were still capable of undergoing gene conversion and may have been derived from small numbers of surviving bursal stem cells. In contrast, the B cells in the small follicles were considered to be derived from more mature B cells that had already undergone gene conversion. These data suggest that the ability to recover from IBDV-induced suppression of antibody production and diversity is based on the proportion of small versus large follicles developing after infection. Biró et al. [87] reported that B cell maturation may be negatively affected by IBDV-induced changes in the extracellular matrix of the antigen-trapping regions of the spleen, which may contribute to permanent immunosuppression.

In addition to replication in B cells, IBDV can also infect and replicate in macrophages [88,89] and mesenchymal stem cells [90]. Khatri et al. [89] found viral RNA and proteins by reverse transcriptase (RT)-PCR and immunohistochemistry, respectively, in bursal macrophages between 1 and 7 days PI. The absolute number of macrophages in the bursa was decreased significantly at 3 and 5 days PI. Similarly, the number of macrophages was also decreased in spleens after IBDV infection [91]. The actual impact on the immune response of these observations is not clear. Proinflammatory cytokines such as IL-6, IL-1 β , and CXCLi2 are increased at the transcriptional level in the bursa and spleen, while the antiinflammatory cytokine transforming growth factor (TGF)- β 4 is decreased [77,89,91,92]. These different groups of authors reported somewhat conflicting results for the production of type I IFNs and IFN- γ transcription, but the differences could be the result of using different virus strains and/or infecting different age groups. IBDV infection also increased the production of inducible NOS (iNOS) mRNA in bursal macrophages.

Khatri and Sharma [93] showed that the activation of macrophages is through the p38 mitogen-activated protein

kinase and nuclear factor (NF)- κ B pathway, but it is not clear whether these pathways are activated through specific viral proteins. The alterations in cytokine transcription are compatible with the inflammation in the bursa during the acute infection. However, it is less clear how these changes play a role in IBDV-associated immunosuppression. It will also be important to determine if the changes occur in IBDV-infected macrophages before they undergo lysis or whether infection and cytokine deregulation occur in different subpopulations.

Although T cells are not susceptible to infection with IBDV, these cells form an important component of the overall immunopathogenesis of IBD. There is an influx of CD4⁺ and CD8⁺ T cells into the bursa between 1 and 10 days PI [63,64]. Chicken macrophage migration inhibitory factor is upregulated in vivo and in primary bursal cell cultures after infection with vvIBDV, which may be responsible for the influx of T cells and macrophages [94]. The CD4⁺ cells may also contribute to the production of iNOS by IFN- γ production or other soluble factors stimulating iNOS transcription in macrophages and subsequent nitric oxide (NO) production. NO can contribute to the inflammatory lesion development but may also be involved in downregulation of splenic T cell responses to mitogens, which is associated with the acute phase of IBDV infection [95]. The infiltrating T cells showed a strong upregulation of mRNA for several cytolytic molecules (e.g., perforin and granzyme A [96]), suggesting a role in the clearance of virus-infected cells and thus a contribution to bursal atrophy.

IBD is controlled by vaccination with live attenuated IBDV strains, some of which have residual pathogenicity. Especially the so-called “intermediate plus” but also less pathogenic vaccine strains can cause apoptosis of B cells in the bursa of Fabricius [97–99]. The vaccine-induced immunosuppression may aggravate immunosuppression caused by CIAV, which is primarily controlled by virus-neutralizing (VN) antibodies (see next section). The vaccine-associated immunosuppression is perhaps one of the main driving force behind the successful introduction of recombinant vaccines expressing IBDV VP2 [100].

In conclusion, IBDV infection causes a complex set of interactions between B cells, macrophages, and T cells leading to destruction and subsequent partial recovery of the bursa and long-lasting suppression of primary antibody responses. Recent studies by several groups have provided a better insight into the immunopathogenesis of IBDV in young birds; however, important questions remain. For example, how can we explain the recovery from infection in the face of suppression of primary antibody responses if neutralizing antibodies are the key to recovery? Do we have to postulate that natural killer (NK) responses and cell-mediated immunity are far more important than previously believed, as suggested by the

findings by Raul et al. [96]? Another unresolved question is why young birds mostly develop the subclinical disease, while infection of older, antibody-negative birds results in clear-cut pathology including hemorrhages. There may be similarities between the situation in birds with IBDV and in mice with influenza virus, where the immune response is responsible for most tissue damage, in which case the damage can be reduced by treatment with TGF- β [60].

Chicken infectious anemia virus

CIAV is a small DNA virus of approximately 25 nm belonging to the *Anelloviridae*, genus *Gyrovirus* (reviewed in [101]). It has a single-stranded covalently closed DNA genome of 2.3 kb, which produces one polycistronic transcript coding for three proteins. The virus is extremely resistant to disinfectants and can resist heat treatment at 80°C for 15 minutes. Due to its ubiquitous presence in chicken flocks, its small size, and its resistance to physical and chemical treatments, it can be present as a contaminant in other viruses especially if these agents are propagated in embryonated chicken eggs. As such it can become a confounding factor in studies on immunosuppressive properties of other pathogens CIAV was originally isolated in 1979 by Yuasa et al. from a contaminated vaccine [102]. It is surprising that papers are still being published between 2014 and 2019 indicating the production of CIAV-contaminated vaccines [103–105].

The pathogenesis and immunosuppression caused by CIAV have been reviewed [5,101,106,107]. Infection of chicks may result in clinical disease after vertical transmission, which occurs when hens first become infected during egg production, or by horizontal transmission during the first few weeks of age. However, most chicks are protected against early infection by maternal antibodies and clinical disease is not frequently seen. Infection after 3 weeks of age is mostly subclinical but may result in significant immunosuppression. The development of VN antibodies is essential to curtail virus replication, and immunosuppression caused by IBDV, for example, has been implicated in prolonged replication of CIAV.

The small genome coding for only three proteins, VP1, VP2, and VP3 or apoptin, requires the infection of dividing cells in order to use the cellular machinery for viral DNA replication. VP1 is the capsid and only protein present in the virions, while VP2 has several roles in viral replication. Apoptin causes apoptosis of infected cells, and the mechanisms of the induction of apoptosis have been studied in detail in mammalian cells (reviewed in [101]), in part because apoptin may be used as a potential anticancer treatment in humans [108,109]. Dividing cells that are susceptible to infection are hemocytoblasts in the

bone marrow, T cell precursors in the thymus, or dividing T cells in response to antigenic stimulation. Infection of hemocytoblasts results in a decrease in erythrocytes, thrombocytes, and granulocytes. The loss of the latter two cell types is important because thrombocytes and granulocytes are both important effector cells during bacterial infections and, as a consequence, secondary bacterial infections (e.g., “blue-wing disease”) are frequently associated with CIAV-induced immunosuppression.

Adair et al. [110] reported that $CD3^+CD8^+TCR\alpha\beta$ spleen cells constitute the major CIAV-infected population in the spleen. Haridy et al. [111] found that infection in 4-week-old chickens resulted in a moderate loss of $CD4^+$ and $CD8^+$ cells in the spleen and thymus. Infection in 1-day-old chickens causes a more severe depletion of $CD4^+$ and $CD8^+$ cells (e.g., [112]). The effect of virus replication in these cells is especially important when replication of CIAV occurs at the same time that cytotoxic T cells (CTL) are generated in response to vaccination or infection with a second pathogen. Markowski-Grimsrud and Schat [113] reported the absence of REV-specific CTL 7 days after birds were coinfecting with REV and CIAV, at which time CIAV was actively replicating, based on real-time quantitative RT-PCR analysis. Because there was no effect of CIAV infection on transcription of IL-2 or IFN- γ at 7 days PI, it was suggested that the lack of REV-specific CTL was caused by CIAV-induced apoptosis of $CD8^+$ cells during the generation of CTL. In contrast to the effect on CTL, NK cells were not affected by CIAV infection [114]. Based on the studies in MSB-1 cells, Peters et al. [115] suggested that VP2 may also play a role in immunosuppression through downregulation of major histocompatibility complex (MHC) class I antigens. The importance of this observation for immunosuppression is difficult to evaluate because the assumption is that CIAV-infected cells will become apoptotic.

CIAV-induced immunosuppression has been causally linked to increased incidence of other diseases [5]. For example, infection with CIAV can aggravate IBV-induced disease and avian influenza (H9N2) [116,117], likely by affecting both CMI and antibody responses. Van Ginkel et al. [118] demonstrated reduced local antibody responses to IBV in the Harderian gland and lacrimal fluids in CIAV-infected chickens. This effect was most likely caused by a decrease in $CD4^+$ Th cells as a consequence of CIAV infection. The impact of CIAV infection on increased incidence of MDV in vaccinated and non-vaccinated birds has been extensively documented (reviewed in [5,101,119]). In many instances MD vaccination breaks are associated with the isolation of CIAV (e.g., [120]).

The impact of CIAV infection on cytokines has not been well studied and early investigations relied on bio-

assays representing the state-of-the-art at the time (reviewed in [121]). More recently, quantitative (q)RT-PCR assays have been used to investigate the effects of CIAV infection on cytokines in relation to virus replication. Unfortunately, the few published studies have not included the effect of virus replication prior to 7 days PI, when high levels of virus replication occur [113,122]. At that time, immunosuppressive effects are already evident with impairment of macrophages [123] and CTL [113], but IFN- γ , IL-2, and IL-1 β mRNA levels were not affected at that time point [113]. Giotis et al. [124] conducted a transcriptome analysis of thymus, spleen, bone marrow, and bursa of Fabricius between 4 and 11 days PI of 1-day-old SPF chickens. Although there was major variation in the findings between days PI and the four tissues, the general conclusion was that cytokines associated with Th1, Th2, and Treg populations were downregulated. Exceptions were IL-2, IL-10, IL-13, and IFN- γ which were upregulated in the thymus and bone marrow. They subsequently examined gene expression in CIAV-infected MDV-transformed chicken T-cell line MSB-1 and demonstrated inhibition of the induction of IFN- α and IFN-stimulated genes and modulation of genes involved in the T-cell, inflammation, and NF-kappaB signaling pathways [125].

Clearly, additional studies using quantitative RT-PCR assays, transcriptome profiling, or enzyme-linked immunoassays (ELISA) are needed to further determine the impact of CIAV infection on cytokines starting at 2–3 days PI, because viral antigens can be detected in lymphoid tissues and bone marrow as early as 3–4 days PI [126]. Interestingly, the damage to the thymus and bone marrow was quite extensive between 3 and 12 days PI, although relatively few cells in these organs were positive for viral antigens. The collapse of the thymus is probably the result of damage to the cytokine network needed for T cell maturation. Detailed studies on early cytokine changes during CIAV infection are essential to gain an understanding of the subsequent immunosuppression.

Reovirus

Avian orthoreovirus (ARV) belongs to the genus Orthoreovirus of the subfamily of Spinareovirinae of the *Reoviridae* [127]. ARV have a genome consisting of 10 dsRNA fragments. ARV infections can cause tenosynovitis in chickens and other diseases, or result in a subclinical infection. Although horizontal transmission is the main route for infection, egg transmission may occur infrequently in chickens [128,129]. Infections with pathogenic, but not nonpathogenic, strains have been associated with depletion of lymphoid cells in the bursa and thymus and decreased serological responses to inactivated NDV [130]. However, Montgomery et al. [131] were unable to

find a significant impact of reovirus infection on antibody responses to NDV, SRBC, and *Brucella abortus* antigen, although a decrease in relative bursa weight and some lymphocyte depletion in the bursa was noted. The cause of the latter is not clear because reovirus replicates in macrophages but not in B cells [132,133].

Reovirus infection decreased the responses of peripheral blood monocytes and splenocytes to mitogens at 7 days PI but not afterward [131,134–136]. Removal of plastic-adherent cells primed to produce NO restored mitogen responsiveness of T lymphocytes to some degree and suggested that suppressor macrophages were responsible for the immunosuppression [134–136]. However, NO can also have a beneficial effect by significantly reducing reovirus replication [137]. The pathways of the antiviral and immunosuppressive actions of NO are very complex and have been reviewed [138]. The immunosuppressive pathways of NO include modulation of the Th1/Th2 balance through Treg cells and a reduction in T cell proliferation.

The S1 genome segment of avian and mammalian reovirus codes for 3 ORFs, two of which are translated in non-structural proteins. These two proteins have pleiotropic functions, and many of these interfere with cellular signaling pathways [139]. The S1 proteins have been linked to immunosuppression in mammalian reoviruses [140], perhaps through interference with the innate immune response [141]. The avian reovirus S2-coded σ A protein has anti-IFN (presumably anti-Type I) activity, which in addition to immunoevasive properties may contribute to immunosuppression associated with avian reovirus infections [142].

Poult enteritis and mortality syndrome (PEMS) is characterized by diarrhea, growth depression, and immunosuppression with a multifactorial etiology [143]. Turkey reovirus has been associated with some of the aspects of PEMS, including a decrease in thymus and bursa weights [144]. However, this specific reovirus isolate, ARV-CU98, did not infect macrophages or B and T cells [145]. Using a different isolate, NC/SEP-R44/03, in 3-day-old turkeys, Day et al. [146] reported moderate to severe bursal atrophy, a decreased antibody response to NDV, and decreased cutaneous basophil hypersensitivity. It is not clear whether these effects are caused directly by the virus infection or indirectly by interfering with the uptake of nutrients and water.

Adenovirus

Adenoviruses consist of nonenveloped, icosahedral particles containing a linear, double-stranded DNA genome of 26–48 kb. Commercially important avian adenoviruses belong to the Aviadenovirus, Atadenovirus, and Siadenovirus genera of the *Adenoviridae*. Currently, 12

species are recognized in the Aviadenovirus genus. Fowl aviadenovirus (FAdV) A–E are mostly infecting chickens and some, but not all, are important pathogens. Other species in the genus Aviadenovirus [duck aviadenovirus (DAdV-B) and turkey aviadenovirus (TAdV) B–D] are thus far not important for the duck and turkey industry. A total of 12 serotypes are recognized within the FAdV-A–E groups. The genus Atadenovirus has only one species: DAdV-1 which is associated with egg drop syndrome in chickens. The pathogenic turkey adenovirus (TAdV-3) belongs to the genus Siadenovirus (reviewed in [147]).

Although the literature often mentions that FAdV's are immunosuppressive, the immunosuppressive potential of FAdV's has not been fully examined. Some of the FAdV species are associated with clinical diseases such as inclusion body hepatitis (IBH), hydropericardium syndrome, gizzard erosion and necrotizing pancreatitis, and respiratory disease (reviewed in [147–149]). Steer et al. [150] infected 1-day-old SPF chickens with FAdV-A serotype 1, FAdV-E serotype 8b, and FAdV-D serotype 11. In addition to clinical disease in the liver and kidneys, serotypes 8 and 11 also caused thymic and bursal atrophy and virus could be detected in these organs. In contrast, serotype 1 did not cause significant lesions in the thymus and bursa of Fabricius and virus was only detected in 2/6 chickens. In contrast, infection of 6-week-old chickens with serotypes 8 and 11 did not cause significant lesions in the bursa of Fabricius and thymus [151]. Some serotype 1 strains, associated with IBH, induced a significant decrease in IgM responses to *B. abortus* antigen, but not against sheep red blood cells, when administered 1 week PI with FAdV. In addition the response to stimulation with phytohemagglutinin was also significantly reduced at 2 and 3 weeks PI [152]. Unfortunately, there is no information provided on the presence of IBDV and/or CIAV in the experimental chickens. FAdV-C serotype 4 caused atrophy of the thymus and bursa of Fabricius at 1 and 2 weeks PI, which was associated with an increase in apoptosis. In addition, mRNA transcription of several cytokines (IL-6, IL-8, and TNF- α) was significantly increased at 3 days PI in the spleen, thymus, and bursa of Fabricius [153]. When birds were vaccinated against Newcastle disease and avian influenza 7 days PI with FAdV, when the birds were recovering from clinical disease, titers were significantly reduced 2–3 weeks postvaccination [153,154].

Thus far, there is very little information available on the role of the different viral proteins that are responsible for the immunosuppression. Corredor and Nagy [147] suggested that the early genes ORF22 and *Gam-1* may be involved. *Gam-1* protein, encoded by FAdV-A serotype 1, activates heat shock protein (hsp) 40 and hsp70 [155], which is essential for virus replication and may induce apoptotic pathways.

Tumor viruses

The three tumor viruses associated with lymphoid tumors, MDV, ALV, and REV, are discussed in more detail in Chapter 17. In this chapter only the immunosuppressive aspects are reviewed. Extensive reviews on the diseases caused by these viruses are available in the 12th and 13th editions of *Diseases of Poultry* [52,53].

Marek's disease virus

MDV, or Gallid herpesvirus 2, belonging to the genus *Mardivirus* in the subfamily of *Alphaherpesvirinae*, is causing tumors of T lymphocytes in chickens. It has attracted the attention of virologists and immunologists because highly successful vaccines protecting against the disease became available during the early 1970s. Since then, the virus has evolved from virulent to vv and, more recently, to vv + pathotypes [156]. This change in pathogenicity not only increased the incidence of tumors but also was linked to some new neurological syndromes [157]. In addition, the vv + strains caused more severe damage to the lymphoid organs than did virulent and vv pathotypes [158] probably leading to increased immunosuppression.

Gimeno and Schat [119] recently reviewed immunosuppression caused by MDV. In this chapter we present only the most important aspects of MDV immunosuppression and immunoevasion (see Section 14.4). MDV-associated immunosuppression is often divided into an early immunosuppression phase during the cytolytic infection and a late immunosuppression phase when virus is reactivated with or without tumor development. One of the key characteristics of the early immunosuppression is the destruction of lymphocytes in the lymphoid organs during the first 2 weeks of infection causing severe atrophy of the thymus and bursa of Fabricius; this may be permanent or transient depending on the pathotype of MDV.

The model for the pathogenesis of Marek's disease, as originally outlined by Calnek [159] and Schat [160], provides the basis for our understanding of how MDV causes both B and T cell depletion. Briefly, chickens become infected through cell-free virus present in feather-follicle dander. The virus is transferred to the lymphoid organs, probably through macrophages, and replicates first in B lymphocytes, causing a productive–restrictive infection in which cell-associated, but not cell-free MDV, is produced and the infected cells are destined to die. This phase is often referred to as the cytolytic phase.

As a consequence of the production of viral antigens and subsequent immune responses, T cells become activated, expressing MHC class II antigens and other activation markers. In contrast to resting T cells, activated T cells are susceptible to MDV infection and become

infected. Normally, MDV establishes a latent infection in activated T cells through poorly understood mechanisms [161]. Cytokines [162] and microRNA (miRNA) [163] most likely play a role in the process. Depending on the pathotype of MDV and the genetic resistance of the host, latency may be permanent, temporarily followed by a secondary cytolytic cycle, additional immunosuppression, and tumor development, or absent with continuous virus replication and, frequently, early mortality.

As early as 3 days PI, the expression of IFN- γ is upregulated [164], which led Schat and Markowski-Grimsrud [162] to suggest that this may upregulate the expression of IL-8 receptors. The upregulation of IL-8 receptors is likely an essential step in the transfer of cell-associated MDV from B cells to activated T cells, allowing viral IL-8 produced during the lytic infection [165] to attract activated T cells. Increased cytolytic infection, and thus increased damage to the lymphoid organs associated with some of the vv + pathotypes, may be caused by a more effective transfer of MDV from B to T cells due to increased levels of vIL-8 production combined with the inability to establish latency after 7 days PI.

Jarosinski et al. [166] reported that vv + strains produced higher levels of vIL-8 than less virulent strains, but this is likely the consequence of increased virus replication [167] rather than an intrinsic ability to enhance vIL-8 production. The importance of vIL-8 for the cytolytic infection is further shown by the fact that tissue culture-attenuated strains have no or very low vIL-8 transcript levels [167] and that deletion of vIL-8 [168,169] or deletion of exon 1 of vIL-8 [170] results in attenuation of the cytolytic infection. The reasons for the continued replication of vv + MDV instead of the establishment of latency have not been elucidated. It is of interest to note that vv + MDV causes a highly significant upregulation of proinflammatory cytokines as early as 4 days PI in spleen and brain tissues [171,172]. It is certainly feasible that the distortion in cytokine profile impacts the production of cytokines involved in the induction and or maintenance of latency [173] with the consequence of a prolonged cytolytic infection leading to more profound immunosuppression.

Apoptosis is the most likely mechanism responsible for cell death during the cytolytic infection in the lymphoid organs [174,175] affecting CD4⁺CD8⁺ thymocytes. Interestingly, during the early cytolytic infection MDV-antigen positive cells in the thymus are mostly B lymphocytes [176] with only less than 0.1% of the cells in the thymus expressing pp38, which is associated with the lytic infection [177]. What needs to be resolved is whether MDV-infected thymocytes become apoptotic or does MDV-induced cytokine deregulation affecting thymocyte maturation cause apoptosis of noninfected cells and a collapse of thymus architecture [178]. The latter is certainly possible, based on the evidence that cytokines

can be deregulated, especially after infection with vv + strains. MDV infection can also cause apoptosis in CD4⁺ peripheral T cells, but CD8⁺ T cells are apparently not affected [179]. The reasons for the latter are not clear because CD8⁺ T cells can be infected with and transformed by MDV [180,181]. Berthault et al. [182] examined thymus and bursa of Fabricius for the degree of apoptosis between 6 and 14 days PI. Their results indicated that apoptosis in the bursa was mostly in noninfected cells at 6 days PI, while apoptosis in the thymus occurred in virus-infected cells and to some degree in noninfected cells. These data are somewhat different from the results reported by Baigent et al. [177]. These authors suggested that infected cells in the bursa would undergo apoptosis because the expression of pp38 is linked to cell death. Clearly, additional studies are needed to further resolve this question and it will be important to include the phenotype of the cells in the analysis.

Thus far, it has not been resolved which MDV gene products are responsible for the immunosuppression. Basically, any protein involved in the lytic infection cycle will be involved in causing cell death and thus destruction of lymphocytes. In addition to LORF4, VP23, and Meq, vIL-8 [170], LORF9 [183], and UL5 [184] have been implicated in the development of bursal and thymus atrophy. It is also likely that the SORF2 gene plays a role, based on two studies using opposing experimental strategies. Deletion mutants lacking several ORFs, including SORF2, caused decreased cytolytic infection without preventing tumor formation [185]. Upregulation of SORF2 expression through the insertion of the REV long terminal repeat (LTR) resulted in an enhanced cytolytic infection in the absence of tumor development [186].

MDV infection can result in alterations of T cell functions by different mechanisms. Downregulation of MHC class I [187] apparently through the UL49.5 gene product [188] may interfere with recognition of target cells by CTL but could increase recognition by NK cells. MHC class II can be upregulated or downregulated (reviewed in [119]) by MDV but the importance for immunosuppression is not clear. Gurung et al. [189] described a new type of Treg cell expressing TGF- β which are present at elevated levels in the lungs as early as 4 days PI and during the development of tumors. TGF- β can downregulate immune responses and increased levels of TGF- β may be, at least in part, involved in MDV-induced immunosuppression.

In addition to the destruction of lymphoid tissues and T cell deregulation, MDV induces immune suppression through activation of macrophages. These macrophages were able to inhibit mitogen stimulation of T cells obtained from noninfected chickens [190]. Schat and Markowski-Grimsrud [162] suggested that this inhibition was likely the consequence of MDV-induced NO production by the macrophages and was protective rather than

immunosuppressive by reducing the pool of activated T cells. The concept of immunosuppression around 7 day PI is conflicting because there is destruction of lymphoid tissue, potential immunoevasion, and the activation of "suppressor" macrophages, while, at the same time CTL against several MDV proteins are present [162]. In contrast with the more classical strains, vv + strains may actually infect macrophages [191] and macrophages have been associated with MDV lesions in the brain [192] (and B. L. Njaa, K. W. Jarosinski, and K. A. Schat, unpublished data). The importance of these observations for immunosuppression is not clear; more likely the macrophages are part of the enhanced proinflammatory response in the brain [171].

Late immunosuppression can be caused by lytic infection of lymphoid cells upon reactivation of MDV from latency, causing effects on the immune system similar to those during early lytic infection. The former is difficult to study, but Faiz et al. [193,194] used the degree of protection against infectious laryngotracheitis (ILT) after vaccination against ILT in chickens previously infected with different pathotypes of MDV. Challenge with vv + MDV in MDV vaccinated birds protected against tumor development but not against ILT. Using different attenuated forms of the vv + 686 isolate and wild-type 686 it was learned that late immunosuppression was attenuated before attenuation of tumor development. In addition tumor cells may cause immunosuppression (reviewed in [119]). However, tumor-induced immunosuppression is the direct consequence of a clinical disease and so is not discussed here.

Feather follicle epithelium (FFE) is the source of cell-free MDV [195] but little attention has been given to the immune responses in the skin. Heidari et al. [196,197] analyzed the FFE for MDV-associated changes. Suppressor of cytokine signaling (SOCS)1 and -3, with known immunosuppressive properties, were upregulated in the skins of infected chickens. CTL were marginally increased in virus-positive FFE compared to control FFE. Unfortunately, quantitative data were not provided. Two possible mechanisms were proposed: inhibition of migration of CTL to the infected areas and/or downregulation of CD8 glycoprotein. A better understanding of the absence of a strong immune response in the FFE will be important to develop vaccines that can reduce MDV replication in the skin.

MDV vaccine strains can also cause immunosuppression. Friedman et al. [198] showed that vaccination with SB-1 (a serotype 2 MDV strain), together with HVT or CVI988 (a serotype 1 vaccine strain), caused a significant decrease in B lymphocyte activation and antibody production after in vitro stimulation with *Salmonella typhimurium* or bovine serum albumin (BSA) inoculation, respectively. *Escherichia coli*-caused mortality was increased in

vaccinated chicks independently of the MDV vaccine used, when challenged at 11 or 14 days postvaccination, but it occurred only in chicks vaccinated with CVI988 when these were challenged at 21 days postvaccination.

Islam et al. [199] reported that HVT did not cause increased mortality when chickens were challenged with *E. coli* at 28 days postvaccination. However, at 3–10 days HVT caused a significant decrease in B and T lymphocyte numbers. Vaccination with CVI988 precipitated lesions, when chicks were challenged with *C. baileyi* 4 days postvaccination (see Coccidia-induced immunosuppression section). The importance of vaccine-induced immunosuppression is not clear in commercial flocks and is far less important than the protection against MDV challenge. The possibility that in ovo vaccination with HVT might induce tolerance was investigated by Zhang and Sharma [200]. Inoculation at 0–14 days of embryonation induced tolerance to HVT but not to *B. abortus* antigen. In contrast, vaccination at 18 days of embryonation, which is the appropriate time for in ovo vaccination, did not induce tolerance to HVT.

Avian leukosis virus and reticuloendotheliosis virus

ALV and REV belong to the *Retroviridae*. ALV are classified as exogenous or endogenous (or subgroup E) viruses and the former are further divided into subgroups A, B, C, D, and J. Subgroups A–D are associated with infection of Leghorn type chickens, but subgroups C and D are of little practical importance for the poultry industry. ALV-J was first described during the late 1980s and is mostly associated with meat-type birds. ALV and REV can also be divided into defective viruses and nondefective (nd) or helper viruses. The defective viruses have often acquired cellular *onc* genes and cause a rapid onset of tumors and death. These viruses have little importance for immunosuppression due to the rapid mortality. The nd viruses can also cause tumors, but the transformation occurs through activation of cellular *onc* genes by the viral LTRs [201]. Recently, a putative new subgroup (ALV-K) was identified in China [202] which apparently uses the same cellular receptor as ALV-A [203]. Whether it competes with ALV-A for that receptor, which would mean that it therefore actually represents a subtype of ALV-A, has not yet been established.

REV and exogenous ALV can be transmitted congenitally or horizontally. Chicks hatched from congenitally infected eggs are tolerant and unable to produce VN antibodies to the virus; they are viremic, antibody-negative (V^+A^-) and likely to develop tumors or, in the case of REV, tumors and/or runting and stunting. Horizontal transmission during, or shortly after, hatching can also result in tolerance [201,204]. This risk is enhanced in

chicks when subgroup E or glycoprotein (gp) 85 of subgroup E is expressed during embryonal development [205] or after IBDV infection at 1 day or 6 weeks of age. IBDV infection decreased the frequency of VN antibody-positive chickens, and increased virus shedding and viremia levels significantly, although these effects were dependent on the genetic background of the birds. In contrast, infection with REV at 1 day of age or MDV at 2 weeks of age had no significant impact on ALV shedding, viremia, and antibody development [206]. However, exposure to IBDV at 2 weeks of age did not influence the incidence of ALV-J [207]. The induction of tolerance after congenital infection is virus-specific and is not a general inability to produce antibodies against other pathogens than ALV or REV. For example, chickens congenitally infected with a specific strain of ALV-A were able to produce antibodies to other subgroup A strains [208] and influenza virus but were partly tolerant to ALV-B [209].

The importance of ALV-A–D as immunosuppressive viruses is not clear and probably depends on the subgroup. Fadly et al. [210] and Rup et al. [211] reported that infection with RAV-1, a subgroup A virus, did not suppress antibody responses, mitogen stimulation, or the phytohemagglutinin (PHA) skin test, even in birds developing tumors. In contrast to ALV-A, subgroup B viruses have been linked to suppression of CMI responses. In ovo infection with MAV-2(O) resulted in severe damage to lymphoid organs, suppressed antibody responses, and suppressed mitogenic responsiveness [212]. In addition, MAV-2(O) causes a temporary dysfunction of macrophages, suppressing the responses of spleen cells to mitogens. The depressed responses were present as early as 3 days PI and lasted until 21 days PI [211,213]. Macrophage dysfunction reduced the ability to clear bacteria such as *Listeria monocytogenes* [214]. A second subgroup B virus, avian erythroblastosis virus (an acute transforming, defective virus), and its associated helper virus, also suppressed T cell responses to mitogens, but in this case, the suppression was directly related to T cells and not macrophages [215].

Coinfection of ALV-J with MDV increased the viral load of both viruses and caused more severe lesions compared to infections with ALV-J or MDV alone. Moreover, dually infected CEF promoted the expression of IL-6, IL-10, and TGF- β suggesting that ALV-J and or MDV cause immunosuppression enhancing the replication of both viruses [216]. Several groups have previously shown that ALV-J infection does not affect cell-mediated and humoral immune responses to a significant degree. However, several assays for immune responses and resistance to other diseases were marginally lower in ALV-J infected than in control chickens [217–219], suggesting, but not proving, that immunosuppression may be

associated with ALV-J infection. Cui et al. [220] reported that some ALV-J isolates may cause thymus and bursa atrophy and reduced antibody responses to NDV vaccines in chicks when infected at 1 day of age and vaccinated at 7 days. However, the reduced response was not detected when the chickens were vaccinated at 30 days of age. Moreover, ALV-J infection did not reduce the antibody response to IBDV vaccine. Guo et al. [221] reported decreased CTL responses against MDCC-CU147 at 17 days PI but not at 37 days PI. Coinfection with REV did not further increase the reduction, although REV alone caused a significant further reduction at 17 days PI compared to ALV-J alone or the combined infection. The reduced response is most likely not caused by antigen-specific CTL because CU147 is a B¹³B¹³, CD4⁻CD8⁺, $\alpha\beta$ 1TCR⁺ MDV-transformed cell line [222]. It is possible that the effect is caused by NK cells. Landman et al. [218] had reported a reduction in NK cell activity after in ovo infection with ALV-J although the difference with uninfected control birds was not significant. The timing of ALV-J-induced immunosuppression may also depend on the type of assay used because Wang et al. [223] indicated that viral titers increased between 3 and 4 weeks PI, while relative bursa and thymus weight decreased significantly starting at 3 weeks of age. The molecular mechanisms of ALV-J-induced immunosuppression have not been elucidated. In vivo experiments showed an increased transcription of IL-6, IL-18, IFN- α , and IFN- γ in the spleen coinciding with the start of a drastic increase in ALV-J virus in the PBMCs at 6 days PI [224]. This is in contrast with the decreased production of IFN- α in the HD11 cell line infected with ALV-J which was caused by blocking of I κ B phosphorylation and subsequent downregulation of NF- κ B p65 [225]. The relevance of this observation for understanding immunosuppression is not clear because HD11 is an MC29 (RAV2) ALV transformed chicken macrophage cell line [226], which produces many growth factors.

There is very little information on the immunosuppressive potential of the recently described ALV-K virus group. Wu et al. [227] reported a significant reduction in antibody titers after vaccination with a live NDV vaccine but no effect on the titers after vaccination with an inactivated avian influenza vaccine.

REV infection resulted in decreased antibody responses to HVT [228], MDV [229], NDV [e.g., [230,231]], and H5N1 vaccines [232]. In contrast, Witter et al. [233,234] found only decreased primary antibody responses to SRBC and *B. abortus*, but no effect on MDV antibodies, although protective immunity to Marek's disease was decreased. Embryo inoculation with the T strain, but not with the CS strain, also impaired the secondary immune responses to SRBC and *B. abortus*. REV infection increased ALV infection levels in a commercial line [233].

The effect of REV infection on MDV was questioned by Buscaglia et al. [235] because REV infection did not impair the establishment of MDV latency or reactivation from latency. These authors suggested that REV may actually reduce the pool of susceptible cells for MDV replication, which would be compatible with the REV-associated atrophy of the lymphoid organ [234]. Filardo et al. [236] suggested that the lymphoid atrophy is strain specific and is caused by a combination of *env* and *gag* genes.

The immunosuppressive effects of REV infection on cell-mediated immunity have been studied by several groups, mostly using mitogen stimulation assays [233,235,237]. The immunosuppressive effects were linked to the development of suppressor cells by Rup et al. [211,238], which are probably, but not proven, the same as Treg cells. Interestingly, Tiwari et al. [239] reported that REV increases the production of TGF- β 2 and 3, which is an important cytokine for the development of Treg cells [240] and in general for the regulation of immune responses [241]. The importance of this type of cell-mediated suppression needs to be further analyzed especially in view of the development of REV-specific CD8⁺ CTL starting at 6 days PI [242,243]. REV-induced immunosuppression may have important practical consequences because REV infection has often contaminated Marek's disease vaccines [244,245]. It has been known for a long time that fowlpox viruses (FWPV) can carry replication-competent REV genomes, which may be expressed in vivo [246,247]. It is therefore surprising that FWPV vaccines are still frequently contaminated with REV [248,249] suggesting a lack of quality control. The presence of REV in FWPV or FWPV vaccines can result in immunosuppression causing outbreaks of fowlpox as suggested by Motha and Egerton [250]. Wang et al. [251] compared FWPV vaccine strains with and without REV inserts and wild-type FWPV containing REV. REV-positive FWPV strains caused decreased antibody titers to BSA and SRBC and decreased responses to PHA-P skin tests and in vitro lymphocyte stimulation in contrast to FWPV lacking REV.

The effects of REV infection on cytokines have been reported in a few papers using different approaches. Xue et al. [252] infected 1-day-old SPF chickens with a REV-A isolate and used PBMCs at 7–28 days PI for mRNA expression of different cytokines. Viral genome copies were detected starting at 6 days PI with a significantly higher level of genome copies present at 14–28 days PI. Expression of IL-4, IL-10, IL-13, IL-8, and TNF- α mRNA was upregulated but only at significant levels for IL-4 and IL-10 at 7 days PI and for IL-8 and TNF- α at 28 days PI. It is not clear if the authors indeed described the effect of REV-A on TNF- α because TNF- α was not characterized until 2018 [253]. It is more likely that they

described the effect on lipopolysaccharide-induced tumor necrosis factor- α factor. IFN- γ , IL-2, and IL-18 mRNA levels were significantly decreased between 7 and 28 days PI. INF- α and - β , IL-3, and CSF-1 mRNAs were also downregulated but only significantly at 14 days PI. In addition IL-15 and IL-17F were significantly downregulated at 7 and 28 days PI, respectively. Zavala [reviewed in [254]] mentioned that the REV-associated immunosuppressive peptide is located in the transmembrane TM region of the envelope gene. Using a synthetic peptide, CSK-17 which is homologous to the TM peptide, to treat a human monocyte cell line and human peripheral blood mononuclear cells, it was possible to shift the Th1/Th2 cytokine balance resulting in the upregulation of IL-10 and the downregulation of IL-2, IL-12, and TNF- α resulting in the suppression of cell-mediated immunity [255]. In addition to the cytokine changes, thymic atrophy was associated with REV infection most likely caused by an increase in apoptosis in the virus-infected chickens [256]. The increased apoptosis was linked to changes in mRNA and protein expression of cyclinD1 and Bcl-2. There was also a change in the CD4⁺/CD8⁺ ratio with a shift to increased CD8⁺ cells relative to CD4⁺ cells. Starting at 35 days PI the thymus started to recover.

The poultry industry in China has frequent problems with mixed infections of REV and ALV-J. Coinfection of SPF chickens resulted in significantly increased mortality, decreased weight gain, and decreased antibody titers after to NDV and AIV-H9 compared to single infections with REV or ALV-J. Relative bursa and thymus weights were not different between chickens infected with REV, ALV-J, or REV + ALV-J [257]. Infection of 1-day-old broiler chickens with REV + ALV-J also resulted in significantly decreased growth rates and antibody responses to NDV and AIV-H9 vaccines compared to birds infected with REV or ALV-J. The coinfecting birds also have higher levels of *E. coli*-associated mortality than birds receiving only REV or ALV-J [258].

14.3 Mechanisms of immunosuppression

In the previous section three broad categories causing immunosuppression were identified: (1) corticosteroids in relation to stress, (2) programmed cell death (PCD) by apoptosis, which needs to include necroptosis and pyroptosis, and (3) virus-induced changes in the regulation of immune responses. In many instances, the actual molecular interactions between virus proteins and host cells are poorly characterized. In order to use cytokines or TLR ligands as adjuvants to improve vaccines as suggested by Asif et al. [259] and Gupta et al. [260], future research needs to be focused on a better understanding of the interactions between viruses and cytokine regulation.

14.3.1 Corticosteroids and stress-induced immunosuppression

It has been known for a long time that CORT is the major GC in poultry although very low levels of cortisol are also produced and that CORT levels are increased as a consequence of stress (reviewed in [261]). Dohms and Metz [10] treated chickens with CORTs or related GCs, which resulted in lymphopenia and atrophy of the lymphoid organs suggesting immunosuppression. Measuring several immunological parameters, El-Lethey et al. [262] compared the effects of treating chickens with corticosteroids with the effects of lack of foraging materials. Interestingly, antibody titers against SRBC and tetanus toxoid were decreased after both treatments, but antibodies to human serum albumin were not influenced by either treatment. In addition, cell-mediated immune responses such as cutaneous response to PHA and delayed-type hypersensitivity responses to mycobacterium antigen were decreased with both treatments. The authors of both papers indicated that the mechanisms of stress-induced immunosuppression were poorly understood at the time especially in birds. Since then the pathway for the production of GC has been largely elucidated (reviewed in [263]). Mostly based on research in mammalian species, the common pathway for stressors involves the HPA axis ultimately resulting in the release of GCs. Briefly, stress or inflammatory cytokines like IL-1, IL-6, and TNF interact with the hypothalamus resulting in the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). Both, CRH and AVP induce the anterior pituitary gland to secrete adrenocorticotropin hormone, which, in turn, will bind to receptors on the adrenocortical cells in the adrenal gland. These activated cells will use cholesterol to produce CORT in mice and presumably chickens. In mammalian species, TLR 2 and 4 can also activate these cells to produce CORT. Low levels of CORT can actually stimulate immune responses, but high levels induce apoptosis of thymocytes and dendritic cells (DC) through several pathways. In addition, high levels of CORT can downregulate DC activity and interfere with TCR signaling, causing downregulation in T cell activation. In mice, CORT can affect different subpopulations of T cells such as Th1 and Th17 cells, but it is not known if it has the same effect on chicken T cells (reviewed in [263]).

14.3.2 Apoptosis, necroptosis, and pyroptosis

Virus infection can lead to PCD of the infected cell, which can be beneficial for the host if it occurs prior to completing the virus replication cycle. If PCD occurs after the virus replication cycle has been completed it will result in release of infectious virus. Virus replication in lymphoid

cells (e.g., in chickens IBDV, CIAV, MDV) resulting in PCD will cause (sub)clinical immunosuppression.

Currently, there are three mechanisms of PCD described: apoptosis, necroptosis, and pyroptosis (reviewed in [264–266]). The latter two mechanisms of PCD are inflammatory, while apoptosis is a noninflammatory process. Apoptosis can be triggered by cell-intrinsic and cell-extrinsic pathways. The intrinsic pathway is triggered by intracellular stress signals resulting in the permeabilization of the outer mitochondrial membrane by activated Bax or Bak proteins. Cytochrome C and other mitochondrial components are released in the cytosol leading to the formation of the apoptosome. Caspase-9 is recruited to apoptosome, becomes activated, and cleaves caspase-3 and -7 which in turn leads to cell death. Although great progress has been made in understanding the intrinsic pathways there are still many unanswered questions that need to be addressed [267]. The extrinsic pathway is triggered by extracellular stress signals resulting in the activation of death receptors of the TNF family forming the death-inducing signaling complex (DISC). DISC activates caspase-8 which in turn activates caspase-3 and -7 leading to cell death.

Necroptosis is a tightly regulated process of cell death that does not require caspases and can be triggered by external and internal stimuli similar to apoptosis. Viruses can trigger necroptosis by both types of stimuli ultimately resulting in cell death by causing swelling of the cells, loss of plasma membrane permeability, and release of cytosolic contents. Pyroptosis is also a tightly regulated process of cell death resulting in a strong inflammatory response. Danger signals triggered in the cytosol result in the formation of an inflammasome which activates caspase-1 and starts an immediate strong innate type of immune response. Thus far, necroptosis and pyroptosis have not been described for the avian viruses mentioned in this chapter and will not be further discussed.

As mentioned in the previous sections, virus replication in lymphoid cells is the major cause of cell death for IBDV, CIAV, reovirus, MDV and to some degree REV. In most of these infections, apoptosis is the cause of cell death although necrotic lesions have been reported for MDV [268]. It would be of interest to determine if the necrotic lesions are actually caused by necroptosis. The induction of apoptosis by VP5 and VP2 of IBDV was mentioned before, but it is not clear how these proteins actually interact with the apoptotic pathway. Liu and Vakharia [71] showed that IBDV infection *in vitro* activates effector caspase-3 and the initiation caspase-9 as well as nuclear factor-kappa B (NF κ B) resulting in apoptosis late in the infective cycle. These authors suggested that NF κ B may be activated through the accumulation of reactive oxygen species as has been shown for reovirus-induced apoptosis.

CIAV provides another example for viral induction of apoptosis [108]. VP3 (or apoptin) is the major viral protein inducing apoptosis although VP2 may also be a minor cause of apoptosis. Early after transfection of MSB-1 cells with a VP3-expressing plasmid VP3 is present in the cytoplasm and nucleus as fine granular structures. At the time that cells become apoptotic VP3 forms discrete nuclear structures. The carboxy terminus is positively charged and, based on work with deletion mutants, it is suggested that the positive charge is important for the interaction with the nuclear DNA. Noteborn [108] suggested that this may lead to alterations in the supercoiled organization of the cellular DNA causing apoptosis or alternatively that VP3 acts as a transcriptional regulator of genes involved in the apoptotic process.

Although MDV induces apoptosis, little is known about the viral proteins and mechanisms involved in the induction of apoptosis. Thus far, no proteins preventing or inducing apoptosis have been found in MDV, although Kingham et al. [269] identified in the related HVT an ORF coding for a protein with significant similarity to the putative quail antiapoptotic gene NR-13. Piepenbrink et al. [270] found that pp38 causes decreased production of cytochrome C oxidase subunit 1 and ATP in mitochondria, which could be a consequence of release of Cytochrome C into the cytosol. It will be of interest to further examine the relevance of the regulation of mitochondria-associated apoptosis for the pathogenesis of MD especially since Chu et al. [271] noted changes in mitochondrial DNA content in susceptible line 7₂ but not in resistant line 6₃ chickens after infection with MDV.

14.3.3 Virus-induced changes in the regulation of immune responses

Thus far, there are few studies describing quantitative changes in cytokine production as a consequence of virus infections in chickens. It is not clear how many of the changes are related to immunosuppression rather than changes associated with the induction of immune responses. It is clear from the work by Jarosinski et al., [171] using vv + MDV strains that strong proinflammatory responses may cause pathology. This observation has important consequences for genetic selection for Marek's disease resistance, because the resistant N2a line had a very strong proinflammatory response causing neurological disease with significantly higher levels of cytokine and NO production in the brain. Additional studies on cytokine deregulation leading to immunosuppression are urgently needed using RNAseq analysis, real-time RT-PCR assays, proteomics, and preferably ELISA to examine the impact on transcription and actual production of cytokines.

miRNAs are short (approximately 22 nt), noncoding, single-stranded RNAs which are present in all eukaryotes. Briefly, miRNA is loaded into the Argonaut protein forming the RNA interference silencing complex (RISC). The miRNA in RISC binds to the corresponding mRNA, which is then cut by Dicer. miRNAs are important regulators of gene expression including genes involved in immune responses (reviewed in [272]). Many DNA viruses carry miRNAs (e.g., MDV) and these miRNAs play an important role in viral replication, pathogenesis, and in the case of MDV oncogenesis. Virus infection can also cause upregulation of cellular miRNAs, especially in MDV-infected cells many of which may contribute to tumor formation (reviewed in [273,274]). Thus far, cellular or viral miRNAs have not been linked to immunosuppression after infection with MDV, ALV, and REV [274].

One of the common immunosuppressive effects described for MDV, reovirus, IBDV, and REV infections is the generation of “suppressor” macrophages. These cells suppress T lymphocyte blastogenesis using mitogen responses and are frequently detected between approximately 3 and 15 days pi. Removal of these cells restores the responsiveness of the T cells (e.g., [135,190,238]). Interestingly, this immunosuppression has been linked to NO production by macrophages. NO is a highly versatile molecule with immunosuppressive as well as antitumor, antibacterial, and antiviral activity. NO can influence gene expression probably through the NF κ B pathway and, at high levels, can be part of proinflammatory pathology (reviewed in [275]).

14.4 Immuno evasion

14.4.1 Introduction

As we have learned more about the molecular interactions between viruses and their hosts, it has become apparent that most, if not all, viruses employ immuno evasion mechanisms to survive even the innate immune responses of the host. Those viruses with a persistent lifestyle also have to deploy a spectrum of subtle, refined mechanisms to survive in the face of potential acquired immune responses. The factors involved in immuno evasion were most easily identified in the mammalian large DNA viruses (poxviruses and herpesviruses). With the exception of the molluscipoxviruses, which have no direct relatives in birds but which have some similarities with the avian poxviruses, poxviruses cause acute infections. Their immuno evasion mechanisms, therefore, only need to counter (or are only capable of countering) the innate immune responses, and possibly delay the acquired responses.

Herpesviruses are able to become latent, a state that requires the expression of very few genes. They do, however, appear to be able to prevent the induction of

acquired responses, even though the immune system is regularly open to stimulation by episodic, localized reactivation of latent virus. The recognition that large complicated viruses needed to evolve specific mechanisms to counter innate immune responses arguably drove the suspicion that the smaller, “simple” viruses (often with an RNA genome) must have evolved equivalent mechanisms to survive the same pressures. There are now several examples, particularly in the paramyxoviruses, of small accessory proteins of RNA viruses or of multifaceted proteins, playing a role in immuno evasion, particularly in suppressing the type I IFN pathway.

The relative dearth of reagents available for many aspects of avian biology means that a detailed study of the mode of action of many of the immuno evasion mechanisms of avian viruses remains considerably behind that of mammalian viruses. Indeed, many of the mechanisms remain presumptive, but the situation has improved since the derivation (and ongoing improvement) of the chicken genome sequence. This in turn has allowed identification of hundreds of IFN-regulated (or stimulated) genes (IRGs; ISGs) using RNA-seq, thereby characterizing the primary antiviral innate immune responses [276–279]. This facilitates identification of instances of modulation of those innate responses by viruses, whether as a broad mechanism, by suppression of IFN induction, or more specifically, where viral immunomodulators might target induction or activation of particular IRGs/ISGs.

Recognition of the importance of avian viruses as emerging zoonotic agents (for instance, West Nile virus and H5N1 AIV) is also likely to drive much more work in this area. We consider immuno evasion mechanisms, both known and postulated, of avian herpesviruses, poxviruses, orthomyxoviruses, paramyxoviruses, and reoviruses, as well as reviewing prospects for future work on avian adenoviruses and coronaviruses, orthomyxoviruses and paramyxoviruses.

14.4.2 Immuno evasion by viral proteases

It has long been recognized for mammalian picornaviruses, such as poliovirus, that the viral proteases, which are required to cleave the encoded virus polyprotein, are capable of cleaving components of the cellular transcription and translation machinery. Amongst other effects, one consequence of this is suppression of host innate immune responses. This has received little attention in the avian field but it is likely that the avian picornaviruses (such as Avian encephalomyelitis virus and Duck hepatitis A) utilize the same strategy. It may also be used by other avian viruses that use viral proteases (such as IBDV VP4) to cleave their polyproteins. Although the activity has not yet been attributed to any of its three proteases,

Yu et al. [280] have shown that IBV inhibits induction of IFN- β by cleaving mitochondrial antiviral-signaling protein, an adapter protein in the signaling pathway downstream of MDA5. In a preprint (<https://www.biorxiv.org/content/10.1101/751578v1.full.pdf>), the deubiquitinase motif of the papain-like protease (PLpro) of the Middle East respiratory syndrome beta-coronavirus appears to modulate induction of the innate response. That protease is conserved in IBV, a gammacoronavirus.

14.4.3 Immuno-evasion mechanisms of avian coronaviruses

The accessory protein 5b of IBV has been shown to limit type I IFN production by inducing host shutdown [281]. In that regard it appears to be a functional equivalent of Nsp1, found in alpha- and beta coronaviruses, which induces degradation of host, but not viral, RNA [282].

14.4.4 Immuno-evasion mechanisms of the avian herpesviruses

Study of the biology of MDV teaches us that it uses at least two strategies of immuno-evasion, even though no specific mechanisms have as yet been attributed to these observations. Morimura et al. [179] showed that MDV infection downregulates transcription of CD8 α and β . Hunt et al. [187] reported that MHC class I expression was downregulated in two cell lines, MDV-infected OU2 cells and in MDV-transformed MSB-1 cells, treated with 5-bromo-2'-deoxyuridine—most likely through a block in the transport of the MHC molecules to the cell surface. Morgan et al. [283], using microarray analysis, found that MDV upregulated MHC class I mRNA, but this is likely to be IFN-driven. A mechanism that may favor the survival of MDV in its latent state is promotion of the survival of MDV-transformed tumor cells. Whether this is achieved directly by enhanced expression of chicken CD30, previously identified as the AV37 antigen [284], or by the downregulation of CD28 seen in cells overexpressing CD30 [285] is not clear, nor is the actual viral mechanism of achieving these perturbations in cell surface marker expression.

The herpesviruses have proved a rich hunting ground for those seeking the mediators of immuno-evasion, hereafter referred to as immunomodulators, and the avian herpesviruses, such as MDV, prove no exception. The most interesting immunomodulator found in MDV is the IL-8 mimic (vIL-8), identified initially by its sequence homology with host IL-8. More informatively referred by its synonym, eotaxin, IL-8 is a chemokine capable of attracting eosinophils. Deletion of vIL-8 [168,169] or exon 1 of vIL-8 [170] from the RB-1B strain of MDV resulted in a

lower frequency of birds with tumors and smaller, less invasive tumors. This suggests that vIL-8 affects virus replication rather than tumorigenesis. The possibility of immunomodulatory functions for vIL-8 cannot be eliminated, but the discovery that it can also be expressed as a fusion protein with the MDV oncoprotein Meq presents the likelihood of a more complicated situation [286].

Recently, Sun et al. [287] demonstrated that infection of chickens with two strains of MDV resulted in the downregulation of IFN- α and - β mRNA and protein in the thymus and bursa of Fabricius during the early and secondary lytic replication cycle but not during the latent phase. Several MDV proteins (e.g., VP23, Meq, and RLORF4) have been implicated in inhibiting the production of IFN- β . Deletion of *meq* abrogates lymphoma formation but not the lytic cycle [288], while RLORF4 deletion attenuates the MDV lytic cycle and significantly reduces lymphoma formation [289]. VP23 and MEQ interfere with the DNA sensor cGMP-AMP synthase (cGAS), which detects viral DNA in the cytosol. cGAS activates STING (stimulator of interferon genes) which ultimately leads to activation of interferon regulatory factor (IRF)3 and -7 [290,291]. Sting also activates NF- κ B which together with IRF7 initiates transcription of type I IFNs. RLORF4 inhibits the activation of NF- κ B but not through IRF7 [292].

14.4.5 Immuno-evasion mechanism of the avian poxviruses

The avian poxviruses make up one genus (Avipoxvirus) of the *Chordopoxvirinae* subfamily. The other eight genera comprise only mammalian poxviruses. Recent phylogenetic studies, based on only a couple of conserved genes, reveal that the avipoxviruses show considerable diversity, equivalent to that observed between the Capripoxvirus, Suipoxvirus, Yatapoxvirus, and Leporipoxvirus genera of the mammalian poxviruses. They appear to form three major clusters (or clades) of related viruses, one broadly related to FWPV, one to canarypox virus (CNPV), and the other comprising the psittacine poxviruses [293]. Various phylogenetic studies have indicated that the mammalian poxvirus most closely related to the avipoxviruses is the human Molluscum contagiosum virus, with which avipoxviruses share many common features of molecular biology; these include key aspects of gene complement and organization, as well as aspects of pathogenesis.

The avipoxviruses have some of the largest DNA viral genomes: up to more than 300 kbp and encoding up to 300 genes. The complete genome sequences of pathogenic and attenuated vaccine strains of FWPV [294,295] and of a pathogenic strain of CNPV have been determined

[296]. Avipoxviruses cause diseases ranging in severity and mortality from relatively mild cutaneous infections (e.g., fowlpox in chickens) through the more severe diphtheritic infections (also seen in some cases of fowlpox) to disseminated systemic or pneumonia-like infections with high mortality (e.g., CNPV infections). The nature of the infection is probably related to host-virus adaptation, in which immunoevasion mechanisms and especially immunomodulators play an important role. The less severe infections likely reflect long-standing adaptations of virus and host, whereas the more severe infections probably reflect more recent introductions of viruses to novel hosts.

The derivation of the first sequences brought with it the surprise that FWPV encoded no obvious candidates for modulators of the type-I IFN or IFN- γ pathways or any of the antiviral effectors which these pathways induce [294–296]. This was in contrast to the mammalian poxviruses, which encode: dsRNA binding proteins (e.g., E3 of vaccinia virus); mimics of eIF2 α , the substrate for activated dsRNA-dependent protein kinase, PKR (e.g., vaccinia virus K3); and secreted, soluble binding proteins for IFN-I and IFN- γ . However, FWPV and CNPV appear to encode mimics of TGF- β , and CNPV encodes an IL-10-like protein. Assuming they act as agonists, both of these candidate immunomodulators are predicted to downgrade the host's inflammatory responses by stimulation of T regulator cells, thereby protecting the virus-infected cell.

Two groups independently predicted that two different FWPV genes (fpv073 and fpv214) would encode candidate IL-18 binding proteins [294,295]. Only one of the predicted genes (fpv214) encoded a protein with a conserved IL-18 binding motif. Interestingly, a knockout of this gene in a recombinant FWPV expressing IBDV VP2 resulted in an enhanced CMI response against IBDV when chickens vaccinated with the recombinant were challenged with IBDV. The enhancement was comparable to, but less dramatic than, the enhancement observed when the VP2 recombinant FWPV coexpressed chicken IL-18, whether fpv214 was intact or not [297].

The avipoxviruses also encode multiple chemokine-like molecules (e.g., fpv060, fpv061, fpv116, and fpv121). These appear to be secreted, although as yet no biochemical activity [294] has yet been attributed to any of them, partly because of the lack of identified chicken chemokine and chemokine receptor reagents [298]. It is therefore unclear whether they behave as agonists or antagonists and whether they will target bona fide chemokine receptors or similar receptors for other host ligands. Avipoxviruses also encode serpentine molecules with seven transmembrane segments resembling G-protein-coupled receptors. These proteins (fpv021, fpv027, and fpv206) are candidate chemokine receptors [294]. Whether they actually bind host chemokines or other host ligands, and whether they are signaling-competent or

merely decoy receptors remains to be tested when chicken chemokine reagents become available.

Although no gene encoding IFN- γ -binding protein was predicted from the sequence, a binding protein was identified biochemically by its interaction with recombinant-tagged IFN- γ . It proved to be the product of fpv016 [299]. Type I IFN-binding proteins are not predicted, and it remains to be seen whether alternative approaches will identify genes encoding such proteins. The lack of obvious mechanisms for evasion of the type I IFN responses is intriguing, particularly as FWPV is resistant to recombinant chicken type I IFN at concentrations up to 1000-fold higher than is required to inhibit the chicken cell-adapted vaccinia virus strain MVA [300]. It is also clear that FWPV, unlike other chicken viruses (e.g., IBDV) does not induce chicken type I IFN in CEF culture. Moreover, it can even block the induction of expression of the chicken type I IFN β promoter normally seen upon transfection with the dsRNA analog, polyI:C [301].

The avipoxvirus genomes encode many as yet unassigned proteins, which might function in immunoevasion. For instance, one feature that distinguishes avipoxviruses from mammalian poxviruses is that the former encode several families of related proteins. Most notable is the family of proteins containing multiple ankyrin repeats. These repeats are found in many types of host proteins and their presence normally denotes a protein–protein interaction. Up to 31 of the proteins of this family are encoded by FWPV, with 51 (15% of the total gene complement) encoded by the related CNPV [302]. We have no idea of their targets but presume they are likely to be host proteins and that at least some probably play a role in immunoevasion. Most of these proteins have C-terminal F-box motifs, which in other poxviruses have been shown to interact with the ubiquitin ligase complex via Skp1a and Cullin-1 [303]. By screening chimeric vaccinia MVA carrying inserts of FWPV genomic DNA for enhanced resistance to chicken type I IFN, Buttigieg et al. [300] identified fpv014, an ankyrin repeat protein, as contributing to IFN resistance. They also demonstrated that fpv014 interacted with Cullin-1, but were unable to establish the identity of any cellular proteins bound by the ankyrin repeat domain and presumably destined for ubiquitination and likely degradation by the proteasome. Similarly, Laidlaw et al. [301] screened a library of FWPV mutants, each carrying a single nonessential gene knock-out, for reduced ability to restrict polyI:C-mediated induction of expression of the chicken type I IFN β promoter. They identified another ankyrin repeat protein, fpv012, as contributing to restricting IFN β expression induced by dsRNA, but were also unable to identify the cellular ligand for the ankyrin repeat domain of fpv012.

Identification of two members of this family of proteins from the same virus that appears to modulate

chicken IFN responses at different points provides tools to assist ongoing analysis and description of the chicken IFN responses, which though broadly similar to those of mammals, clearly differ in significant ways. The best example of this is the absence from chickens, and indeed from all galliforms but not from anseriforms [304], of the cytoplasmic dsRNA sensor, RIGI, which presumably leaves MDA5 to perform all cytoplasmic sensing of dsRNA undertaken in mammals by RIGI and MDA-5 [305–307]. The complete consequences of this are still not fully apparent or understood.

14.4.6 Immuno-evasion mechanism of the avian orthomyxoviruses

Viruses of the *Orthomyxoviridae* family are enveloped viruses enclosing eight segments of negative-strand RNA. The best known are of the Influenza virus A genus, mostly as a consequence of their role in human pandemics. As is now widely appreciated since the emergence of the H5N1 strain, influenza A viruses are primarily an infection of birds, originating in waterfowl, and readily passing to a wide range of birds both wild and domestic.

It was using influenza virus infection that IFN was discovered in 1957 [308]. Subsequently, it was one of the first viruses for which a resistance mechanism to IFN (activation of host protein p58) was elucidated [309]. Since 1997 and the emergence of the highly pathogenic H5N1 AIV, which is capable of causing high mortality in humans, NS1 has been identified as the major viral IFN-resistance protein [310–313]. Moreover, a determinant of virulence of H5N1 (D92E) has been located on NS1 [314]. These studies have been performed in the context of mammalian IFN responses, which is appropriate because of the clinical threat and practical because the avian IFN response is not fully understood and there is a lack of avian reagents.

Perhaps because of the lack of biochemical assays and reagents, one of the few direct studies of the interaction between AIV and avian IFN was performed with live virus [315]. The study concluded that there was considerable heterogeneity within and between virus populations in their ability to induce and resist avian type I IFN. This heterogeneity was ascribed to their presence in the population of subpopulations that had packaged multiple genome segments. It was presumed that those particles which had packaged multiple segments encoding IFN resistance protein(s) displayed higher resistance, although this characteristic was not necessarily inherited. Such heterogeneity illustrates one of the potential complications of using live-recombinant influenza viruses to dissect out the role of particular molecular determinants of IFN resistance.

Studies with the mammalian system have revealed that NS1 is a very complex, multifunctional protein. It has at least three major functions: (1) binding dsRNA to block IFN induction, (2) inhibiting host gene expression by preventing mRNA splicing and nuclear export, and (3) enhancing viral mRNA translation. All of these functions merit further investigation in mammalian and avian hosts.

Resistance of influenza virus (influenza A virus unless otherwise specified) to IFN was initially believed to be primarily due to activation of a cellular inhibitor, p58 (IPK), of PKR by NS1 [309]. Others have suggested that M2 [316] and nucleoprotein [317] bind host HSP40 which is an inhibitor of p58(IPK). Another mechanism involved the ubiquitin-like host protein, ISG15, which is one of the most predominant proteins induced by type-I IFN [318]. Influenza B virus strongly induces ISG15, but a specific region of the influenza B virus NS1 protein (NS1B), which includes part of its effector domain, blocks the ability of ISG15 to become covalently linked to its target proteins by inhibiting its UBE1L-mediated activation. The influenza A virus NS1 protein does not bind ISG15 but inhibits its synthesis [319].

Subsequently, however, NS1 was shown to play a major role in resistance to IFN. Specifically, NS1 mutants were able to replicate only in IFN-defective cell lines [311]. NS1, normally 230 amino acids long in influenza A virus, is encoded by virus RNA segment 8. The amino terminus (73 residues) of NS1, which dimerizes [320], is capable of binding dsRNA [321]—albeit at relatively low affinity [319]—thereby preventing activation of the dsRNA-dependent protein kinase, PKR [322]. NS1 also binds poly-adenylated RNA, inhibiting nuclear export of mRNAs [323], and a stem bulge in U6 snRNA, inhibiting pre-mRNA splicing in vitro and in vivo [323]. Both of these activities can downregulate the expression of cellular genes. They can also prevent IFN production by binding to IRF-3, blocking its kinase-mediated activation [313], and by blocking NF- κ B activation [324]. Although expression of NS1 alone was reported to induce apoptosis in MDCK and HeLa cells [325], in the context of a viral infection of mammalian or avian cells, its IFN-regulatory activity makes it antiapoptotic [326]. NS1 selectively enhances translation of viral but not cellular mRNAs by binding eIF4GI, PABP1, and the 5' UTRs of vmRNAs [327,328].

It is interesting that the region of NS1 (aa 81–131) binding eIF4GI spans the location of the known virulence mutation (aa 92) and that the region of PABP1 to which NS1 binds is not conserved evolutionarily. The C terminus, or effector domain, downregulates the formation and export of cellular mRNAs, by binding to the 30 kDa subunit of CPSF and to PABII [329,330]. The dsRNA-binding activity of NS1 can be abrogated by mutating two basic residues (R38 and K41 to A). In MDCK cells, virus thus mutated

[331] failed to inhibit IFN- β and replicated to lower titers. On passage, a better replicating virus emerged with an S42G mutation that did not improve dsRNA binding, but which had intermediate virulence in mice.

Recent studies of modulation of avian IFN by AIV have focused on the role of NS1 and of PB2. There are many difficulties with studying the induction of IFN responses *in vivo*, not least in standardizing the nature of the inducing virus; this can depend on the strain and on its passage history. Penski et al. [332] showed that NS1 deletion mutants of HPAIV (H5N1 and H7N7) induced higher levels of IFN β in CEFs than did parental virus. Liniger et al. [333] showed that NS1 from HPAIV also controls IFN β expression in chicken macrophage HD-11 cell line. However, NS1 deletion mutants of HPAIV still manage to control IFN β levels in infected chickens [332], suggesting that other mechanisms are involved. Liniger et al. [333] demonstrated that HPAIV PB2 could block induction of IFN β expression in the immortalized chicken fibroblast DF-1 cell line when induced by overexpression of the chicken homologs of VISA/CARDIF or MDA-5.

The role of an ESEV motif found at the C-terminus of HPAIV NS1 proteins, but not in seasonal human AIV (which bear the motif RSKV), has been investigated by Zielecki et al. [334]. The motif represents a ligand for PDZ domains of cellular proteins. It attenuates virus replication in human, murine, and duck cells but not in CEFs. Nevertheless, it appears to have little influence on virulence in either mice or chickens.

Most studies on immunomodulation by AIV focus primarily on adaptation to and pathogenicity in the mammalian host but Cui et al. [335] showed that a 113 aa truncation of the NS1 of an H5N6 AIV reduced its ability to inhibit IFN production (but increased apoptosis) in both avian and mammalian cells. *In vivo* studies in chickens showed that virus carrying the deleted NS1 is highly attenuated [336].

The possibility of using NS1 mutant AIV as live attenuated vaccines was explored by Steel et al. [337]. Viruses with internal deletions of NS1 stimulated higher levels of IFN β expression in mice and were attenuated in chickens. They also stimulated protective immunity against HPAIV challenge (the mutants also contained an E residue at position 627 of PB2 to restrict replication and virulence in mammals).

14.4.7 Immuno-evasion mechanism of the avian paramyxoviruses

The *Paramyxoviridae* family of negative-sense RNA viruses, which includes measles virus, has two well-known avian members: NDV (member of the Avulavirus

genus) and turkey rhinotracheitis virus or avian metapneumovirus (member of the Metapneumovirus genus).

There have recently been considerable studies of the mechanism by which paramyxoviruses modulate IFN-I, demonstrating the importance of the V proteins in blocking IFN induction and signaling [338]. These proteins are expressed following RNA editing of the mRNA that encodes P protein. Because of the lack of reagents for the avian IFN-I system, this work has barely extended to avian paramyxoviruses. The likely role of the NDV V protein has, however, been demonstrated using genetically modified viruses [339]. Thus, mutant viruses, defective for V protein expression (V⁻ NDV), replicate poorly in embryonated eggs and CEF. This defect can be complemented by transfection of a plasmid-expressing cDNA encoding the V protein into V⁻ NDV-infected cells [339] or by insertion of the influenza virus NS1 gene into the V⁻ NDV. The NS1 gene allows the modified NDV to replicate better in human cells than does the parental NDV, suggesting that the NDV V protein is more effective in modulating the chicken IFN system than the mammalian IFN system (unlike influenza NS1 which modulates both systems effectively).

These differences are likely to be important for the host-range specificities of the two viruses. All examined V proteins of mammalian paramyxoviruses appear to be involved in modulating the mammalian IFN response via interaction with mammalian mda-5 [305]. They can also modulate the induction of expression of the IFN β promoter by transfected polyI:C in mammalian and avian cells, as can NDV V. However, mda-5 shows species specificity of induction in that mammalian and avian mda-5 can only induce expression of the IFN- β promoter in mammalian and avian cells, respectively [305].

14.4.8 Immuno-evasion mechanism of the avian reoviruses

It is clear that dsRNA is a powerful inducer of the antiviral type-I IFN system. Although the positive-strand RNA viruses, and even poxviruses, generate some dsRNA during their replication and transcription, the dsRNA viruses must be able to prevent recognition of their dsRNA genome by the cell. This is probably largely achieved by ensuring that the genome is never exposed outside of the capsid within the cytoplasm. However, the avian reoviruses, in common with mammalian reoviruses, appear to encode a dsRNA-binding protein that can mask dsRNA from cellular dsRNA-binding proteins such as the dsRNA-dependent protein kinase PKR. The avian reovirus-coded S2-encoded σ A protein functions like poxvirus E3, and can actually replace E3 in vaccinia virus [340].

The crystal structure of bacterially expressed σA has now been solved, elucidating the mechanism of its cooperative binding to minimal lengths of 14–18 base pairs of dsRNA [341]. The cellular distribution of σA has also been studied. Although most of it localizes to the cytoplasmic viral factories, some are able to enter the nucleus, apparently independently of the classical nuclear localization signal recognition by importin, and localizes to the nucleoli in a manner dependent on the presence of two arginine residues required for dsRNA binding [342].

14.4.9 Immuno-evasion mechanism of the avian birnaviruses

IBDV clearly induces expression of the chicken type I IFN promoter in CEFs [301]. The induction of the IFN- β promoter by virus infection alone is relatively strain-independent, but the ability of IBDV to inhibit transfected polyI:C-mediated induction of the promoter is pathotype-dependent (Laidlaw & Skinner, unpublished), as is the induction of the IFN- β promoter in bursal tissue of IBDV-infected chickens [77].

As described above, it has now been reported that VP3 interferes with the production of IFN- β by blocking the binding of viral dsRNA to MDA5 [78]. A study of IBDV inhibition of TNF- α or Sendai virus-induced IFN expression in human HEK293T cells found that VP4 interacts with glucocorticoid-induced leucine zipper (GILZ) in a yeast-two-hybrid screen and colocalizes with it in the cell, and its IFN-suppressive activity is reduced if GILZ expression is reduced by siRNA [343]. It has since been shown that VP4 interferes with the K48-linked ubiquitylation of GILZ [80].

14.5 Conclusions

Immunosuppression as a consequence of (subclinical) virus infections is a common occurrence with important consequences for the poultry industry. Thus far, most studies describe its effects on immune responses without addressing the mechanistic aspects of the immunosuppression, which is the consequence of the lack of reagents and appropriate techniques. However, during the last few years, and especially since the chicken genome has been sequenced, research has started to focus on the interactions between virus infection and the effects on the (de) regulation of the immune responses. It is expected that rapid progress will be made in the next 5–10 years in this area of research.

There has also been a burgeoning interest in the mechanisms by which viruses evade or modulate the innate resistance mechanisms of the host, in particular the type I IFN system. This interest has so far had little impact on the study of avian viruses because of the lack

of essential reagents. The derivation of the chicken genome sequence has opened up easier access to such reagents. It is likely that the coming years will see a rapid increase in the number of studies, and hence our knowledge of the intricacies of avian virus–host systems. Development of systems for manipulating the large and complicated RNA genome of IBV [344], including field strains, H52, and QX [345–348] may also help identify additional viral immunomodulators and hopefully elucidate their mode of action [281,349–353]. Such technology has been available for some time for avian adenoviruses, but the divergence between mammalian and avian adenoviruses has made it difficult to predict candidate immunomodulators. Hopefully, reannotation of the avian adenovirus CELO genome, which identified three putative surface glycoproteins with immunoglobulin-like folds, will be useful for further analysis [354].

That studies of this type are likely to elucidate new paradigms, which may even be relevant beyond the avian host, is illustrated by the relative dearth in avian herpesviruses and poxviruses of obvious immunomodulators of the type observed in their mammalian cousins. The emergence of West Nile virus and of H5N1 AIV as threats to wild birds, farmed poultry, mammalian livestock, and companion animals, as well as to humans will, hopefully, ensure the resources needed to understand the complexity of the interactions of these and other viruses with their avian hosts. It is to be hoped that we will then be better equipped to control and treat, or possibly even eradicate, such viruses.

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Factors modulating the avian immune system

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15.1 Endocrine regulation of immunity

Almost all cells of the immune system have receptors for one or more of the stress hormones, metabolic hormones, sex hormones, and other endocrine-signaling molecules. This implies that hormones have direct effects on the immune system and are responsible for many of the changes in immunocompetence that occur as a result of development and growth, changes in reproductive status, migration, molt, stress, and dietary changes [1]. Although it would be convenient to label a hormone “immunosuppressive” or “immunostimulatory,” there are no clear instances where changes in the level of a hormone within normal physiological ranges result in similar directional changes in all indices of immunity (though this may occur when a hormone is ablated or administered at pharmacological levels). Instead, physiological adjustments in the level of a hormone typically result in a rebalancing of the immune system (i.e., immunomodulation), with some effector mechanisms increasing and others decreasing. This rebalancing of immunity appears to function to integrate the immune system into the life history stages and environmental changes that birds face [2,3].

15.1.1 Stress hormones: epinephrine, norepinephrine, dopamine, and glucosteroids

A stress response occurs when a situation (stressor) threatens a bird's homeostasis. The acute stress response is a coordinated effort to remove the animal from the stressful situation and involves the “fight-or-flight” response driven by catecholamines such as epinephrine (EP), norepinephrine (NE), and dopamine (DA). Simultaneously, hypothalamic corticotrophin-releasing factor and vasopressin secretion

stimulate release of adrenocorticotrophic hormone (ACTH), resulting in glucocorticoid secretion from the adrenal cortex [4–6]. Glucocorticoid levels remain elevated for the extent of the period of stress and are thought to mediate many of the chronic effects of stressors. The catecholamines are a group of neurotransmitters and hormones consisting of NE, EP, and DA. NE and EP (noradrenaline and adrenaline) are integral in maintaining behavioral and physiological homeostasis during both aversive and rewarding events. Together, the stress hormones initiate a series of behavioral, physiological, metabolic, and immunological adjustments that redistribute resources in order for the animal to immediately survive, continually adapt, and ultimately return to homeostasis. Stress hormones can exert direct effects on the immune system. Catecholamines are released into lymphoid tissue as a result of direct innervation by sympathetic nervous systems. Lymphocytes have high-affinity receptors for ACTH and glucocorticoids, which are upregulated by immune stimulation [7]. Immune cells also produce stress hormones; bursal and thymic cells produce and release corticosteroids (CORT) [8], and several types of leukocytes can also synthesize ACTH, corticosterone, and other neuroendocrine mediators [9]. Modulation of the immune response by stress hormones can also occur indirectly as a result of hormone-induced changes in cytokine production by other cell types in the tissues where leukocytes reside. Finally, stress hormones can have direct effects on bacteria in the gastrointestinal tract, respiratory tract, or skin that change their growth rates, motility, and gene expression [5]. These important direct effects on potential pathogens are often initiated by quorum sensing and affect a transition from commensal to pathogenic phenotype.

The effect of corticosterone on the immune system of birds is highly dependent on the level and duration of

CORT. Different aspects of the immune system are studied in association with stress [10–14]. During the early response, corticosterone causes trafficking of lymphocytes out of the blood to lymphoid tissues and sites of ongoing or de novo immune activation. At the same time, heterophils are mobilized from bone marrow and accumulate in blood, resulting in an increased heterophil-to-lymphocyte ratio. During the early response to corticosterone, both heterophils and lymphocytes increase the expression of mRNA for pro-inflammatory cytokines and chemokines [14]. These phenotypic changes, even in the absence of stimulation by antigens or pathogen-associated molecular patterns, indicate a higher state of activation and likely contribute to the greater resistance to some types of bacteria during acute stress [5,14]. Following more chronic exposure to corticosterone, heterophils and lymphocytes decrease expression of pro-inflammatory cytokines and take on an antiinflammatory phenotype. Together, changes in organ sizes, cell numbers, gene expression, and functionality indicate a transition of the immune system from a higher state of activation during acute stress to an antiinflammatory and immunosuppressed state during chronic stress.

The bidirectional communication between the central nervous system and the enteric nervous system, known as the gut–brain axis, represents the link between emotional and cognitive centers of the brain with peripheral intestinal functions [15]. Recent evidence suggested the prominent role of the gut microbial population in such interaction, leading to the new concept of “microbiota–gut–brain axis,” and thereby the relation with the immune system. The gut microbiota can act both directly and indirectly on the brain through different pathways of communication, including the activation of afferent sensory fibers of the vagus nerve, neuroimmune and neuroendocrine pathways, microbial metabolites and microbially derived neurotransmitters [16]. Recent studies showed in broilers via probiotic treatment that basal CORT level did not change in unpleasantly handled broilers [17], broilers [18] or laying hens, but it reduced basal ACTH level in laying hens [19]. Similarly, prebiotic treatment did not alter basal CORT level in broilers [20]. Yet, pro-, pre-, and synbiotic treatment reduced basal CORT levels in heat-stressed broilers [21–23]. Thus, findings are inconsistent with regard to microbiota effects on basal CORT level but did not investigate the relation with immune system and how this was affected. *In ovo* exposure to stress hormones is also relevant to future immune responses. First, chick exposure is affected by the glucocorticoid status of the hen, and hens with elevated glucocorticoids incorporate higher levels in the yolk of their eggs [24]. Second, chicks hatched from eggs with elevated corticosterone have slower growth rates, increased adrenocortical responses to acute stress, and reduced cutaneous basophil

hypersensitivity (CBH) responses [25]. Zaytsoff and colleagues [26] demonstrated physiological stress via administration of CORT in chickens modulated host metabolism and systemic responses, which indicated that energy potentials are diverted from muscle anabolism during periods of stress.

The adaptive significance of chronic stress-induced immunosuppression has been the subject of investigation [27,28] and converges on two nonexclusive ultimate causes: immunopathology avoidance and resource limitation. The “outcome” of chronically induced stress and immune response could be dependent on the coping style of chickens [29]. A proactive coping style is, among others, associated with active behavioral responses, low baseline activity and stress reactivity of the hypothalamus–pituitary–adrenal (HPA) axis, low humoral immunity, but high cellular immunity and innate immune activity compared to a reactive coping style [30,31]. In studies of hens with high feather pecking phenotype, as a chronic stressor, it was shown that nitric oxide (NO) production in monocytes and IgY titers differed from the low feather pecking phenotype [32]. The complex interaction of chronic stress could also result in less active birds. A study showed that explorative birds had an increased complement activity and natural antibodies [33]. In feathers of birds the total amount of corticosterone deposited in time could be measured and is used as a predictor for chronic stress [34]. Chronic stress could lead to immune responses that are maladaptive; they can have profound fitness effects by causing serious damage to host tissue, sometimes leading to loss of function and eventually death. The second posits that immune responses are expensive in terms of energy and nutrients and that these costs compete with and impair other important fitness traits such as growth, reproduction, migration, and predator avoidance (Table 15.1). Certainly, autoimmunity and accelerated aging resulting from immunopathology, as well as the cachexia that results from hyperinflammatory responses, are untoward consequences of uncontrolled immune responses.

15.1.2 Sex hormones

Males and females differ in their immune response and resistance to many infectious diseases, with females often more resistant than males [38]. This generality is often attributed to differing effects of androgens and estrogens on the immune system, but the data in different bird species are conflicting. The basis for variable effects of sex hormones across the many studies may be partly due to differences in the age or sex used [39,40], life-history traits of the species in question (e.g., life span, breeding system, parental investment [8,41,42]), environmental effects (e.g., free-living vs captive), interactive effects of

TABLE 15.1 Generalized parsing of costs of innate and adaptive immunity. [35–37]

Component	State	Cost		Protection and effectiveness	
		Nutritional resources	Pathological damage	Novel pathogen	Repeated exposure
Innate	Development	Low	–	–	–
	Constitutive	Intermediate	Low	Good (rapid)	Good (rapid)
	Activated	High	High	Very good (rapid)	Very good (rapid)
Adaptive	Development	High	–	–	–
	Constitutive	Low	Low	Poor	Good
	Activated	Low	Variable	Good (slow)	Excellent (rapid)

Source: Adapted from Iseri VA. The amino acid content of the systemic components of the chicken (*Gallus gallus domesticus*) immune system. Davis, CA: University of California; Klasing KC, Calvert CC. The care and feeding of an immune system: an analysis of lysine needs. In: Lobley GE, White A, MacRae JC, editors. Proceedings of the VIIIth International Symposium on Protein Metabolism and Nutrition. Wageningen: Wageningen Press; 2000. p. 253–264; and Klasing KC. The costs of immunity. *Acta Zool Sin* 2004;50:961–69.

sex hormones with other hormones [43,44] (e.g., glucocorticoids) or with immunomodulatory nutrients [45] (e.g., carotenoids), physiological state [46] and methodological constraints. Disentangling changes in immunity due to hormones from secondary effects is difficult [28,47]. For example, a large body of evidence indicates that high reproductive effort in birds has adverse effects on immune function accompanied by decreased resistance to pathogens such as parasites [48]. However, it has been difficult to determine the extent to which immunosuppression is a result of sex hormones versus changes induced by an elevated workload and associated higher glucocorticoids, tissue damage, and energy expenditure. Finally, the majority of recent work on sex hormones and immunity has examined nondomestic birds, which necessitates reliance on simple assays of immune function that is often very difficult to interpret relative to the array of functional and mechanistic assays used in domestic species.

Androgens

Androgen receptors are located in the bursa of immature chickens [43], and development of the thymus and bursa is influenced by androgen levels [49–51]. For example, embryonic exposure to high levels of testosterone completely halts the development of the bursa [49] and exposing chicks to high levels during their first 3 weeks posthatch markedly increases their susceptibility to an experimental bacterial challenge [52]. At the opposite extreme, caponizing male chickens blocks the normal involution of the bursa that occurs during sexual maturation [50,51]. Interestingly, it was hypothesized that immunomodulatory effects by *in ovo* testosterone exposure may involve the conversion of testosterone to estradiol by the enzyme aromatase. When using fadrozole to inhibit aromatization of endogenous testosterone in chickens during a critical period of embryogenesis, changes in some immune parameters were observed and it was concluded

that aromatization at least in part contributes to testosterone modulation of developing immunity [53].

Although testosterone has clear and repeatable effects on the development of the immune system at very high levels of administration, the effects of dosing at physiological levels are often conflicting [2]. Both *in vivo* and *in vitro* studies clearly show that the effects of testosterone on the immune system are highly dose- and sex-dependent [52,54].

Many *in vivo* studies have used testosterone implants to experimentally increase testosterone levels, but the effects of these implants are often contradictory to the effects of testosterone in nonimplanted animals. For example, testosterone implants depressed humoral immunity in superb fairywrens (*Malurus cyaneus*), but testosterone levels in nonimplanted males were positively correlated with humoral immunity [55]. Testosterone implants may affect immunity by increasing corticosterone levels [43], and the interactive effects of these hormones are often different from their individual effects [44].

In a well-controlled study using Japanese quail with testosterone delivered at various levels via implants [44], testosterone had little or no effect on indices of local inflammation induced by phytohemagglutinin (PHA) or an antibody response. However, location of the birds in the battery (i.e., distance from floor) did have effects. This is instructive at several levels. First, it indicates that relatively minor environmental differences modulate immunity to a much greater extent than either moderate or high levels of testosterone. Second, it demonstrates the need for careful control and experimental design to sort out subtle immunomodulatory effects of hormones.

As with corticosterone, the amount of androgen deposited in eggs affects the immune responses of progeny. However, the results are very contradictory likely because of the large numbers of species, sexes, supplemental doses, and immunological endpoints used [56–58]. A study designed to test the hypothesis that exposure to

elevated yolk androgen levels would benefit chicks sired by immunocompetent fathers but would be detrimental to chicks sired by less immunocompetent fathers did not prove any paternal effect [59].

Estrogen

Although the effect of androgens on the development of the bursa has long been known, an appreciation for estrogen in the development and function of the immune system has been the focus of renewed study. Receptors for estrogen are expressed in lymphocytes from the thymus, bursa, and spleen of chickens [60]. Expression is highest in the bursa after embryonic day 15 [61]. The bursa expresses the enzymes necessary to synthesize estrogen, and it is likely that estrogen acts on developing B lymphocytes in a paracrine fashion [61]. When exogenous estrogen is added to eggs, the effect on bursal weight and antibody production of hatched chicks can be either stimulatory or suppressive depending on the day of estrogen treatment and the dose. The size, follicular area, and cellularity of the bursa are markedly decreased when the egg is exposed during the early developmental period [62–64]. Surprisingly, early exposure reduced the normal regression of the bursa during sexual maturation [62].

Estrogen also has effects on the immune system post-development. It enhances the phagocytic and cytostatic function of macrophages but decreases chemoattractant expression (reviewed in Ref. [63]). Like testosterone, estrogen at pharmacological levels inhibits the mitogen-induced proliferation of lymphocytes. However, unlike testosterone, it increases lymphocyte proliferation to either a B cell mitogen (LPS) or the T cell mitogen, concanavalin A (ConA) [54].

15.1.3 Metabolic hormones: thyroid hormone, growth hormone, and leptin

Thyroid hormone is a primary regulator of basal metabolic rate in animals, but it also affects immune function via direct action on leukocytes, which have nuclear receptors for thyroid hormones [65]. In birds, thyroid hormone positively affects thymus growth [49] and affects humoral immunity and the number of circulating lymphocytes; it is positively correlated to IL-2-like activity in chickens [65]. However, thyroid hormone responsiveness differs between species and/or age and may be more demonstrable during growing stages [66].

Growth hormone can influence thymic growth and maturation of T lymphocytes [67]. Growth hormone mRNA has been identified in the thymus, spleen, and bursa of chickens, although the amount present in these tissues is low relative to that in the pituitary [68]. These same tissues also express growth hormone receptors, indicating a

paracrine action of endogenously produced growth hormone [69]. Interestingly, macrophages, not lymphocytes, are the primary cells possessing the receptor, so effects on lymphocyte development and activity may be secondary.

Leptin, a peptide hormone synthesized and secreted by adipose tissue, is also implicated in immunomodulation. Addition of leptin to cultures of turkey lymphocytes increases their proliferation in response to ConA [70]. Injecting or infusing leptin into Asian blue quail, greenfinches, or zebra finches increases local inflammatory responses to PHA injection [70–72].

15.1.4 Environmentally responsive hormones: melatonin

Several avian biological functions such as reproduction, metabolism, and immunity follow daily and seasonal patterns [73]. These processes are under control of an endogenous biological clock, which is organized slightly different in birds compared to mammals [74]. The mammalian master circadian clock is located in the suprachiasmatic nuclei (SCN) of the hypothalamus, whereas the avian master clock is composed of the avian SCN equivalent but also the pineal gland and the retina. Furthermore, the avian pineal gland exhibits direct photosensitivity, which was lost during evolution and not seen in mammals [75]. Between 2 and 8 weeks of age a transitory lymphoid infiltration of the chicken pineal gland is observed indicating special neuroendocrine–immune interactions [76].

A number of hormones have been implicated in the cyclicity of the immune response, including CORT, sex steroids, and melatonin [10,77]. Although all of these hormones are likely important regulators of immunity during the annual cycle, melatonin appears to predominate during the daily cycle [78]. Like other hormones, the primary action of melatonin involves differential modulation of diverse types of immune responses, increasing some types of responses and decreasing others.

The major systemic source for melatonin is the chicken pineal gland, and melatonin undergoes a distinct daily rhythm that is determined by light [79]. Cells in the chicken thymus, bursa, and spleen express three types of melatonin receptors [80]. The level of receptor expression in the thymus, bursa, and spleen is higher during the light period than the dark period [78,81]. In the Indian Jungle Bush Quail (*Perdica asiatica*), the rhythms of the two different receptors, Mel1a and Mel1b, are about 4 hours out of phase [78]. Assuming that different leukocyte types express differing proportions of these melatonin receptors, they may follow different daily rhythms.

Pinealectomy during embryogenesis reduces thymic and bursal growth and cell numbers and reduces parameters of humoral and cell-mediated immunity in

combination with changes in neuroendocrine networks in 17-day-old embryos [82]. Likewise, pinealectomy in the first week of life impacts the circadian rhythm of heterophil counts and lysozyme concentration in peripheral blood of 5-week old chickens, and restoration was obtained by injection of exogenous melatonin [83]. Similarly, accelerated development of cellular and humoral immune responses was observed when newly hatched turkey poults were administered melatonin or when melatonin was administered *in ovo* just prior to hatching. Later in life, melatonin levels and binding decrease [81]. Older ringdoves respond to supplemented melatonin with increased heterophil phagocytosis but less so than younger birds do [84].

In Japanese quail the cellular inflammatory response to PHA is greatest and the antibody response to foreign red blood cells is lowest when these challenges are made during the daytime; this reverses when the challenge is at night [85]. However, in chickens injected with thioglycollate at the beginning of the light period it was shown that inflammation occurred faster than compared to chickens treated just before the dark period [86]. In young chickens, splenic mRNA expression of IL-1 β , IL-6, and IL-18 occurred in a rhythmic (but variable by cytokine) profile over a 24-hour period, and when birds were challenged with LPS, the dynamics of cytokine mRNA induction also appeared to be under circadian clock control. When melatonin was administered just prior to immune challenge, cytokine mRNA expression was altered, resulting in significantly greater IL-1 β and IL-6 expression post-LPS but not postsaline, which suggests additive or synergistic effects of melatonin and LPS that are variably modulated depending on the cytokine in question [87]. However, the effects of melatonin on various types of immune responses are strongly interactive with other hormones, including glucocorticoids, thyroxine, and androgens, and they are species-specific [88–90].

Taken together, significant evidence lead to the conclusion that endogenous melatonin is an important factor for regulating rhythms in avian immunity. Mechanisms by which melatonin influences immune system functions are complex, but may include participation of mediators (endogenous opioids, cytokines, hormones, zinc pool) [79]. Moreover, direct interactions between opioid peptides and cytokines (e.g., IFN- γ and IL-2) have been suggested [81] as have effects on Ca²⁺ channels and intracellular Ca²⁺ concentration [79].

As in mammals, the relationships between the immune system and the pineal biological clock in chickens appear bidirectional. It was reported that in an inflammation model where peritonitis was induced by thioglycollate, sleepiness was evoked suggesting that immune activation was perceived by the master clock [91]. Later studies showed that cytokines produced in response to

inflammatory reactions modulate melatonin synthesis in the pineal gland with IL-6, in particular, has been found to be a strong modulator of melatonin synthesis [92].

15.2 Physiological states

Social, environmental, and housing stresses are common in the life of birds and influence the immune system and susceptibility to diseases. Changes in immunity are thought to be orchestrated primarily by hormones, although direct neural modulation may also be involved. The diet itself is also a major determinant of the type and/or magnitude of immune response. Although nutrient deficiencies receive the most attention, there are many nutrients that modulate immunity even at levels between the dietary requirement and toxicity. In fact, many nutrients that are not normally considered to be either required or toxic are immunomodulatory.

15.2.1 Temperature and housing as immune modulators

Stress has many definitions, but in this context is defined as an adaptive response to threats to an animal's homeostasis [6]. Stressors may be external or internal stimuli, and severity, duration, novelty, and host status all affect the response to them. Many environmental stressors affect immune responses; this is a topic that has been extensively reviewed [93]. Potential stressors include temperature, light (e.g., ultraviolet light), air quality (e.g., ammonia, ozone), general sanitation including infectious agents, environmental contaminants (e.g., mycotoxins, pesticides), and nutrients.

Temperature

Exposure of birds to extreme temperatures can impact immune responses, and the effect of heat or cold stress is generally inhibitory toward lymphocyte-mediated responses [6], particularly in terms of CBH responses and lymphocyte proliferation (*ex vivo*), with variable effects on humoral responses [94]. Chronic heat stress causes inflammation of the jejunal epithelium and increases the pathology resulting from a *Salmonella* Enteritidis challenge, possibly as the result of altered macrophage function [95,96]. The mechanism by which heat stress modulates immunity is in part due to the extent of induction of heat shock proteins in lymphocytes, heterophils, and macrophages [93], while cold stress suppresses plasma corticosterone levels and enhances thyroid hormone levels [97]. Even mild heat stress can cause a redistribution of lymphocytes and immune system changes, although these changes may have minimal impact on resistance to some disease challenges [98].

Housing

The physical environment can affect the stress response and thus the immune response. For example, chickens raised on slatted floors have elevated heterophil-to-lymphocyte ratios and duration of tonic immobility, as well as reduced antibody titers and CBH responses, compared with birds in a litter-based environment [99]. Recent studies showed that enrichment of chicken farms with a dark brooder changed the microbiota diversity (not due to feed differences) and the behavior of broiler chicks, and it also affected the intestinal immune system (De Jong et al., personal communication). Elevated stocking density is negatively correlated with the growth of immune organs [100]. Similarly, increasing the breeding density of wild birds reduces CBH responses, although this is also correlated with body size and presumably food availability [101]. In laying hens, providing access to perches and the ability to engage in natural behaviors decreases heterophil-to-lymphocyte ratios [102]. On poultry farms, environmental stress influences serum corticosterone levels and affects the susceptibility of chicken to infectious diseases, possibly through effects on the immune system. Overcrowding stress could lead to decreased macrophage phagocytosis intensity and increased *Salmonella* Enteritidis counts in the livers of birds challenged with *S. enteritidis*. Overcrowding stress could also influence via the HPA axis, an increase in CORT, thereby influencing the intestinal integrity leading to transmigration of *S. enteritidis*.

The geographical environment in which a bird lives also affects immune responses; thus, the same species may have different responses depending on location. Baseline corticosterone levels, for example, were elevated to a much greater degree in house sparrows (*Passer domesticus*) from temperate environments than in those from tropical environments [2,10]. These effects may be due to environment (e.g., photoperiod can affect corticosterone levels and immune function), or they may be part of differing evolutionary selection pressures in different environments. Additionally, it has been proposed that birds living in tropical environments have increased parasite loads and thus mount lower corticosterone responses in order to maintain adequate immune defenses [2,10]. In contrast, birds living in temperate environments may mount corticosterone responses when challenged and when other resources (e.g., nutritional) are available to allow this response to occur.

Light in broiler housing could affect the immune development. When comparing incandescent, compact fluorescent, neutral, cool and warm light or comparing white and white supplemented with green/blue or red light, majority of studies show that light spectrum does not affect immunity. Although some studies showed

effects, other studies report a change in heterophil/lymphocyte ratio or changed T cell proliferation [103].

On-farm hatching systems for broiler chicks are increasingly used in practice. In these systems birds are not exposed to chick handling, delayed feed and water access and transport. This affects welfare and health [104]. This early feeding could also influence the immune development, although no difference in villus-to-crypt ratio was found due to on-farm hatching [105].

15.3 Dietary effects on immunity

The interaction between poultry nutrition and nutrients and the bird's immune response has long been known and has been reviewed extensively [106–110]. Studies on feed restriction have brought further insights into the effect of nutrition on the immune competency of poultry [111]. Klasing's [106] seminal paper classifies nutritional modulation of immune function via seven broad categories: (1) immune system development, (2) immune cell substrates, (3) leukocyte regulatory activity, (4) reduction of immune pathology, (5) regulation of hormonal activity, (6) physiological actions on the intestinal tract, and (7) limiting nutrient availability to pathogens during infections. The field of nutritional immunology has become a specialty within avian immunology over the past decade. There is a large body of evidence that the impact of nutritional components on the avian gut and systemic immunity has been increasingly recognized. Furthermore, the implications of nutrition and nutritional intervention on prevention of disease and even corrections of pathophysiological conditions have become established concepts [109,112,113].

Despite the number of papers demonstrating the ability of nutrients to influence the avian immune response, relatively few studies have been successful using nutritional modulation of immunity to induce protection against infectious diseases [114–116]. Recent studies [110,116] have argued that part of the problem is that most research has used only a single immune measurement as a marker for immune effectiveness. On the other hand, despite recent advances in the field, the interplay between nutritional processes and immune system is incompletely understood. Particularly, specific cellular and molecular immune responses invoked by feed components and the role of the gut barrier and microbiota on the interaction between the immune system and nutrition remains to be fully elucidated. Some of the areas have been either neglected when studying the effects of nutrition and nutrients on immune function (the effect of the gut microbiome) or the nonintentional effects of over-feeding various nutrients on the avian immune response (feed-induced inflammation and metabolic inflammation [meta-inflammation]).

15.3.1 Contribution of the microbiome

Within an animal's interactive physiological networks, the complexity of nutritional interactions is not solely confronted by the host but is made substantially greater since the animal plays host to entire communities of commensal and symbiotic microbes that derive nutrients from the host diet and provide vital nutrients to the host [117]. Furthermore, the gut microbiome is involved in the development and maturation of the host immune response [118–121]. As a “second genome” of the vertebrate host, the gut microbiome acts as a critical regulator of immune responses, influencing the differentiation, function, and maturation of immune cells of both the innate and acquired arms of mucosal immunity [122]. This accumulation of immune cells, physical barriers, and soluble mediators contains and controls the microbiota [122–124] by “molecular firewalls” [124] that prevent microbiota-specific acquired responses against commensal microbes. These firewalls include: (1) the epithelial cell layer, which produces mucus, generates antimicrobial peptides, and continuously secretes IgA that contain commensals and neutralize invasive pathogens, (2) subepithelial macrophages that phagocytize and kill commensals that attempt to penetrate the intestine, and (3) regulatory dendritic cells and regulatory T cells with immunosuppressive activity to control effector immune cells [124,125]. Under the influence of diet, the composition of the gut microbiome, as well as the commensal-derived nutrients and metabolites (lipids, short chain fatty acids, amino acids, vitamins), are altered [126,127]. These diet-induced shifts in microbiome composition and commensal-derived nutrients and metabolites have profound direct and indirect effects on host immunity via alterations in signaling pathways and gene transcription of effector immune cells, development of immune cells, and receptor recognition/sensing of immune cells [118,128,129]. The most glaring issue facing using nutrition/diet to modulate host immunity is that virtually all previous work has ignored the effect of the gut microbiome. Many studies have concluded a direct effect of a nutrient (protein, fatty acids, carbohydrates) on the immune system without determining the direct effect on the gut microbiome, leading to an indirect effect on immunity. Therefore, it is apparent that diet and commensal microbiota interaction has a remarkable influence on the host immune response, suggesting that many studies indicating poultry diets and their components directly affect the avian immune response may have to be reevaluated, since the role of the gut microbiota was not considered in their interpretations of the results [130–134]. Although much is still to be learned about the microorganisms that comprise the avian gut microbiota, it is paramount that avian immunologists must consider the role of

the gut microbiota in every live bird experiment, especially when investigating the role of the diet and dietary components on avian immune function [135].

15.3.2 Immunomodulatory nutrients and feed additives

Amino acids

Amino acids are of paramount importance in maintaining the integrity and function of the immune system in poultry. To support these functions, nutritionists must understand the intestinal immune system and its relationship with the intestinal microbial community as a distinct organ with specific nutrient needs. In practice, nutrient profiles used in feed formulations for broiler chickens are typically based on economically important production outcomes such as weight gain, feed intake, feed conversion ratio, and carcass yield [108]. The nutrient and energy needs for immunity or disease resistance, however, may be understudied. Several different enteric infections may alter the development of the immune response, and certain nutrients such as amino acids may become limiting factors to produce key proteins required for appropriate immune function. In the last few years, a great number of publications have demonstrated the effects of increasing the dietary concentration of certain amino acids to support the development and well-functioning of the intestinal immune system [136–140]. Therefore, for a more complete review on the effects of different amino acids on the immune system and endogenous losses of amino acids due to enteric challenges see the recent reviews [141,142].

In poultry, particular interest has been given to amino acids known for having effects on metabolic functions such as those involved in the proper functioning of the immune system and in the intestinal mucosal repair processes [143]. According to Wu et al. [144], dietary amino acids promote intestinal repair through induction of enzymes needed for the mitotic process, such as ornithine-decarboxylase required for the synthesis of polyamines, as well as through altering the expression of genes involved in antiinflammatory and reparative processes. Few examples of amino acids that exert important immune function include threonine (Thr), arginine (Arg), and glutamine (Gln).

Threonine is a nutritionally essential amino acid for broiler chickens, and in diets based on corn and soybean meal, it is considered the third most limiting amino acid after methionine and lysine. Threonine participates in the synthesis of protein, and its catabolism generates many products important for metabolism, such as glycine, acetyl-CoA, and pyruvate [144]. Broiler chickens have a high Thr requirement for maintenance due to its rapid

turnover rate and high abundance in intestinal secretions [145], for instance, as part of the protein mucin [146]. Studies have indicated that the components of the immune system are responsive to manipulations in dietary Thr [139,147–149]. In addition to mucin production, Thr is a major component of immunoglobulins (Ig), particularly IgA, which is secreted by the intestinal mucosa and accounts for more than two-third of all immunoglobulins in the body [150]. In chickens, using an *ex vivo* explant, Zhang et al. [149] showed that Thr deprivation to the medium upregulated the expression of IL-8, MUC2, and IgA, which was reversed by Thr addition, and downregulated the expression of the inflammatory genes IFN- γ and IL-1 β [139]. As stated by Faure et al. [151], in pathological situations, the defense and repair will increase the demand for amino acids, specially Thr, and if the extra requirement for Thr is not met by the diet, muscle protein will be mobilized. Threonine may also modulate the intestinal immune system by modulating its microbiome [139,146,152]. The reduction of pathogens observed with higher dietary Thr concentrations may be due to its indirect effect on the host, as observed by upregulation of mRNA expression of MUC2, and downregulated IL-1 β expression [139].

Chickens lack key enzymes involved in the metabolism of Arg synthesis, which makes this amino acid of particular importance in poultry. The genetic material of birds does not encode for the enzyme carbamoyl phosphate synthetase which catalyzes the first step of ammonia detoxification involved in the production of citrulline from ornithine [153]. Citrulline can ultimately be converted to Arg through urea cycle enzymes, and as such, citrulline but not ornithine can spare dietary Arg in chickens [154]. Additionally, chickens lack the enzymes necessary for citrulline production in the small intestine [155]. In addition to its function as a protein constituent, Arg is a precursor for the synthesis of creatine, polyamines, and NO and stimulates secretion of insulin-like growth factors [156]. NO has several biological functions, but it primarily acts as a cytotoxic mediator of immune-activated cells and regulator of the immune system [157]. Depletion of Arg may be observed in coccidial-infected chickens, due to the high expression of inducible NO synthase (iNOS) in an attempt to limit the replication of *Eimeria* in the intestinal epithelia [136]. Indeed, a 40% reduction in dietary Arg reduced plasma NO concentrations in unchallenged birds but did not limit the increased NO production elicited by a coccidial infection, reflecting the high metabolic prioritization of Arg for NO synthesis during coccidiosis [158]. Additionally, Tan et al. [136] showed that coccidiosis downregulated the expression of MUC-2 and IgA, but upregulated β -Defensin-8, and inflammatory genes (iNOS, IL-1 β , IL-8, TLR4) mRNA expression. Meanwhile, Arg linearly diminished the

expression of TLR4, suggesting that the antiinflammatory effect of Arg is via suppression of TLR4 pathway, which was verified when the inflammation was stimulated by lipopolysaccharide [136].

Glutamine serves as an important source of energy for enterocytes, particularly during periods of increased proliferation. As such, Gln may reduce the intestinal atrophy and support mucosal repair following an insult. Gln is also a component of glutathione, a key molecule in the defense against free radicals [108,159,160]. Evidence suggests that the intestine competes with other organs for Gln, as the intestinal mucosa has a high capacity to remove Gln from both arterial blood and dietary supply [161]. Gln may be considered an essential amino acid under inflammatory conditions, disease challenge, or surgery [162]. Santos et al. [163] found that Gln reduced intestinal permeability and bacterial translocation to physiological levels and preserved mucosal integrity. In a later study, Santos et al. [164] reported that Gln plus NO synthase inhibitor increased bacterial translocation to mesenteric lymph nodes, liver, spleen, and lungs, but Gln alone reduced the translocation similarly to the control group. In addition, Gln increased levels of IL-10 in the serum and sIgA in the intestinal mucosa. These findings indicate that Gln improves the intestinal barrier and affects the intestinal bacteria community through its effect on NO synthesis.

Glutamine supplementation of 1% to the diet did not decrease *Salmonella* Typhimurium shedding in broiler chickens but improved BW gain [165] and antibody (IgA) production [166]. Oxford and Selvaraj [167] found that Gln downregulated the expression of IFN- γ and IL-10 and upregulated the expression of tight junction genes in the intestine of chickens induced to coccidiosis, which indicates the immunomodulatory role of Gln during stressful situations.

Microminerals

Microminerals have long been known for their influence on the growth performance, metabolism, and immunity of poultry flocks. For instance, zinc (Zn) and copper (Cu) are acknowledged to be important modulators of the mechanisms against infections, due to the antimicrobial effects, inflammation, and oxidative stress. Trace minerals supplementation is not new in poultry nutrition, but higher levels may be supplemented to maximize performance. On the other hand, concerns related to the increased excretion of these minerals into the environment are driving the industry to use more bioavailable sources of minerals, such as organic minerals, which would reduce the concentration of minerals added into the feed.

Zinc is an essential micromineral required for many biological functions, including growth, reproduction, meat

quality, and immune response against pathogens [168]. The dynamic distribution of Zn in the body of chickens changes during infections. As shown by Bortoluzzi et al. [169], the concentration of Zn in the serum decreased and increased in the liver 7 days after challenge with *Eimeria maxima*. Additionally, Zn concentration in the serum slightly increased in birds supplemented with 90 mg/kg of ZnSO₄ compared to nonsupplemented birds. Indeed, severity of growth depression has been observed to be lessened when supplemental Zn was increased to 85–90 mg Zn/kg diet [170,171]. Additionally, organic Zn induced higher expression of A20, an antiinflammatory regulator, downregulated the expression of inflammatory inducers, including NF-κB p65 [172,173], and promoted MUC2 and IgA production, when compared to its inorganic counterpart [172]. Epigenetic mechanisms alter gene expression without changes in DNA sequence and can explain the effects of Zn on the cell. It has also been demonstrated that organic Zn had a significant impact in lessening the inflammatory response in the intestine by downregulating the expression of pro-inflammatory cytokines, such as IL-8 and IFN-γ, TLR-2 and iNOS [169,174].

Copper is also an essential trace element in animals for exercising several important roles. The poultry industry has used prophylactic concentrations of dietary Cu for its ability to improve feed efficiency for a long time. One of the first reports of Cu supplementation having a growth-promoting effect was that of Mehring et al. [175], suggesting an effect similar to that of the antibiotics. High concentrations of Cu were originally thought to having benefits in prevention of crop mycosis, but addition of up to 250 mg of Cu/kg of diet results in increased erosion to the lining of the gizzard [176,177] and an “inhibition of normal fermentation” in the ceca of the chick [177]. A more recent study [178] has reported that high dietary supplementation of Cu (up to 330 mg/kg) led to reduced antioxidant capacity and higher oxidative stress in chickens. Additionally, it was shown that the excess of dietary Cu upregulated the mRNA levels of TNF-α, IFN-γ, IL-1, IL-1β, IL-2, iNOS, COX-2 and the concentrations of TNF-α, IL-1, IL-1β and the protein expression levels of TNF-α and IFN-γ in the spleen, thymus, and bursa of Fabricius. This suggests that excessive Cu can induce inflammatory responses and provides evidence of Cu toxicity mechanisms in broiler chickens [178]. On the other hand, lower dietary supplementation of Cu (20 mg/kg) reduced cholesterol and triglycerides, increased IL-6, IgA, and IgY, and the glutathione peroxidase activity in the serum of broilers. These results show that low dietary supplementation plays an important role in blood lipids, and immune and antioxidant defenses in chickens [179].

Manganese (Mn) is described as a trace mineral associated with better immunity, or to functions that support immunity [180], such as the synthesis of mucopolysaccharides

through its activation of glycosyltransferase [181]. It has been reported in broiler chickens that on day 6 postchallenge with *Eimeria acervulina* there was a reduction of Mn absorption by 23% and 34% in two consecutive studies, respectively [182]; however, there was an increase in Mn absorption compared to uninfected control birds by day 10 after challenge, and then it returned to normal rates as the unchallenged birds. Although there is a lack of studies relating Mn supplementation and its different presentation forms on the immune response against enteric diseases in poultry, one can assume that Mn is beneficial during enteric challenges due to its role in the production of mucopolysaccharides. Burin Junior et al. [183] have shown that birds fed organic Mn had a more efficient response against a *S. Enteritidis* vaccine when compared to birds fed its inorganic counterpart. Zhang et al. [184] found that the supplementation of 50 or 100 mg/kg of Mn to broilers under *S. Typhimurium* reduced the intestinal permeability caused by the challenge. Unlike the effects observed in *Salmonella*-treated birds, serum inflammatory cytokines IL-1, IL-6, and TNF-α were not altered by Mn itself. The authors speculate that dietary Mn promotes inflammatory response in the spleen that plays an important role for the fight against *S. Typhimurium* challenge for broilers [184].

15.3.3 Immunometabolism

Cellular metabolism is a collective phrase to describe how cells generate the energy and metabolites required for cell growth and maintenance. Immunometabolism refers to the central role that metabolism plays in shaping the development, differentiation, and function of immune cells [185,186]. The foundation of immunometabolism is the assertion that the engagement of specific metabolic pathways, the production or utilization of specific metabolites, or the noncanonical functions of metabolic enzymes can be decisive in the balance between inflammation, immune tolerance, or resolution.

The immune system provides defense against pathogens and functions to maintain tissue homeostasis for the life of the host by continually sensing and responding to environmental threats. However, this process carries a considerable bioenergetic cost. For instance, the sensing of a pathogen or environmental insult results in secretion of cytokines, chemokines, and inflammatory mediators by the innate immune cells, and the clonal expansion of adaptive immune cells. Since immune cells lack significant stores of nutrients, these effector responses can only be sustained if immune cells can dramatically increase the uptake of glucose, amino acids, and fatty acids from their microenvironment that requires the strict control of cellular metabolic pathways. Collectively, the metabolic pathways of oxidative metabolism, glycolysis, and glutaminolysis, preferentially fuel the cell fate decisions and effector functions of

immune cells [185]. For example, an intricate relationship exists between macrophages and host metabolism [187]. A pro-inflammatory phenotype of M1 macrophages is characterized by an increase in aerobic glycolysis in chickens [188–191]. Alternatively, the antiinflammatory phenotype of M2 macrophages and T regulatory cells is characterized by oxidative phosphorylation-mediated metabolism [190,191].

Within poultry production, a consideration of the immuno-metabolic consequences would be invaluable. A focus solely on maximizing animal growth can reduce immune potential, while a strong immune response has negative consequences on growth [132]. A consideration of the integrated whole may allow one to maximize growth and animal production without having a detrimental impact on animal health and immune potential. We have shown the nearly innumerable links between cellular signaling proteins classically characterized as members of either the immune or metabolic functional groups [192,193]. Further insights into how metabolism fuels and regulates the maturation of immune responses and understanding of the metabolic checkpoints that control these transitions might provide new methods for modulating immunity in infection using diet and nutrition. However, understanding how the nutrient supply in the chicken can influence the metabolism and function of immune cells in diverse tissues such as the intestine and lung is a major challenge for utilizing immunometabolism as a means of modulating the avian immune response.

15.4 Assessment of immunocompetence

There are a variety of approaches available to evaluate the status and functional capacity of the avian immune system [194,195]. Regrettably, there is no single definitive marker of either status or functional capacity of the avian immune system. The activity of most elements of the immune system can be measured separately, typically by scrutinizing that element of interest under controlled *ex vivo* conditions. An immune response can be assessed *in vivo* during a controlled challenge (toxin, vaccination, infection). In avian studies, these functional immune activities can be investigated by isolating and differentiating cells from the blood, thymus, spleen, gut-associated lymphoid tissue, and the bone marrow [144,196–201].

Under most conditions, circulating cell numbers, their activation state, and their responses to *ex vivo* challenge can be measured. However, it is imperative to realize normal ranges have not been established for circulating immune cell numbers, circulating immunoglobulin concentrations, or for immune cell functional responses in poultry. Assessments of immune status have frequently involved the measurement of various leukocyte numbers in the bloodstream including the total number of

leukocytes (heterophils, monocytes, and lymphocytes) [202] and of the subclasses of leukocytes (T lymphocytes, B lymphocytes, CD4⁺ cells, CD8⁺ cells) in the blood can be determined using immunological staining procedures and analytical techniques such as flow cytometry [203–205]. The absolute number of CD4⁺ (T helper) to CD8⁺ (T cytotoxic) cells and the ratio of CD4⁺ to CD8⁺ cells are often reported as a measure of immune responsiveness; however, without functional analysis of these T cell subpopulations, the true meaning of this ratio as a measure of immunity is unclear.

15.4.1 Functional activity of the immune response

Assessments of the functional capacity of the immune response can be made by measuring specific cell functions *ex vivo* or measuring *in vivo* responses to challenge combined with measuring the incidence and severity of infections such as pathogen shedding or host survival.

Ex vivo measures

Ex vivo measures allow the functional responses of specific immune cell types to be determined. The following *ex vivo* measurements are possible: phagocytosis, generation of an oxidative burst, microbicidal killing, and chemotaxis by monocytes and heterophils; natural killer cell activity; T and B lymphocyte proliferation; total and antigen-specific immunoglobulin (Ig) production; cell surface molecule expression; and cytokine mRNA transcription and/or cytokine production by lymphocytes, macrophages, monocytes, dendritic cells, and heterophils.

In vivo measures

In vivo measurements of immune functional activities include circulating total Ig and Ig subclasses concentration levels; circulating antigen-specific Ig concentration levels; fecal IgA concentration; and plasma cytokine concentrations.

Regardless, of what measures are made of immune function, it must be remembered that the responses being measured are dynamic in nature; thus, the response measured may be different at different time points. Furthermore, different responses may follow different time courses. Both issues must be kept in mind when evaluating the functional immune responsiveness to infectious or environmental (physiological stressors, dietary changes) stimuli.

The sequencing of the chicken genome has enabled a greater understanding of the genes and molecules involved in the avian immune response and, thus, access to new tools required to understand the biology of that response in far greater detail. The advancement of high-throughput technologies, together with the extensive

identification of new genes, proteins, and other biomolecules in the “omics” era, has facilitated large-scale biological measurements of the avian immune response. Methodologies to study the impact of nutrition and diseases on the immune system development of chickens have been developed, including molecular and genomic technologies such as RNA sequencing, proteomics, transcriptomics, metabolomics, and kinomics. For example, utilizing transcriptomics allows for the identification of the level of mRNA transcripts in response to different stimuli, while proteomics facilitates the study of proteins and posttranslational modifications under specific conditions, and metabolomics to study either endogenous or exogenous metabolites [206]. Kinome analysis using peptide arrays provides site-specific information, displays similar biochemical properties to the full-length protein, and provides a means for defining phosphorylation-mediated events [207]. Phosphorylation is the predominant mechanism of posttranslational modification regulating protein function, has a central role in virtually every cellular event, is essential for all cell signaling networks, and regulates fundamental biological processes [208,209]. Global analysis of the kinome provides information on the abundance, activity, substrate specificity, phosphorylation pattern, and mutational status of a given peptide [210], and chicken-specific kinome arrays are available [35]. Therefore, these technologies will drive a deeper understanding of the functioning of the immune system in avian species and how it can respond to different stimuli.

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Autoimmune diseases of poultry

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16.1 General characteristics of autoimmune diseases

The immune system has developed many effective ways to protect an individual from environmental insults and disease. Adaptive (specific) immunity will focus these defensive efforts very specifically on a given antigen to remove and/or destroy it. However, when this specific response is directed against self-antigen, the result is autoimmune disease. Autoimmune disease is defined as a disease caused by a breakdown of self-tolerance such that the adaptive immune system responds to a self (autologous)-antigen and causes cell and tissue damage.

Tolerance to self-antigens is normally maintained by selective processes that prevent the maturation of self-antigen-specific lymphocytes or suppress self-reactive mature lymphocytes that entered the periphery. Loss of self-tolerance may result from abnormal selection or regulation of self-reactive lymphocytes, as well as from abnormalities in the way self-antigens are modified, processed, and/or presented to the immune system.

Self-tolerance is primarily inherent in the T cell compartment. This is due in part to the rigorous selection processes T cells encounter during maturation in the thymus, the important regulatory function of T cells in adaptive immune responses, and the restriction of T cells to recognition of antigenic peptides in association with self-MHC molecules. Failures of self-tolerance within the T cell compartment can result in autoimmune diseases in which the autoimmune lesion is caused by cell-mediated and/or humoral immune responses.

Autoimmune diseases are typically multifactorial, requiring several components for expression, such as genetic susceptibility, immunological responses, and environmental factors. Unfortunately, the relative contribution of these factors to the development of autoimmune disease is not clear-cut and cannot be easily dissected. In organ-specific autoimmune diseases, genetic susceptibility is

frequently associated with an inherent target cell defect that predisposes the cell to immune recognition, and may include aberrant immunological activity at various levels (e.g., dendritic cells/macrophages, B cells, and T cells). The autoimmune destruction of cells is associated with a lack of regulatory function within the immune system, aberrant innate and adaptive immune activity, and altered responsiveness of immune components to self-antigens, especially if the production of targeted-self antigen is site- or development-specific. The role of environmental factors in the loss of specific self-tolerance and development of autoimmune disease is also multifaceted. Triggers may include exogenous factors, such as microbial infections and exposure to chemicals, and/or endogenous factors, ranging from altered activities of various physiological systems to intracellular molecular pathways.

The consequences of having adaptive immune mechanisms specifically focused on a self-antigen are clearly destructive. It is puzzling; however, that autoimmune attacks against components such as the myelin sheath in multiple sclerosis, pigment cells in vitiligo, and thyroid cells in autoimmune thyroiditis do not necessarily destroy all of these target cells, as would be implied by the specificity of the recognition. Rather, cell and tissue destruction tend to be progressive, suggesting an important interplay between the target tissue and the antigens it displays, immune recognition, and immunoregulation.

Another interesting aspect of autoimmune disease is the association of one disease with other autoimmune diseases, the so-called kaleidoscope of autoimmune disease [1]. For example, roughly 30% of patients with autoimmune vitiligo will also express thyroid disease or one of a multitude of other organ-specific autoimmune disorders that have been associated with vitiligo, albeit with a lower association incidence [2,3]. Understanding the interrelationships between the multiple factors leading to autoimmune disease expression and expression of associated disorders is important in the prevention and treatment of autoimmune disease.

The complex nature of multifactorial, noncommunicable diseases poses a challenge for poultry breeders because, in the absence of environmental triggers, disease susceptibility often is not apparent and may be coselected with desirable production traits. This may have devastating consequences should commercial conditions favor disease expression. Although autoimmune diseases do not appear to be of widespread concern in poultry production, one may ask whether autoimmune mechanisms may play a role in the observed noncommunicable disorders associated with high-intensity poultry production (e.g., leg, endocrine, neuronal, metabolic, integumental, pulmonary, alimentary tract and reproduction-related problems).

In fact, an immune system component has been implicated in complex, noncommunicable diseases in poultry such as wooden breast [4–7], idiopathic pulmonary arterial hypertension (ascites) [8], lameness [9], ovarian autoimmune disease [10], epididymal lithiasis in roosters [11], and Chagas-like heart disease [12]. Thus, poultry breeding programs will need to pay attention to a wide variety of complex, noncommunicable diseases that may appear in certain flocks, locations, and environmental conditions, and may be a reflection of inherent weakness in target cells not evident until the appropriate environmental factors precipitate disease expression. For this reason, breeding effectiveness may best be assessed in ideal as well as challenging conditions to gain insight into susceptibility of poultry to complex disease.

Autoimmunity in humans also is on the rise, particularly in developed countries, with estimates of 5%–8% of the U.S. population affected, and 80–100 different autoimmune diseases identified [13,14]. Over the years, the chicken has made significant contributions to the understanding of the components and mechanisms involved in autoimmune diseases. For example, the Smyth-line (SL) chicken is the only animal model for the pigmentation disorder vitiligo, manifesting all clinical and biological symptoms of the human disease (Figs. 16.1–16.3). The Obese-strain (OS) chicken (Fig. 16.4) is one of the most valued and best models for spontaneously occurring Hashimoto's thyroiditis. Finally, the University of California, Davis (UCD) 200/206 chicken lines are the only model for spontaneously occurring scleroderma/systemic sclerosis (SSc) that presents the combination of symptoms observed in humans (Figs. 16.5 and 16.6). It should be noted that the type and combination of autoimmune disease represented in these avian lines are among the most prevalent autoimmune diseases in the United States population based on 2016 estimates [14]. Specifically, Hashimoto's thyroiditis in the OS strain is the second most prevalent autoimmune disease, affecting 792 individuals per 100,000 people; vitiligo in the SL model is the sixth most prevalent and scleroderma/SSc in the UCD 200/206 lines is in 16th place, with,



FIGURE 16.1 Chicks from SL and parental control BL showing that pigment loss is a posthatch phenomenon. BL, Brown line; SL, Smyth line.



FIGURE 16.2 BL rooster showing normal plumage colors. BL, Brown line.



FIGURE 16.3 Young SL roosters showing pigmentation loss. SL, Smyth line.



FIGURE 16.4 Example of CS (euthyroid) and OS (hypothyroid) chicken. OS, Obese strain.

respectively, 400 and 24 per 100,000 people affected. Moreover, in SL chickens, associated autoimmune diseases include Hashimoto's thyroiditis and alopecia areata, the second and ninth most prevalent autoimmune diseases observed in humans [14].

While the medical research community prefers the mouse model, mouse strains with spontaneously occurring autoimmune disease are rare. Induced mouse models for autoimmune disease can be developed rapidly using experimental approaches to break tolerance (e.g., by hyperimmunization, genetic engineering of transgenic mice with self-antigen specific T cells, adoptive transfer of transgenic T cells or antibodies, and various combinations thereof) [15]. These disease models are, however, missing the underlying, complex disease susceptibility and the factors involved in natural disease expression.



FIGURE 16.5 Gradual necrosis of comb in UCD 200 scleroderma.



FIGURE 16.6 Skin lesion in UCD 200 scleroderma/systemic sclerosis.

Similarly, global gene-sequencing and -expression studies using samples from human populations with autoimmune diseases [2,3] yielded important insights and potential biomarkers for disease susceptibility. But as disease was already present at the time of detection, a direct connection to disease etiology is not possible.

The hidden susceptibility to autoimmune disease also is becoming a major concern in cancer immunotherapy, where attack of the tumor cells with cell-specific or non-specifically activated T cells may lead to target cell-specific autoimmunity in some patients; i.e., autoimmune disease susceptible patients [16,17]. Hence, the avian models do fill a unique niche as an important intermediate biomedical model with spontaneously, naturally occurring autoimmune disease. Inclusion of the avian models offers critical insights into genetic susceptibility, the role of epigenetic, immunological, and environmental factors in disease onset and progression, as well as the development of effective treatment and prevention strategies.

This chapter will describe the characteristics of the autoimmune disorders in SL, OS, and UCD 200/206 lines of chickens and highlight the many scientific contributions these research lines made to our understanding of the cause–effect relationship between a genetically controlled disease,

immune function, and environmental factors. Emphasis also will be on the opportunities that these lines continue to provide in advancing knowledge regarding complex diseases, as well as on the importance of preserving these genetic research lines for the scientific community. It should be noted that the only known remaining breeding populations of these lines are currently maintained at the University of Arkansas, with OS and UCD 200/206 imported in 2015.

In order to update current genetic information on the remaining SL, OS, and UCD 200/206 lines, their original development more than 50 years ago will only briefly be addressed in this edition, but for each line (Sections 16.2–16.4) the interested reader will be referred to comprehensive reviews in the literature.

16.2 Autoimmune vitiligo in Smyth-line chickens

16.2.1 Introduction

Vitiligo is a common dermatological disorder affecting at least 1% of the world's population. It is characterized by postnatal, autoimmune destruction of melanocytes in the skin, generally resulting in patches of depigmentation and, in some individuals, complete depigmentation. Additionally, there is a recognized association between autoimmune vitiligo and a variety of other autoimmune diseases, and the cosmetic disfigurement resulting from this condition leads to psychosocial effects that are particularly severe in the young and in people with dark skin [2,3,18].

The mutant SL chicken is an important animal model for autoimmune vitiligo. Chickens from this line develop a spontaneous, vitiligo-like, posthatch loss of melanocytes in feather and ocular tissue (choroids). The incidence of SL vitiligo (SLV) within a population of SL chickens is typically 80%–95%, with the majority developing SLV at 6–16 weeks of age. Studies examining the basic defects manifested in the SL melanocyte described the presence of a competent pigment system at hatch (Fig. 16.1). Prior to visible signs of SLV, the earliest abnormality detected in SL melanocytes are irregularly shaped melanosomes containing pigmented membrane extensions, hyperactive melanization, and selective autophagocytosis of melanosomes. These aberrant processes precede the degeneration of SL melanocytes but are not sufficient to cause vitiligo without a functioning immune system. They do, however, appear to be involved in provoking an immune response resulting in autoimmune destruction of melanocytes. Loss of melanocytes in the feather is associated with infiltration of lymphocytes and cell-mediated immune activity. Lastly, an environmental component (i.e., herpesvirus of turkeys, or HVT) also appears to play a role in the development of this disorder in SL chickens [15,19,20].

SL chickens are unique for studies on the etiopathology of autoimmune diseases, in part because the target tissue (feathers) is easily accessible and regenerates (allowing for repeated sampling in the same individual throughout SLV development), the incidence of the disorder is highly predictable and occurs at a young age, and onset and progression of the disease can be visually monitored. Moreover, this avian model for vitiligo offers a unique opportunity to study autoimmune diseases in the context of genetic susceptibility and environmental influences.

16.2.2 The Smyth line chicken model for autoimmune vitiligo

The mutant SL chicken (previously known as the delayed amelanosis chicken) was developed by Dr. J. Robert Smyth Jr. at the University of Massachusetts, Amherst, starting with one vitiliginous female hatched in 1971 from the Massachusetts Brown line (BL). The selection and breeding strategies employed by Dr. Smyth to establish various SL and control sublines are comprehensively described in several reviews [19,21–23].

Unfortunately, most of these valuable lines no longer exist following the closure of the Poultry Farm at the University of Massachusetts and the retirement of Dr. Smyth. The lines homozygous for the major histocompatibility complex (MHC; B-locus, see Chapter 7) haplotype B101 are the only lines remaining and are currently maintained by the author at the University of Arkansas, Division of Agriculture, Fayetteville (UA). Specifically, in 1996, three MHC-matched (B101) lines were imported from Massachusetts to Arkansas; these were the vitiligo-prone SL101, the parental BL101, and the normally pigmented Light-brown Leghorn (LBL101). Together, the SL, BL, and LBL chickens constitute the current animal model for autoimmune vitiligo.

Within this animal model, the SL is considered vitiligo-susceptible with most individuals expressing vitiligo; the BL is the parental control and is considered vitiligo-susceptible with a very low incidence of vitiligo (<2%); and the LBL line constitutes the vitiligo-resistant control. The genetic susceptibility/resistance of BL and LBL chickens to vitiligo, respectively, has been further underscored by their response to treatment with the DNA methylation inhibitor 5-azacytidine. Following treatment with 5-azacytidine, 71% of BL chickens developed vitiligo, whereas vitiligo was not observed in treated LBL chickens [24]. Although this treatment caused similar alterations in lymphocyte profiles in primary and secondary lymphoid organs in chickens from the three lines, the only vitiligo-associated effect of 5-azacytidine noted was the infiltration of lymphocytes into the feathers. The phenotypic characteristics of these feather-infiltrating

lymphocytes were similar to those observed in vitiliginous feathers from untreated SL chickens [25]. Hence, alterations in gene expression triggered by treatment with 5-azacytidine resulted in recruitment of lymphocytes to the target tissue, loss of self-tolerance to melanocytes, and development of an autoimmune response to melanocytes.

As reported in 2001, the SL and BL B101 sublines had already a high level of inbreeding within lines (0.948 ± 0.015 for SL; 0.902 ± 0.071 for BL) and high genetic similarity between SL101 and BL101 lines (similarity index: 0.949 ± 0.006 between the lines) [26]. The SL, BL, and LBL B101 populations in Arkansas were started with 20–40 hen families with 10–20 roosters, and although several bottlenecks were encountered for each of the lines during the past 25 years, every effort has been made during pedigree breeding to avoid pairing males and females that share a great-grandparent and to match 25–30 hens with 14–20 roosters. Fertility remains excellent, although SL hens come into egg production 3–4 weeks later than BL and LBL hens. Eggshells from SL with SLV tend to be whiter-color, dirtier, and of lower quality than the light-brown eggs from nonvitiliginous SL, BL, and LBL hens.

The genetic basis of autoimmune vitiligo and line-associated traits have long been described as being under the control of multiple autosomal genes [21]. High-resolution quantitative trait loci (QTL) mapping carried out at Uppsala University on F2 offspring from SLxBL crosses led to the identification of three candidate gene loci [27], including one on chromosome 12 with high association. Next-generation whole-genome resequencing of SL chickens with SLV and nonvitiliginous BL chickens was carried out by Jang et al. [28]. Alignment of sequence data to the chicken reference genome sequence of Red Jungle Fowl identified various potential genetic markers with amino acid changes that may play a role in SLV development. Bioinformatic analysis of the genomic data identified SNPs for genes involved in dermatological disorders, including melanoma and vitiligo. Moreover, intermolecular gene network analysis revealed that the candidate genes identified in SLV chickens play a role in networks centered on protein kinases, phosphatase, ubiquitinylation, and amyloid production, some of which were also identified in the analysis of genome-wide association studies in vitiligo patients [2,3]. Hence, similar gene mutations may be underlying vitiligo susceptibility in SL chickens and humans, underscoring the relevance of the SL animal model for biomedical research.

In retrospect, comparisons to the parental BL, rather than a nonvitiligo susceptible, pigmented population (e.g., LBL or Red Jungle Fowl), may not have been the best approach for the genetic studies, as both SL and BL are vitiligo-susceptible. However, the shared genetic background and disease susceptibility in SL and BL offer a

unique opportunity to study epigenetic regulation in the context of vitiligo susceptibility and expression, vitiligo susceptibility without expression, and vitiligo resistance. Considering the minimally invasive access to the target tissue, activities in GF samples collected prior to and throughout SLV can be compared to age-matched samples from nonvitiliginous SL, BL, and LBL chickens.

16.2.3 Characteristics of the Smyth-line chicken

Without experimental manipulation, using standard rearing protocols, 80%–95% of the Arkansas SL chickens spontaneously express the vitiligo-like, posthatch loss of melanin-producing pigment cells (melanocytes) in feather and choroidal tissue. Destruction of melanocytes, which usually is first seen during adolescence and early adulthood in humans, occurs in SL chickens at 6–16 weeks of age but may develop as early as 4 weeks or as late as 18–20 weeks (Figs. 16.1–16.3). Because growing/regenerating feathers are rare in mature chickens, 20 weeks of age is considered a natural endpoint for SLV studies. In both humans and SL chickens, amelanosis may be either partial or complete and remelanization of amelanotic tissue may occur, although complete pigment loss, as well as phases of repigmentation, are more common in the chicken. Little or no gender preference is reported for vitiligo in humans and SL chickens; however, a greater incidence of early onset has been observed in SL females compared to males [15].

In addition to SLV, SL chickens exhibit uveitis (often resulting in blindness), hypothyroidism (Fig. 16.7), and an alopecia areata-like feathering defect. Similarly, in humans it is not uncommon to find thyroidal, ocular, and integumental defects associated with vitiligo [2,3]. In the SL chicken, the incidence of these associated autoimmune diseases can vary greatly from hatch to hatch and can be increased by selection. In the last 5–10 years, the incidence of uveitis has been high, with 10%–20% of



FIGURE 16.7 Hypothyroidism also occurs in SL but is independent of the development of vitiligo. SL, Smyth line.

vitiliginous individuals exhibiting signs of visual impairment or blindness. The incidence of hypothyroidism in growing SL chickens ranged between 2% and 6% and occurred with or without SLV (Fig. 16.7) [19]. Interestingly, examination of thyroid tissue from reproductively active SL hens without overt clinical symptoms of hypothyroidism revealed mononuclear cell infiltration into the thyroid suggesting onset of thyroid autoimmunity in developed adults [29]. Similarly, the alopecia areata-like feathering defect described by Smyth [19] was thought to be lost in the Arkansas populations, but in recent years reemerged in young adult vitiliginous SL females (20%–30%), with varying degrees of severity. Lastly, observations by Dr. Smyth and the author suggest that the sex-linked homozygous recessive dermal shank pigmentation trait (*id* + ; gray shanks) may suppress the expression of SLV. Selection for this trait continues to be included in the pedigree breeding of SL chickens.

16.2.4 Pigmentation and normal melanocyte function

In most breeds of chickens with pigmented plumage, the epidermis of the skin is essentially devoid of melanin and melanocytes. However, melanin is found in other integumental tissues, most notably in feathers, and may be present in the beak and scales of the shanks. The melanin-producing cells, destined for integumental or eye tissues (e.g., the choroid and the anterior surface of the iris), originate in the embryonic neural crest, whereas the iridial and retinal pigment epithelia originate from the outer layer of the optic cup. Undifferentiated melanoblasts from the neural crest migrate early in embryonic development (about 90 hours). Those migrating to the feathers populate stem cell reservoirs located near the base and/or in the collar bulge epithelium of the feather follicles [30]. In growing feathers (GFs), melanocyte precursors proliferate, differentiate into melanin-producing melanocytes, and populate the barb ridge (modified epidermis), where they transfer pigment to columns of keratinocytes (barbule cells).

Production of melanin (melanogenesis) by melanocytes is similar in birds and mammals, involves key enzymes, such as tyrosinase, tyrosinase-related protein-1 (Tyrp1), and dopachrome tautomerase (Dct), and occurs in membrane-bound organelles called melanosomes. Once situated in the barb ridge, the cell bodies of melanin-producing epidermal melanocytes can be observed aligned with the interface between the dermis of the feather pulp and the barb ridge, whereas their dendrites extend along the barb ridge barbule cells (Figs. 16.8 and 16.9). Pigment (eumelanin and pheomelanin) containing melanosomes are then transferred from the melanocyte dendrites to the barbule cells. As the feather grows and pigment is



FIGURE 16.8 Regenerating (growing) feathers are evaluated by a scoring system and can be used for tissue collection.

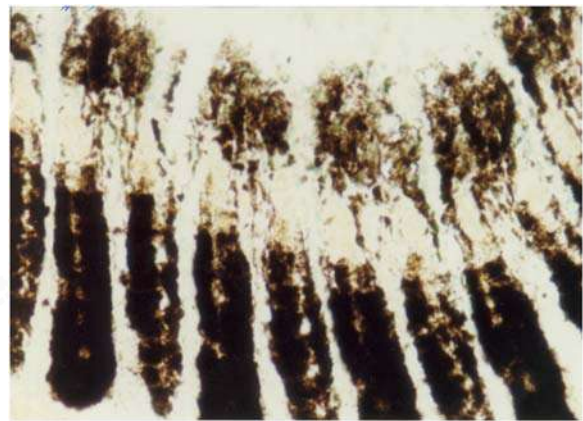


FIGURE 16.9 Cross-section of feather barb ridge with normal pigmentation; melanocyte cell bodies and dendrites are visible, as is the pigment that has been deposited in the keratinocytes.

deposited in the barbule cells, the melanocytes degenerate and are phagocytosed by keratinocytes (reviewed in Ref. [31]). The barbule cells become keratinized to form the barbs of the feather. The epidermal layer that extends beyond the ramogenic zone does not contain melanocytes and envelopes the entire dermis of the pulp.

16.2.5 Target cell defects

Although SL chicks hatch with a competent pigment system [19], ultrastructural studies conducted by Boissy et al. [32] revealed that after hatching, regenerating feather melanocytes produced abnormal melanosomes in SL chickens compared to controls. The abnormal melanosomes had irregularly shaped surfaces containing pigmented extensions that were continuous with the outer rim of the melanosome and appeared to be delimiting an electron-lucent region. Based on histochemical analysis of feather tissue, SL melanocytes have an aberrant and widespread distribution of tyrosinase compared to controls,

suggestive of a hyperactive process of melanization in SL melanocytes.

In the feather epithelial barb ridge, one of the earliest manifestations of SLV detected by light microscopy was the appearance of histologically abnormal melanocytes. These melanocytes had thickened, partially retracted dendrites and an irregular shape. Pigment cell transfer from melanocyte dendrites to barbule cells was reduced at this stage. More advanced stages were represented by marked clumping or the absence of melanocytes and further reduction in pigment transfer. Intracellular changes correlating with these abnormal melanocytes included fewer pigmented extensions on melanosomes and melanosome aggregation. Eventually, aggregated melanosomes were inside a single large autophagocytic complex, presumably trying to contain the cytotoxic melanin precursors. Once autophagocytosis was initiated, melanogenesis effectively stopped, as indicated by the disappearance of tyrosinase activity and the concurrent increase of acid phosphatase activity in the abnormal, compartmentalized SL melanocyte [33]. Amelanotic feather tissue that developed after this state was completely devoid of melanocytes [32]. Similar degenerative processes in melanocytes could also be observed in situ in immunosuppressed SL chickens and in vitro in neural crest-derived melanocytes from embryos of SL chickens [33,34].

One frequently reported difference between melanocytes derived from nonaffected skin of vitiligo patients and normal melanocytes is a heightened sensitivity of vitiligo melanocytes to oxidative stress due to an imbalance and/or a deficiency in their antioxidant system [35]. Considering the extensive generation of reactive oxygen species (ROS) during melanogenesis in both humans and chickens [36], a genetic defect in the antioxidant protection system of melanocytes may significantly contribute to the development and progression of the pathological lesion in autoimmune vitiligo. More recent studies conducted in our laboratory using SL, BL, and LBL embryo-derived melanocyte cultures and GF tissue revealed heightened ROS generation, oxidative damage, and altered redox status in SL melanocyte cultures and feather tissue compared to BL and LBL controls [37].

Additionally, we tested the response of SL and control melanocytes to in vitro exposure to 4-tertiary-butyl-phenol (4-TBP) [38], a phenolic compound known to trigger vitiligo in susceptible humans [39]. For this, GFs were collected from HVT-positive and HVT-negative SL and control chickens at 1, 4, 7, and 12 weeks of age. The melanocyte-containing portion of the feathers (bottom 3 mm) was exposed to 4-TBP in culture, and the generation of ROS was examined [40]. Compared to BL and LBL controls, ROS generation was significantly higher in feather tissue from vitiligo-prone SL chickens, independent of age and HVT status. (HVT is a known environmental

trigger of SLV expression; see Section 16.2.7). The same trends in 4-TBP-induced ROS generation were observed in cultured melanocytes derived from GFs or from the neural crest of 72-hour embryos. The heightened sensitivity of SL melanocytes to 4-TBP is in line with observations in melanocytes derived from nonlesional skin of human vitiligo patients, suggesting aberrant ability of SL melanocytes to cope with cellular stress [38].

Impaired ability of SL melanocytes to cope with oxidative stress also was demonstrated by Falcon [41] in feather-derived SL and BL melanocytes cultured in the presence of hydrogen peroxide (100 and 200 $\mu\text{g H}_2\text{O}_2/\text{mL}$). In this study, SL melanocytes exhibited not only altered mRNA expression of antioxidant-related genes but also increased expression of CD40 and Fas mRNA compared to BL melanocytes. Additionally, H_2O_2 treatment increased levels of MHC class II molecules and B7-1 and CXCL8 mRNA in both SL and BL melanocytes in a dose-dependent manner [41]. Collectively, these results further support the role of oxidative stress and cell-mediated immune mechanisms in SLV.

The reports described earlier clearly established the existence of inherent melanocyte defects in SLV. However, the inherent melanocyte abnormalities/sensitivities alone do not appear sufficient for the pathological progression of pigment cell degeneration and the appearance of vitiligo.

16.2.6 Immunological mechanisms

From the initial research into the etiopathology of SLV, it became evident that the immune system plays an important role in the loss of melanocytes. Histological studies of the lesion revealed a strong association of mononuclear cell infiltration and the degeneration of melanocytes. When melanocyte loss was complete, the mononuclear cells could no longer be observed in the affected feathers [32]. Additionally, the presence of melanocyte-specific autoantibodies was described in SLV chickens, as were differences in immune functions in SL chickens compared to controls. These differences included increased antibody production to T-dependent (sheep red blood cells or SRBC) and T-independent antigens (*Brucella abortus*) [42], lower graft-versus-host responses and wing web swelling in response to phytohemagglutinin injection [43], lower in vitro proliferative responses to stimulation with concanavalin A [44], and altered blood lymphocyte profiles in SL compared to BL controls [45]. It should be noted, however, that the differences in antibody responses to SRBC observed in the Lamont and Smyth [42] study were no longer observed when MHC sublines B101 and B102 were examined [46].

The importance of the immune system in melanocyte destruction in SLV was further demonstrated through

immunosuppression studies using neonatal bursectomy [47], inhibition of T cell activity via cyclosporin A [48,49], and suppression of inflammatory immune activity with corticosterone [50]. It was concluded that in the absence of a functional immune system, melanocyte abnormalities, although present in immunosuppressed individuals, were insufficient for the pathological progression of pigment cell degeneration and the appearance of SLV. Additionally, these studies demonstrated the involvement of both humoral and cell-mediated immunity (CMI) in SLV but attributed a more critical role to CMI.

Humoral immunity

Maternal melanocyte-specific autoantibodies can be detected in eggs and during the first week of life in chicks from vitiliginous hens. After this time, melanocyte-specific autoantibodies appear again in the peripheral circulation 1–2 weeks before the onset of SLV (Erf, unpublished observations). However, the contribution of these autoantibodies to the onset and progression of SLV has not been defined. The autoantibodies cross-react with mouse and human melanocytes, bind to melanocytes within tissues, and recognize antigens expressed in the cytoplasm and on the surface of melanocytes and melanoblasts [51]. Specifically, SL autoantibodies recognize mammalian Tyrp1 and, based on molecular studies, the avian homolog of TYRP1 [52]. Preliminary studies in our lab examining the specificity of these autoantibodies by 2-D gel electrophoresis and mass spectrometric analyses revealed recognition of members of the heat-shock protein families (i.e., HSP 70 and 90) [53]. These cellular stress-related proteins have also been implicated in human vitiligo in the initiation of the melanocyte-specific autoimmune response.

Cell-mediated immunity

At the light microscopy level, one of the earliest manifestations of SLV is the morphological alteration in feather and choroid melanocytes. These cytological changes in melanocytes were associated with large numbers of infiltrating mononuclear leukocytes (IML) into the pulp of GFs and choroid. When melanocyte destruction is complete, the number of IML in the feather pulp returns to the levels in the BL and LBL controls [32].

Over the years several studies conducted by Erf and collaborators examined immune system activities in the evolving autoimmune lesion. A time-course approach was used for all, owing to the easy, minimally invasive access to the target tissue (GF), with samples initially collected every 2 weeks for identification and localization of T cells in frozen cross-sections of the melanocyte containing portions of GF [54,55]. These studies were followed by temporal, qualitative, and quantitative assessment of mononuclear cells infiltration, targeted mRNA-based

cytokine gene expression analyses, and global gene expression analyses at the transcriptome level by microarray using the entire target tissue (GF pulp) collected weekly or twice per week starting as early as 1 day old and throughout SLV development (before onset, at onset, and during active and complete melanocyte destruction) [38,41,56–58].

Collectively, after aligning the data for each individual SL chicken with respect to its SLV onset time (0 days), the following picture on events occurring before and during SLV development in the evolving lesion is emerging [38,41,55–58]. T cell infiltration into the pulp of GF can be observed 3–4 weeks before visible signs of SLV onset (i.e., signs of depigmentation in the melanocyte-containing 3–4 mm portion of the newest growth of GF). T cell infiltration reached the highest levels (10- to 12-fold increase) near SLV onset, remained elevated throughout active SLV (4–8 weeks), and returned to near baseline levels when melanocyte destruction was complete. All three T cell receptor (TCR)-defined T cell subsets are represented in the infiltrate, with levels of $\alpha\beta 1$ T cells dominating, followed by $\alpha\beta 2$ T cells and $\gamma\delta$ T cells representing 70%, 20%, and 10% of T cells, respectively. An infiltration profile different from that of total T cells emerged for T helper cells (CD4⁺) and cytotoxic lymphocytes (CD8⁺). T helper cells reached peak levels 3–7 days before SLV onset, remained at peak levels for 3–7 days after SLV onset, and steadily declined thereafter; however, cytotoxic cells reached maximal infiltration levels within days after onset and remained elevated at high levels throughout the complete loss of melanocytes. At onset of SLV, the ratio between CD4⁺ and CD8⁺ lymphocytes in the feather pulp was near 1.0 and decreased to below 0.4 because of increasing presence of CD8⁺ cells as melanocyte destruction progressed. T cell infiltration was accompanied by a sharp and short-lived increase in B cells (Bu-1⁺, IgM⁺) 3 days before onset, reaching peak levels 3–7 days after onset and declined rapidly thereafter.

In the tissue, infiltrating lymphocytes can be seen in perivascular aggregates and, as SLV develops, dispersed throughout the dermis and in the barb ridge. During the active phase of vitiligo, CD8⁺ cells can be observed in close physical association with apoptotic epidermal melanocytes (Figs. 16.9 and 16.10) [42,55]. Additionally, the amount of cells expressing MHC class II in the pulp increased with SLV onset, primarily due to increased numbers of B cells and macrophages, but also MHC class II⁺ T cells. In tissue sections, MHC class II-staining was also localized to venules (likely endothelial cells) involved in transmigration of leukocytes, and within the barb ridge. Although melanocytes and keratinocytes may express MHC class II in chronic inflammatory situations like vitiligo, the MHC class II staining in the barb ridge could not clearly be attributed to these cells [54–56].

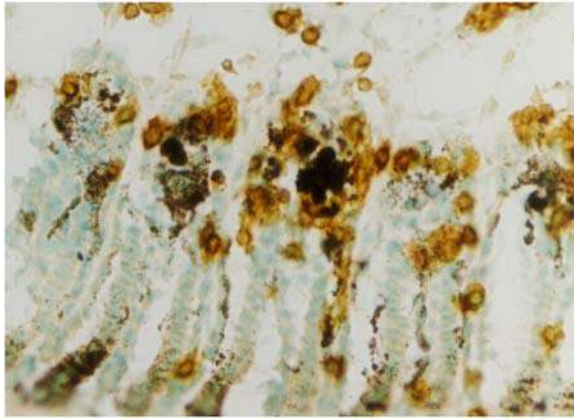


FIGURE 16.10 Cross-section of a barb ridge from a vitiliginous feather; brown cells are CD8⁺ lymphocytes associated with melanocyte cell bodies that are mostly destroyed; only a little pigment deposition has occurred.

A T helper-type 1 (Th₁), interferon (IFN)- γ polarized cell-mediated response in SLV was confirmed by Northern blot, Western blot, and quantitative real-time RT-PCR analyses. Expression of IFN- γ paralleled increases in T cell infiltration, reaching maximal levels at SLV onset and remained at high levels throughout active melanocytes destruction [38,41,57,59]. IFN- γ expression is likely due to effector T helper cells and cytotoxic T cells. Cytotoxic T cell effector activity is further supported by increased expression of granzyme A and Fas-ligand before and throughout active SLV [41]. The expression of the signature cytokine IFN- γ was accompanied by high expression of IL-21 and IL-21 receptor and other T cell-activation/pro-inflammatory cytokines (IL-1, IL-6, CXCL8), but also by antiinflammatory activity as indicated by high IL-10 and CTLA-4 mRNA expression. The strong association of IL-21 and IL-21R with SLV onset is particularly interesting in view of the implication of IL-21 in a number of autoimmune diseases [60,61], and its role in supporting cytotoxic T cell effector function.

Leukocyte- and cytokine-profiles like those reported earlier for SLV development in GF also were observed in eyes from SL chickens with impaired vision or blindness due to autoimmune attack of choroidal melanocytes. Autoimmune activity was not observed in eyes from normally sighted, nonvitiliginous SL and controls, but lymphocyte infiltration and abnormal morphology of choroidal melanocytes could be observed to varying degrees in eyes collected from apparently normal sighted SL chickens with SLV (manuscript in preparation).

The observed leukocyte infiltration in SLV feathers was accompanied by alterations in the proportions and numbers of circulating leukocytes and leukocytes present in dermal lymphoid aggregates in the skin. These alterations were, however, qualitatively different from those observed in the feather lesions [45,62].

Melanocyte-specific CMI in SLV has been demonstrated *in vivo* using the wattle-swelling response, and more recently, using the GF as the cutaneous test site [41,63]. The wattle-swelling response studies demonstrated the presence of melanocyte-specific CMI in SLV chickens but not in nonvitiliginous SL or control chickens [64]. Curiously, this melanocyte-specific CMI in vitiliginous SL chickens was directed only against feather-derived melanocytes, not embryo-derived melanocytes, suggesting different antigenicity of melanocytes that differentiated in GF rather than a controlled culture environment. More recently, Falcon [41] examined temporal, qualitative, and quantitative aspects of the recall/memory response to melanocytes injected into GF. For this, feather-derived melanocytes or vehicles were injected into the dermis of completely depigmented GF (no autoimmune activity) of SL chickens with vitiligo and GF of pigmented controls. Injection of multiple GF in a chicken at the same time, and periodic sampling of GF over 7 days, enabled monitoring of *in vivo* activities by *ex vivo* cell population and gene-expression analyses. Injection of melanocytes in SL chickens resulted in lymphocyte infiltration- and gene-expression-profiles similar to those described during the primary responses in the evolving SLV autoimmune lesion, except the responses occurred very quickly, reaching maximal levels within 6–24 hours postinjection [41]. The immune activities observed following melanocyte-injection into GF of nonvitiliginous controls were transient and not different from vehicle-injection controls. The memory phenotype of the recall response observed in SLV chickens further support Th₁ polarized CMI to melanocytes in chickens with SLV.

Lastly, the complex nature of autoimmune SLV was confirmed by differential gene-expression analysis at the transcriptome level using a 44K microarray [58]. For this study, gene expression in BL feather samples was compared to GF samples collected from SL birds that did not develop vitiligo and from SLV birds at various stages of SLV development (within 2 weeks before visible SLV onset, during active SLV and 2 weeks after complete depigmentation). Functional and network analysis of differently expressed genes highlighted innate and adaptive immunity (both cell-mediated and humoral), as well as neuronal involvement, apoptosis, cellular stress, and melanocyte function [58].

16.2.7 Environmental factors

Evidence for the role of an environmental factor in the expression of SLV in susceptible SL chickens is based on the observation that the incidence of SLV in chickens hatched and raised in isolation at the University of Arkansas Poultry Health Laboratory (PHL, biosecurity level 2) was only 10% by 20 weeks of age (instead of 80%–95%

incidence). This observation was followed by several studies that strongly indicated the presence of HVT as a major “environmental” factor in the expression of SLV [20]. HVT is an alphaherpesvirus commonly used as a commercial vaccine to protect chickens from Marek’s disease caused by serotype 1 Marek’s disease herpesvirus (MDV). Serotype 1 MDV are acute-transforming, cell-associated viruses causing T cell lymphomas, paralysis, and numerous mononuclear cell infiltration-associated lesions (see Chapter 17).

On the other hand, HVT is a nononcogenic serotype 3 MDV isolated from turkeys. In chickens, HVT causes only minor inflammatory lesions characterized by diffuse, light to moderate infiltration by small lymphocytes [65]. Like MDV, HVT exhibits a strong tropism for feather follicles, where it can be detected in its latent stage, at 21–105 days of age [66]; other ages have not been examined. A strong link between HVT and the expression of SLV is further supported by our findings that only vaccination with live, not dead (glutaraldehyde-treated), HVT can trigger the expression of SLV, and by the earlier (day 3 post-HVT vaccination at hatch) and higher immune cell infiltration in skin and feathers in HVT-vaccinated SL compared to HVT-vaccinated BL controls.

Ongoing research confirmed that serotype 1 and 2 MDV are similarly able to trigger the onset of SLV in susceptible SL chickens, that viral DNA levels in SL feathers increase 1–2 weeks prior to SLV onset, and that viral DNA cannot be detected in melanocytes isolated from GFs of HVT-vaccinated chickens. We also established housing conditions that reliably result in low or high SLV incidence. Specifically, a 10% incidence of SLV can be obtained when SL chickens are not vaccinated with live HVT at hatch and are raised in floor pens in a HEPA-filtered isolation room (Erf et al., unpublished).

In contrast, as many as 80%–95% of SL chicks vaccinated with live HVT at hatch and raised on the University of Arkansas Poultry Farm will develop SLV. The ability to greatly reduce SLV incidence offers a unique opportunity to study the effects of other environmental triggers on the etiology of SLV. Examination of the effect of HVT administration at different ages posthatch in SL chicks without vitiligo revealed a stepwise reduction in SLV incidence from 92% to 33% when HVT was administered at hatch compared to 6 weeks of age. HVT administered at 10 weeks of age and onward no longer precipitated SLV expression [38]. Studies are currently underway to further examine the cause–effect relationship between HVT infection and SLV expression.

Considering the tropism of HVT for the feather, it is likely that in newly hatched SL chicks the local, possibly aberrant anti-HVT immune response alters the feather environment. It does so in such a way that the already inherently defective, stressed, potentially immunologically active melanocytes become visible to the immune system,

provoking a melanocyte-specific immune response. This scenario is currently our working hypothesis regarding HVT as an environmental factor in SLV. The report by Grimes et al. [67], on the presence of cytomegalovirus DNA in depigmented and uninvolved skin from some patients with vitiligo, and its absence in control subjects, suggests that vitiligo may be triggered by a viral infection in some patients. Considering that cytomegalovirus is a betaherpesvirus, the herpesvirus connection in the expression of SLV further underlines the similarities between human vitiligo and SLV.

16.2.8 Summary

The SL chicken model (including MHC-matched, susceptible, and control lines) offers unique opportunities to study the interplay between genetic susceptibility, immunological influences, and environmental factors leading to the development of antimelanocyte autoimmune activity. The similarities between the clinical manifestations and the pathological progression between human and SL vitiligo, together with the unique features of the target tissue (easy, noninvasive, repeatable access to the autoimmune lesion), the predictability of the disease, and the ability to study the expression of this disorder in the absence/presence of an environmental component make the SL chicken an excellent model for studies on autoimmune vitiligo and other organ-specific autoimmune diseases.

16.3 Spontaneous autoimmune (Hashimoto’s) thyroiditis in obese-strain chickens

16.3.1 Introduction

Hashimoto’s thyroiditis (autoimmune thyroiditis) is the most common thyroid disease in humans. It is characterized by autoimmune destruction of the thyroid gland whereby the thyroid parenchyma is diffusely replaced by a lymphocytic infiltrate and fibrotic reaction and, frequently, the formation of lymphoid germinal follicles. Depending on the extent of lymphoid infiltration, thyroid function may be only slightly affected, and patients remain euthyroid or, with severe infiltration, physiological and clinical manifestations of hypothyroidism develop. Patients with Hashimoto’s thyroiditis have serum antibodies that react with thyroglobulin (Tg), thyroid peroxidase, and other thyroid proteins. In addition, many patients have CMI directed against thyroid antigens, demonstrable by several techniques. Onset of the disorder is most common in middle-aged individuals. The incidence is about 10–20 times higher in women than in men, with 2% of the general population affected (reviewed in Ref. [68]).

The OS chicken is one of the best and most thoroughly studied animal models for spontaneously occurring autoimmune thyroiditis that closely resembles human Hashimoto's thyroiditis. The onset of thyroiditis is usually before 6 weeks of age and is accompanied by visible effects on growth and development [69]. The effects include small size, abdominal and subcutaneous fat accumulations, and a plump appearance, as well as long, silky feathers that continue to grow and exhibit structural signs of thyroxine deficiency (Fig. 16.4). Delayed maturation and low reproductive performance are also characteristic of this line whereby most females do not lay eggs unless supplemented with thyroid hormones.

Histological examination of the thyroids of OS chickens reveals extensive infiltration by mononuclear cells and germinal center formation, commencing in the second week after hatching and resulting in almost complete destruction of the thyroid architecture by 1–2 months of age. Although originally more prevalent in females than in males, with continued selection for the expression of autoimmune thyroiditis, the gender gap in the OS disappeared and the incidence of spontaneous autoimmune thyroiditis (SAT) is nearly 100% in both genders. Autoantibodies to chicken thyroid antigens, especially Tg, can be detected in the circulation by 2–3 weeks of age. Moreover, the presence of thyroid-specific CMI in chickens with established thyroiditis has been demonstrated using the delayed wattle-swelling response to thyroid extract. Like most autoimmune diseases, the expression of SAT and loss of self-tolerance in OS chickens is a polygenic trait involving many components and systems; these include inherent target cell defects, manifested in part by abnormal MHC class II antigen expression and iodine uptake of thyroid cells; altered immune function (i.e., heightened levels of immune activity and impaired immunosuppression); and altered immunoendocrine communication via the hypothalamic–pituitary axis (e.g., hyporesponsiveness to glucocorticoid-inducing factor). Additionally, iodine levels in food are an important environmental factor in the development of SAT; the severity of SAT can be manipulated with iodine [70] (reviewed in Ref. [23]).

The OS chicken is one of the oldest and best-established models of spontaneous, organ-specific autoimmune diseases. Based on clinical, histopathological, immunological, serological, and endocrinological aspects, it is well accepted by the biomedical community as one of the best animal models for Hashimoto's thyroiditis.

16.3.2 Development and characteristics of OS chickens

Dr. Randall Cole, Poultry Geneticist in the Department of Poultry and Avian Sciences at Cornell University, first identified the abnormalities resembling Hashimoto's

thyroiditis in the Cornell C strain in 1955 [71]. In parallel with Dr. Cole's breeding and selection efforts at Cornell from 1955 to 1995, a flock of OS chickens has been maintained and propagated at the University of Innsbruck since the early 1970s. Since the development of the OS chicken, many excellent, comprehensive reviews have been published by researchers in the United States [70–73] and Austria [23,74–80]. The reviews describe the development of the OS model and the research efforts into dissecting the genetic susceptibility and multifactorial nature of this spontaneous organ-specific autoimmune disease, providing in-depth resources beyond the scope of this chapter.

As mentioned earlier, only one line of the OS chickens remains and is currently maintained at the University of Arkansas, Division of Agriculture in Fayetteville (UA). These OS chickens originated from the Innsbruck populations, were last maintained at the University of Uppsala, Sweden, and transferred to the University of Arkansas in 2015 before termination of the line in Uppsala. Of the fertile eggs sent to UA, 36 hatched from 10 unrelated sires and 11 unrelated dams. Through the efforts of Dr. N. B. Anthony and collaborators [81], a stable OS population has since been established, although the OS line is very difficult to manage and reproduce. Even with thyroxine supplementation (0.5 ppm in the feed) after phenotypic selection at 10 weeks of age, fertility in males remains problematic [81]. All the OS chickens were found to be homozygous B13, hence they likely originated from the OS B¹³B¹³ subline known to exhibit severe disease symptoms [80]. In fact, the visual phenotype of hypothyroidism regarding the growth and development of OS chickens described earlier is expressed in 95%–100% of individuals in the UA population. Because of the fertility issues, experimental studies have only recently been initiated to recharacterize the disorder at the cell and molecular levels. Examination of thyroids and blood collected from OS chickens twice per week from the day of hatch through 6 weeks of age are in progress.

16.3.3 Immunological mechanisms

Histological examination of the thyroid in OS chickens reveals infiltration of mononuclear cells and the appearance of organized lymphoid aggregates and follicles. As thyroiditis progresses, healthy thyroid tissues are replaced by these infiltrates, often resulting in complete destruction of the thyroid glands. Humoral immunity was thought to play a major role in the development of SAT based on greatly reduced mononuclear cell infiltration in bursectomized chicks (either prehatch or posthatch) and the detection of antithyroid autoantibodies. When neonatal thymectomy resulted in more aggressive thyroid infiltration, it first appeared that T cells primarily had a suppressor function

in SAT rather than playing an active destructive role. However, because neonatal thymectomy does not remove T cells that have already entered the periphery, a role for T cells in thyroid destruction cannot be ruled out.

In follow-up studies, neonatal thymectomy was accompanied by the administration of high doses of anti-T cell serum that led to almost complete abrogation of thyroid infiltration. Hence, it appears that in the OS chicken the first cells to leave the thymus and take up residence at peripheral sites are effector T cells capable of producing the pathological changes of thyroiditis. On the other hand, the regulatory T cells responsible for the suppression of the autoimmune attack appear to leave the thymus at a later age. This course of events explains the more aggressive presentation of thyroiditis in neonatally thymectomized OS chicks. Moreover, the observed abrogation of thyroid infiltration as a result of complete removal of the T cells compartment, including effector and regulatory T cells, clearly supports a central role for CMI in SAT (reviewed in Ref. [23,72]).

A phenotypic analysis of thyroid-infiltrating cell populations [82] found that more than 60% of thyroid IML are T cells and 10% are activated T cells. Although B cells made up a significant proportion of thyroid IML (about 20%–30%), they did not appear to be obligatory for the induction of SAT. Moreover, the overwhelming majority (nearly 90%) of infiltrating T cells express TCR $\alpha\beta_1$, with a smaller proportion expressing TCR $\alpha\beta_2$ or TCR $\gamma\delta$. These proportions among the various TCR-defined cell subsets differed greatly from those among circulating T cells. Selective depletion of TCR $\alpha\beta_1^+$ and TCR $\alpha\beta_2^+$ T cells, using repeated injections of mouse monoclonal antibodies specific for either TCR $\alpha\beta_1$ or TCR $\alpha\beta_2$, into embryos and 1–3-week-old chicks, resulted in a selective reduction of 41% and 87% of TCR $\alpha\beta_1^+$ and TCR $\alpha\beta_2^+$ cells, respectively [83]. The reduction in TCR $\alpha\beta_1^+$ cells resulted in a more than 50% decrease in thyroid follicle destruction. Selective reduction of TCR $\alpha\beta_2^+$ cells, on the other hand, did not affect SAT development. These results indicate preferential use of the TCR V β_1 gene fragment by autoreactive T cells in OS-SAT [83].

A similar approach was used to dissect the relative contributions of CD4 $^+$ and CD8 $^+$ lymphocytes in the destruction of OS thyroid follicles [84]. Although in untreated chickens IML had substantially higher proportions of CD8 $^+$ than CD4 $^+$ lymphocytes, anti-CD4 treatment completely prevented the development of SAT in OS chickens. After depletion of CD4 $^+$ T cells, neither residual CD4 $^+$ cells nor any other mononuclear cells infiltrated the thyroid glands. On the other hand, anti-CD8 treatment reduced the severity of SAT but did not prevent the disease. These findings attest to the critical role of CD4 $^+$ cells in the onset and development of SAT while suggesting primary involvement of CD8 $^+$ lymphocytes in

the pathogenesis and progression of thyroid follicle destruction [84].

Because of the absence of thyroid infiltration with selective depletion of CD4 $^+$ cells, it is likely that thyroid-infiltrating CD4 $^+$ autoreactive T cells provide the necessary inflammatory signals to recruit, activate, and retain other cells involved in SAT. To gain insight into the production of inflammatory cytokines and chemokines in OS thyroids, IFN- γ , IL-1 β , IL-2, IL-6, IL-8, IL-15, and IL-18 gene expression analysis was conducted using thyroid tissue collected from OS B ^{13} B ^{13} and controls at various times before SAT development, using 20-day embryos and 3–5-day-old chicks [85]. Although some coordinated expression of IL-1 and IL-8 (a major inflammatory cytokine and chemokine, respectively) was found, the most consistent observation was heightened expression of IL-15 mRNA at all time points in both the spleen and the thyroid. The biological functions of IL-15 are similar to those of IL-2 and include stimulation of growth and proliferation of T cells, intestinal epithelial cells, natural killer cells (NK), and activated B cells.

Considering the biological functions and the early and persistent expression of IL-15 in the OS thyroid, IL-15 appears to play a role in driving the onset of SAT [85]. IFN- γ , which is a NK and Th $_1$ cytokine with a central role in inflammatory CMI activities, was found to be expressed at heightened levels in OS thyroids when the chicks were 5 days of age. The cellular source of IL-15 and IFN- γ in OS thyroids has not been established [85]. Based on the kinetics, one would expect little lymphocyte infiltration by 5 days [77]. However, considering that OS chickens are known to be immunologically hyperactive, it is likely that even a few NK and/or Th $_1$ cells may produce sufficient amounts of IFN- γ to drive the inflammatory cascade toward the development of SAT [86,87]. The initial presence and activation of these effector cells, however, may simply be due to minor stimuli such as minor injury/malfunction of the susceptible target organ or cells.

16.3.4 Target cell/organ defects

In OS thyroids, there appear to be fundamental abnormalities such as reduced growth of thyroid cells in vitro [88] and alterations in the metabolic function of thyroid cells. The OS thyroid gland can function independently of pituitary stimulation, as shown by continued uptake of radioactive iodine during the suppression of thyroid-stimulating hormone (TSH) [73]. A similar defect was observed in the CS parental strain. Although investigations into this phenomenon excluded faulty TSH regulation or thyroid-stimulating antibodies as the underlying cause for this TSH autonomy, the ability of these lines to use the iodine for synthesis of T $_3$, T $_4$, and Tg has not been identified. Evidence also suggests that iodination of

Tg is important in disease induction and that iodine level in the food is an important environmental factor in the development and severity of OS-SAT [75,89,90].

To gain insight into the role of iodine as an environmental factor in the expression of SAT, Bagchi et al. [89] used OS chickens maintained on iodine-deficient diets. Using this approach onset of SAT was avoided until challenge with iodine (NaI). Twelve hours after NaI administration, thyroid injury, but not infiltration, was observed. Subcellular changes included swelling of mitochondria and the rough endoplasmic reticulum. The affected cells often had ruptured luminal cell membranes and showed clumping of chromatin in the nucleus. The damage was greater with higher compared to lower dosages of iodine (250 vs 20 µg, injected ip). There was no evidence of pyknosis, apoptosis, or lipofuscin granules in the thyroid epithelial cells, and the follicular structure remained fully maintained. Infiltration of mononuclear, but not polymorphonuclear, leukocytes could be observed 24 hours after iodine treatment with maximal levels of infiltration occurring at 72 hours and onward [89].

Phenotypic analyses of thyroid IML showed 40% CD8⁺ cells, 20% CD4⁺ cells, 22% B cells, and 17% macrophages. This IML population profile is similar to that observed in OS thyroids from chickens that developed SAT without dietary manipulation. Concurrent treatment with NaI and ethoxyquin, a strong antioxidant, prevented subcellular changes and mononuclear cell infiltration. The protective effect of this antioxidant on acute iodine injury was not due to its potential influence on iodine accumulation, iodine transport, and incorporation into protein. Hence, thyroid injury appears to be a critical event in the induction of OS-SAT by iodine [89].

Other factors that could contribute to the increased susceptibility of the OS thyroid gland to immunological attack include underlying structural alterations of the thyroid architecture and a lower threshold of thyroid epithelial cells for IFN-γ-induced expression of MHC class II molecules that could greatly contribute to the autoimmune recognition and destruction of the thyroid follicles [73,74,91].

16.3.5 Summary

In the OS chicken, minor inborn errors in metabolism and minor aberrancies in the structure, growth, and function of the thyroid tissue may necessitate an influx of macrophages and dendritic cells to regulate tissue homeostasis. This non-infectious influx may, however, be a first step on the road to thyroid-specific autoimmunity. Because of the inherent hyperactive immune responses existing in the OS, an inappropriate high-inflammatory cytokine cascade, including increased IFN-γ production, may stimulate the IFN-γ-sensitive thyroid epithelial cell to become immunologically active (e.g., express MHC II antigen, produce cytokines

and chemokines, present self-antigen). This, together with other known defects in the OS (e.g., early emigration of T effector cells from the thymus into the periphery, an imbalance between effector and regulatory T cells, a disturbed endocrine-immune system balance, including reduced ability of the hypothalamic-hypophyseal axis to stimulate corticosterone production by the adrenal gland), may culminate in autoimmune recognition of thyroid antigens and destruction of the thyroid epithelium [23].

16.4 Scleroderma in UCD 200/206 chickens

16.4.1 Introduction

Scleroderma, also known as SSc, is a complex autoimmune connective tissue disease characterized by pathological remodeling of connective tissues. Clinical and pathological features in humans include microvascular alterations; perivascular inflammatory infiltrates and alterations involving cytokines with either pro- or antifibrotic activity; aberrant activity of innate immunity as well as the T and B cell compartments; presence of multiple autoantibodies; and, ultimately, widespread tissue fibrosis of the skin and several internal organs. The extent and severity of clinical manifestations range widely; however, a progressive thickening and fibrosis of the skin is universally observed in patients with SSc.

Internal organ involvement tends to be subclinical at presentation but may involve fibrosis of the esophagus, lungs, heart and pericardium, kidneys, thyroid, and the male reproductive system. In advanced stages, progression of the vascular and fibrotic changes is accompanied by a decrease in inflammation. Scleroderma is more prominent in females than males and is believed to result from complex interactions between the host's genetic background and the environment. Several other factors have been proposed as agents triggering/modulating the expression of scleroderma, including exposure to organic solvents and toxins both at home and in the workplace, the presence of microchimerism, infectious agents (e.g., human cytomegalovirus), and the inherent tendency for oxidative stress with associated generation of oxidative radicals [92,93].

Chickens from the UCD 200 and UCD 206 lines spontaneously develop an inherited disease closely resembling human SSc (Figs. 16.5 and 16.6). It is considered the best animal model because it occurs naturally and exhibits the whole spectrum of clinical, histopathological, and serological manifestations of human SSc (i.e., vasculopathy, vascular occlusion, severe lymphocytic infiltration of the skin and viscera, fibrosis of the skin and internal organs, antinuclear antibodies (ANA), rheumatoid factors, distal polyarthritis). This animal model provides an excellent opportunity to examine genetic susceptibility and

etiopathological mechanisms underlying SSc and other fibrotic diseases. Moreover, being the only animal model exhibiting the vascular changes involved in human SSc, it is uniquely suited as a model for translational research, including identification of biomarkers and development of preventative and intervention therapies for this devastating disease [23,93]. For poultry, the UCD 200/206 model also is well suited for the study of vasculopathy and fibrotic diseases such as wooden breast and arterial pulmonary hypertension (ascites).

In 2015 both Uppsala and Innsbruck shipped fertile eggs of UCD 200 and UCD 200 and 206 lines, respectively, to the UA, before terminating these research lines at their institutions. Unfortunately, UCD eggs did not handle the transport well; few chicks hatched and only 11 made it to reproductive age to generate the F1 population at UA. Of the 10 UCD 200 adults, all originated from the Swedish colony, and the only surviving chick hatched from the Austrian colony was a female from the UCD 206 line. From these 11 UCD chickens, six males and five females, the current UCD 200 and UCD206 populations were reestablished in Arkansas over the past 5 years [81]. Selection of the sublines was based on MHC haplotype; homozygous B2 for UCD 200, and homozygous B15 for UCD 206 determined by Dr. Janet Fulton, Hy-line International, Dallas Center, IA [94]. Fertility is very good (at nearly 80%), especially considering the small starting population of four unrelated males and four unrelated females. Moreover, the visual SSc phenotype and time-course of comb regression and skin lesions in the UA populations are as described for the original populations, with more than 80% of individuals affected [81]. Studies are ongoing to recharacterize the developing autoimmune lesions at the tissue, cell, and molecular level, and to examine innate cellular responses [95].

16.4.2 Development and characteristics of the UCD 200/206 lines

Dr. Paul Bernier at the Department of Poultry Husbandry, Oregon State University, Corvallis, first reported, in 1942, that some male chickens showed signs of dermal fibrotic disease reminiscent of scleroderma. In 1977 the UCD 200 line was developed at the University of California, Davis by Gershwin et al. [96]. Thereafter, the UCD 206 line, homozygous for the B15 MHC haplotype was developed from the UCD 200 line. In 1988 and 1993 colonies of UCD 200 and UCD 206, respectively, were established at the Experimental Animal Facilities of the Innsbruck Medical University, while the lines were terminated in the USA in early 2000. A population of the UCD 200 line also was transferred from Innsbruck to Uppsala for QTL mapping in 2006 [97].

As with human scleroderma, the incidence of SSc in UCD 200 chickens was higher in the homogametic gender (females in humans, males in chickens). Based on early analyses, the genetic defect responsible for SSc in UCD 200 chickens appears to be autosomal and recessive, exhibits incomplete penetrance [96], and suggests a modulatory role for some MHC haplotypes [98], but not for B15. Genomic analyses of collagen revealed no gross alterations of collagen genes, which is consistent with observations in humans [99]. Recent QTL mapping using a cross of UCD 200 and Red Jungle Fowl chickens suggested disease-predisposing loci on chromosomes 2, 12, and 14 [97]. Included were orthologues of genes suggested to be involved in human SSc and generally related to immune function (chromosome 2: TGFRB1, EXIO2-IRF4, COL1A2, IGFBP3, and CCR8). IGFBP3, which is increased in the serum of SSc patients, was also associated with avian SSc, thus providing a first genetic link of this factor with SSc. SOCS1, another gene with an immunological function, was located in the QTL region of chromosome 14. These candidate genes, which are implicated in avian SSc, further underscore the usefulness of the UCD 200 chicken as a model for human SSc [97].

UCD 200/206 chickens follow a relatively uniform pattern of disease progression [23,98]. The chicks appear relatively normal during the first 1–2 weeks posthatch. The first observable gross abnormalities include severe comb swelling and erythema, leading to necrosis and loss of the comb. This typical “self-dubbing” comb lesion occurs in more than 90% of UCD 200 and 206 chicks (Fig. 16.5). By 3–4 weeks of age, dermal lesions in the dorsal neck region, such as swelling, induration, and loss of feathers, become evident in 20%–40% of chicks (Fig. 16.6). With the progression of the disorder, the skin becomes thickened and tight. Histological examination has shown early skin inflammation that is later replaced by fibrosis of the dermis and subcutaneous fat and muscle. Alterations in internal organs (e.g., esophagus, small intestine, kidney, lung, testis) can also be observed. The age and incidence of internal organ involvement may vary widely, with alterations in renal arterioles occurring in almost all chickens.

16.4.3 Immunological mechanisms

In UCD 200 chickens, examination of both comb and neck integumental tissue revealed prominent mononuclear cell infiltration. Initially, T cells, including both CD4⁺ and CD8⁺ lymphocytes, predominated in the lesions; however, as the lesions progressed, distinct groups of IgM⁺ B cells could be observed. Further phenotypic analysis of skin infiltrates in the early acute phase revealed that the overwhelming majority of skin IML in the deeper dermis and subcutaneous tissue consisted of TCR $\alpha\beta$ 1⁺, CD3⁺, CD4⁺, and MHC class II⁺ T cells, with only

5%–10% expressing the IL-2 receptor (IL-2R). T cells in the inflammatory infiltrate present in the perivascular region of the papillary dermis consisted primarily of TCR $\gamma\delta$ MHC class II⁺ lymphocytes [100,101]. These observations strongly support T cell-mediated immune activity in the SSc lesion.

Compared to healthy control chickens, alterations in the proportions and numbers of T cells in the blood and thymus, as well as altered T cell functional activities (decreased in vitro T cell mitogen-induced proliferation, IL-2 production and IL-2R expression, abnormal function of lymphocyte costimulator molecules, intracellular calcium regulators) also point toward T cell abnormalities and a role for T cells in UCD 200 SSc [101]. Immunohistological study of thymic tissue from UCD 200 chickens revealed alterations prior to visible signs of disease onset, suggesting aberrant T cell maturation and selection that potentially influences establishment, maintenance, and regulation of tolerance [102–104].

Although a major role for T cells in the pathogenesis of human and avian SSc is widely accepted, autoantibodies are also present in both [92]. Circulating autoantibodies in UCD 200/206 chickens consist of ANA, including single-stranded DNA, poly (I) poly (G) and anticardiolipin antibodies, anticytoplasmic antibodies, and rheumatoid factors, as well as antiendothelial cell antibodies (AECA). The levels of these autoantibodies increase with age. Except for AECA, their role in the development of SSc is not clear [105,106].

In both human and avian SSc, endothelial cells have emerged as the primary target of the autoimmune response, with vascular injury occurring before onset of fibrosis. Specifically, AECA in UCD 200/206 chickens are found before the onset of SSc and are involved in endothelial cell injury and endothelial cells apoptosis [107,108]. In human SSc, AECA induce endothelial cell apoptosis as a result of NK cell-mediated antibody-dependent cell cytotoxicity and Fas–Fas ligand interaction [109]. However, in both humans and chickens, the specific autoantigens recognized by AECA autoantibodies have yet to be identified [23].

Endothelial cell apoptosis is followed by the accumulation of mononuclear cells and fibrosis [110]. Compared to controls, fibroblast cell lines prepared from fibrotic skin of UCD 200/206 chickens demonstrated an activated phenotype (increased production of collagen, noncollagenous protein, and glycosaminoglycan) and, similar to neoplastic fibroblasts, increased expression of highly branched *N*-linked oligosaccharides terminating in *N*-acetylglucosamine residues [111,112].

Mononuclear cells isolated from fibrotic skin of UCD 200/206 chickens secrete pro- and antifibrotic cytokines and IgM and thus likely play an important role as effector cells in the development of dermal fibrosis [113]. Among these, transforming growth factor (TGF)- β is thought to be an

important player. There are three isoforms of TGF- β (TGF- β 1, - β 2, and - β 3) with similar signaling pathways but not identical biological functions. Unlike the profibrotic activities of TGF- β 1, TGF- β 2 in the UCD 200 line was found to act as an antifibrotic cytokine in the pathogenesis of SSc.

A recent study by Prelog et al. [114] suggests that diminished TGF- β 2 production leads to increased expression of a profibrotic procollagen alpha 2 type I mRNA variant in embryonic fibroblasts of UCD 200 chickens, providing another glimpse into the complexity of the interrelationships underlying this systemic autoimmune disease. Moreover, based on these studies in the UCD 200 model, TGF- β 2 emerged as a promising candidate for SSc therapy, especially during the early inflammatory stages of the disorder [93,115].

For SSc research in humans, the inability of medical diagnosis at early, presymptomatic stages of SSc presents a major challenge in the development of potentially targetable biomarkers for diagnosis, treatment, and prevention. Thus far it has become apparent that in both human and avian SSc, the early vasculopathy (i.e., apoptosis of endothelial cells, microvascular occlusion, hypoxia, and ischemic injury) is the primary event leading to progressive capillary loss and perivascular accumulation of lymphocytes. Analysis of skin biopsy samples collected from SSc patients during early stages of SSc showed that adaptive angiogenesis is largely defective in SSc and that an impaired interplay between endothelial cells and pericytes appears to lead to a phenotypic switch of pericytes toward myofibroblasts and development of fibrosis instead of normal vascular repair [116].

Recent evaluation of these early pathogenic steps in SSc of UCD 200 chickens compared to human patients provided important new insight into key molecules involved in the link between vascular injury and fibrosis in SSc [116]. Specifically, combs from healthy controls and UCD 200 chickens at different stages of the evolving SSc lesion (erythema, edema, and necrosis) were subjected to immunofluorescence-based histological evaluation along with human skin from healthy controls and patients at early diagnosis of SSc. All aspects examined, from the presence of perivascular fibroblasts to levels of angiogenic molecules, growth factors, and respective receptors (SDF-1, CXCR4; VEGF, ET-1, PDGF, and receptors), were similarly upregulated in SSc tissues from chicken and human lesions compared to healthy control tissues. Moreover, in UCD 200 combs, levels were positively related to the severity of comb involvement, with significant increases in perivascular fibroblasts, ET-1, and ETAR/ETBR receptors already observed in erythema combs. It should be noted that in both the human SSc patients and UCD 200 chickens, plasma levels of ET-1, VEGF, and PDGF were elevated, whereby in chickens their levels increased with severity. Like in tissues,

plasma levels of ET-1 were already significantly elevated in birds with erythema combs, the earliest stage of SSc examined [116].

Similarly, based on observations in SSc patients that hyaluronan (HA) levels in the circulation and skin are elevated and affected by disease severity, Kerje et al. [117] examined HA metabolism in combs from UCD 200 and controls at different stages of comb development. In UCD 200 early stages of inflammation were associated with altered HA metabolism; specifically, accumulation of low-molecular-weight HA and decreased levels of hyaluronidase-1, an enzyme responsible for degradation of low-molecular-weight HA. These alterations in the extracellular matrix, already observed at 2 days of age, may be an early indicator of disease and development of fibrosis [117].

The molecular similarities between human and avian SSc provided important insight into key molecules linking vascular injury, inflammation, and fibrosis in SSc. Collectively these findings further established UCD 200/206 chickens as a relevant and much-needed model for translational research into therapeutic treatments of SSc. In fact, using UCD 206 chickens, Birgani et al. [118] successfully tested the hypothesis that cell-demanded release of fibrin-bound VEGF₁₂₁ leads to the formation of stable blood vessels and clinical improvement of ischemic lesions in combs and skin. Based on their finding, the VEGF₁₂₁ released from the fibrin-matrix controlled angiogenesis by differential regulation VEGF receptor-1 and -2, shifting the balance toward the proangiogenic VEGFR-2. This approach may find direct application in therapy of ischemic lesions (e.g., fingertip ulcers) in human SSc patients [118].

16.4.4 Summary

Considering the many similarities between human and avian SSc, the UCD 200 and UCD 206 chicken lines will continue to make significant contributions to our understanding of SSc in humans. SSc in UCD 200/206 develops spontaneously, predictably, and early, with both dermal and internal organ involvement. This provides excellent opportunities to dissect the complex interrelationship between genetic susceptibility, immune system defects, and environmental factors that drive the onset and progression of this disease. Additionally, information obtained by studying healthy chickens compared to those affected by this multiorgan disorder will greatly contribute to our understanding of poultry biology.

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Tumors of the avian immune system

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17.1 Introduction

The immune system is crucial in protecting the host from infections by either eliminating the invading pathogens or by reducing the negative impact of infections on host fitness [1]. It consists of a complex network of a large number of cell types and cascades of soluble factors, all of which work in concert to induce protective immune responses against the continuous challenge from a wide range of pathogens. As in the case of mammals, a properly functioning immune system is vital to the survival of commercial poultry flocks kept in intensive conditions in the poultry house environment, where they are constantly challenged by a plethora of rapidly spreading infectious pathogens. In all vertebrates, immunity against invading pathogens operates at two levels, referred to as the innate and adaptive responses [1]. Optimum functioning of both systems is also critical to induce effective immune responses to the wide range of live and inactivated vaccines that protect against a very large number of poultry pathogens.

As detailed in other chapters, the avian immune system has made invaluable contributions to the understanding of the many fundamental principles of immunology (see Chapter 1). Among these, perhaps the most significant contribution is the demonstration of the dichotomy of the adaptive immune system into the T cell- and the B cell-dependent compartments associated with the cell-mediated and humoral immune responses, respectively. T and B lymphocytes, the essential component cells of the vertebrate immune system, develop through the primary lymphoid organs, the thymus and the bursa of Fabricius, respectively, before populating the secondary lymphoid organs such as the spleen, bone marrow, and local lymphoid tissues. These cells face up to the challenge of the pathogenic organisms and, especially in a vaccinated host, respond vigorously as cytotoxic T lymphocytes (CTL) or T helper (Th) cells or as antibody-secreting B

cells to eliminate invading pathogens and to provide continuing protection through memory cells [2].

Apart from lymphocytes, other cell types, such as macrophages, dendritic cells, and natural killer (NK) cells, also function as major players in combating infection either as part of the innate immune responses or as antigen-presenting cells. Thus the avian immune system plays a crucial role in preventing disease and maintaining health. However, as is the case with other body systems, the immune system is also vulnerable to different types of diseases, and many pathogens specifically target this system with devastating effects. In this chapter, some neoplastic diseases of the avian immune system are described.

17.2 Tumors of the immune system

Neoplastic diseases in poultry can be of infectious and noninfectious etiology. The noninfectious tumors, although important in terms of animal welfare, are not of major economic significance since they are sporadic, mostly occurring in birds older than the usual life span of production poultry. On the other hand, infectious neoplastic diseases caused by oncogenic viruses are very widespread and hugely important economically. The three neoplastic diseases mainly affecting the avian immune system are (1) Marek's disease (MD)-associated T-cell lymphomas, (2) avian leukosis tumors of B cells and other hematopoietic cells, and (3) reticuloendotheliosis virus (REV)-induced tumors characterized by a variety of syndromes including lymphoid neoplasia [3]. Comprehensive reviews covering these viruses and different aspects of the diseases are available elsewhere [4–8].

17.2.1 Marek's disease

MD, named after the Slovakian-born Hungarian pathologist József Marek, is a lymphoproliferative disease of

domestic chickens and, less commonly, turkeys, quail, and geese. Originally described in 1907 as a polyneuritis affecting the peripheral nerves, it was not until 1926 that MD was recognized as a neoplastic disease associated with tumors in several visceral organs. Although MD exists in all poultry-producing countries of the world, assessment of worldwide incidence and economic impact is difficult because the disease is not notifiable. Nonetheless, MD remains a major problem in many countries [4,9]. Its economic impact on the world poultry industry is estimated to be United States\$ 1–2 billion annually [10]. The causative agent Marek's disease virus (MDV) is a highly contagious alphaherpesvirus belonging to the genus *Mardivirus*. Although MDV is not vertically transmitted through eggs from infected hens, chicks become infected almost immediately after hatching from heavily contaminated poultry houses. The infection of chicks occurs by inhalation of the infected desquamated epithelium in poultry house dust, shed from the feather-follicle epithelium of infected birds, which can remain infectious for long periods because of the high stability of the virus.

Cytolytic infections with MDV occur 3–6 days after infection leading to extensive atrophic changes in the lymphoid organs of bursa of Fabricius and thymus and causing early mortality. After this early cytolytic period, the virus becomes latent, and the clinical expression of paralysis or tumors can occur any time after 3–4 weeks after infection [4,11]. Under field conditions, most of the serious cases begin after 8–9 weeks but sometimes commence well beyond the onset of egg production. Various factors such as virus strain and dosage, gender and genetic resistance of the host, presence of maternal antibodies, infection with immunosuppressive pathogens (as discussed in Chapter 14) such as chicken infectious anemia virus (CIAV), and other environmental factors can affect the outcome of infection.

Clinical signs associated with MD vary according to the specific syndromes and can be divided broadly into different clinical forms based on the various characteristics. In almost all these cases, these involve lymphoid infiltrations into tissue(s) to produce lesions or tumors. In the classical form of the disease, mainly neural lesions occur with mortality rarely exceeding 10%–15% over a period of few weeks or many months [4]. Signs can vary from bird to bird depending on the involvement of the different nerves. The most common clinical sign is the partial or complete paralysis of the legs and wings. When nerves controlling the neck muscles are affected, symptoms such as torticollis are observed. Similarly, the involvement of the vagus nerve can result in the paralysis and dilation of the crop. Such birds can also show symptoms of gasping and respiratory distress. In the acute form of the disease, where there is usually formation of

lymphomas in the visceral organs, the incidence of the disease is frequently between 10%–30% and in major outbreaks can increase to 70%.

Acute cytolytic disease, observed with some of the very virulent MDV pathotypes, results in severe atrophy of the lymphoid organs [4,12] and immunosuppression [13]. This form of the disease, sometimes described as “early mortality syndrome,” results in very high mortality usually at 10–14 days of age. Transient paralysis is a rather uncommon manifestation of MDV infection that occurs at 5–18 weeks of age. Affected birds suddenly develop varying degrees of ataxia, paresis, or paralysis of the legs, wings, and neck. The disease is commonly observed 8–12 days after infection, usually lasts only for about 24–48 hours, and is associated with edema of the brain. The affected organs show lymphoid infiltrations, with the degree of infiltration correlating with the disease manifestations.

The unique epidemiological features of MD, including its highly contagious nature and, widespread distribution, and the long-term infectivity of the poultry house environment, make its eradication almost impossible. Hence control is essentially based on preventive vaccination (see Chapter 18), together with improved biosecurity and selecting for genetic resistance. The development of MD vaccines was a significant landmark both in avian medicine and basic cancer research, as this was the first example of a neoplastic disease controlled by the widespread use of vaccines [14,15]. Live-attenuated vaccines are usually administered as cell-associated vaccines to day-old chicks at the hatchery to provide protection against the natural challenge from the infected poultry house environment. Layers and breeders are normally vaccinated at 1 day of age, while broilers in many parts of the world are vaccinated by in ovo injection [16,17]. MD vaccines, derived from all three MDV serotypes, are highly effective, often achieving over 90% protection under commercial conditions. The most widely used serotype 1 vaccine is derived from the CVI988/Rispens strain and is effective against most MDV pathotypes [18]. Antigenically related serotype 2 strains, such as SB-1 and 301B/1 [19–21], are also used widely in many countries. The serotype 3 FC-126 strain of HVT is available in cell-free and cell-associated forms.

Although many of these vaccines are effective individually, the concept of protective synergism [22,23] has led to the widespread use of polyvalent vaccines with two or more strains administered simultaneously. Recombinant vaccines based on HVT and poxvirus vectors are increasingly used to provide dual protection against MD and other avian viral diseases [24,25]. Although MD vaccines are generally successful in controlling losses, vaccine failures can occur from causes such as improper use of the vaccine, exposure to viruses before immunity develops,

interference by maternally derived antibodies, and the emergence of virulent viruses that can break through the immunity [18,26,27]. Infections with immunosuppressive viruses such as CIAV can also negatively affect the outcome of MD vaccination [28].

17.2.2 Avian leukosis

Avian leukosis embraces several different leukemia-like neoplastic diseases of the hematopoietic system. These tumors are induced by avian leukosis viruses (ALV), members of the *Alpharetrovirus* genus of the subfamily Orthoretrovirinae in the family *Retroviridae* [29]. The ALV genome has from the 5' end to the 3' end, three structural genes, *gag/pro-pol-env*, which encode, respectively, the proteins of the virion group-specific (gs) antigens and protease, the enzyme reverse transcriptase enzyme, and the envelope glycoproteins. Based on the viral envelope, ALV are grouped into 10 subgroups designated A–J [5]. Identification of a new ALV K subgroup has also been reported, although a recent study suggested it uses the same receptor as the ALV A subgroup [30]. Based on the mode of transmission, they can be further grouped into “exogenous” and “endogenous” retroviruses. ALV that are transmitted from bird to bird, either through the egg or contact are termed “exogenous” viruses, and all oncogenic ALV fall into this category. However, some ALV are present as integrated proviruses in the genome and are transmitted genetically as Mendelian genes, either as complete viral genomes (e.g., infectious virus of subgroup E) or, more commonly, as incomplete (defective) genomes coding for retroviral products (e.g., gs-antigen) only. A number of such endogenous retroviruses have been identified in the chicken genome [31,32]. They are generally nononcogenic but may influence the response of the bird to infection by exogenous ALV by inducing immunological tolerance [33]. They are also reported to contribute to induction of bursal tumors in combination with serotype 2 MDV vaccine [34] or stimulate innate antiviral response through Inc-ALVE1-AS1 long noncoding RNA [35].

Depending on the primary cell type transformed, avian leukosis can be grouped into different types of tumors: Lymphoid leukosis (LL), tumors of B lymphocytes, is one of the most common forms of leukosis. LL occurs in chickens from about 4 months of age and is most usually caused by ALV of subgroups A and B. Gross pathological changes include diffuse or nodular enlargement of the bursa of Fabricius, liver, spleen, and other organs due to coalescing foci of extravascular immature lymphoid cells. Erythroid leukosis or erythroblastosis is an uncommon, usually sporadic, tumor of the erythroid cells occurring mainly in adult chickens. In the affected birds, the liver and spleen, and sometimes the kidneys, are moderately

and diffusely enlarged and often of bright cherry-red color. Microscopically the liver shows intra-sinusoidal accumulations of rather uniform, round, erythroblasts, the spleen shows accumulations of erythroblasts in the red pulp, with bone marrow showing enlarged sinusoids filled with erythroblasts.

Myeloid leukosis (ML) broadly involves both myeloblastic (myeloblastosis) and myelocytic (myelocytomatosis) forms. The disease has become particularly prevalent in broiler breeders infected with ALV subgroup J [36]. Although the losses from this disease have reduced significantly in most parts of the world following eradication from the primary breeding flocks [36], ALV-J-induced diseases are still a major threat in countries such as China, where, unlike in the west, they are reported in commercial meat-type and egg-type birds as well as in native chickens [37,38]. ALV are also associated with a variety of solid tumors, including fibrosarcoma, chondroma, hemangioma, histiocytic sarcoma, mesothelioma, myxoma, nephroblastoma, osteoma, and osteopetrosis [5].

ALV infection is dependent on the presence of subgroup-specific cellular receptors, and birds which lack the specific receptors are genetically resistant to infection by the respective ALV subgroup [39,40]. Introduction of genetic resistance to ALV subgroups such as A and B has been used by poultry breeding companies through selective breeding as part of the eradication strategy. More recently, introduction of de novo resistance using gene editing approaches has also been demonstrated for ALV subgroup J infection [41,42].

Tumors induced by ALV occur by two main types of mechanisms. The first one is the activation of a cellular proto-oncogene after ALV integration. The activation of cellular oncogenes leads to neoplastic transformation, the mechanism being described as “insertional mutagenesis” [5]. As this mechanism of induction is usually slow, taking several weeks or months to manifest, these viruses are termed “slowly transforming.” The second mechanism occurs through the transduction of cellular oncogenes into the ALV genome. Such viruses carrying the transduced oncogenes induce tumors rapidly and are termed “acutely transforming.” Examples of such viruses include MC29 and 966 that carry v-myc [43,44] and avian myeloblastosis virus that carries v-myb [45].

ALV can be transmitted either by vertical (congenital or egg) or by horizontal routes through contact. In vertical transmission, eggs become contaminated with the virus within the oviduct leading to the infection of embryos during development. This route of congenital infection results in the birds becoming viremic carriers with shedding of virus in vaginal swabs, egg albumen, and embryos, the detection of which provides the basis for ALV eradication programs. Congenitally infected chicks

are important sources of contact infection in the hatchery and during the brooding period, and meconium and feces from congenitally infected chicks contain high concentrations of ALV. The horizontal mode of spread is responsible for the high incidence of infection in flocks.

Eradication of ALV depends on breaking the virus's vertical transmission from dam to progeny and preventing reinfection. The procedures for eradication depend on the identification and elimination of hens that shed ALV to their egg albumen and hence to their embryos and chicks. Such hens, which are viremic and shedders of the virus, are usually identified by testing of their cloacal/vaginal swabs, semen, or egg albumen by ELISA for the presence of high levels of ALV gs-antigen [46]. Continuous monitoring and elimination of infected birds will break the spreading cycle of the virus and lead to ALV eradication [5,36,47].

17.2.3 Reticuloendotheliosis

Reticuloendotheliosis refers to a group of syndromes in poultry and game birds associated with REV [6,48,49], belonging to the family *Retroviridae*. Syndromes associated with REV include Runting disease syndrome, bursal and thymic atrophy, enlarged peripheral nerves, abnormal feather development, proventriculitis, enteritis, anemia, and necrosis of liver and spleen. Abnormal feathering, in which the barbule of wing feathers are adhered to the feather shaft, is termed "nakanuke" in Japanese and has been seen in flocks vaccinated with REV-contaminated vaccines [6,50]. REV also induces chronic lymphoid neoplasm in the bursa of Fabricius and other organs. These tumors are of B-cell origin and are caused by insertional activation of the cellular myc oncogene. Nonbursal lymphomas of T-cell origin have also been induced experimentally, with latent periods as short as 6 weeks involving the thymus, liver, heart, and spleen.

Reticuloendotheliosis (acute reticulum cell neoplasia) is induced by the defective T strain of REV that carries the v-rel oncogene [51–53]. When injected into young chicks, the T-strain, originally isolated from a turkey with leukotic lesions, induces rapid proliferation of primitive mesenchymal or reticuloendothelial cells and causes death within 1–3 weeks. REV is transmitted both horizontally by contact with infected chickens and turkeys, and vertically from tolerantly infected chicken and turkey dams, as well as from infected males.

Because of the usually sporadic and subclinical nature of REV infections in chickens and turkeys, large-scale control procedures have generally not been considered necessary in commercial poultry production. However, freedom of infection in breeding flocks that produce progeny for export is required by some importing countries. REV eradication could be achieved by detecting and

removing REV gs-antigen ELISA-positive shedding dams and sires, and by rearing the progeny in isolation, using methods similar to those for LL.

17.3 Oncogenic mechanisms of tumor viruses

In eukaryotes, the normal processes of cell division, differentiation, and death are controlled in a tightly regulated and coordinated fashion, thanks to the highly complex integrated, intricate molecular circuitry of growth regulatory pathways. Such tight regulation keeps vital checks on cell proliferation and destroys any damaged or mutated cells by physiological processes such as apoptosis. Neoplastic transformation is the end result of the disruption of one or more of these critical pathways, which leads to the uncontrolled cellular proliferation and inappropriate survival of damaged cells. Understanding the specific molecular pathways associated with oncogenesis is often difficult because of the complexity and the integrated nature of these regulatory networks. As efficient inducers of neoplastic transformation, oncogenic viruses exploit the weaknesses of the regulatory networks to surpass the rigid checks on cell proliferation. This exploitation results in uncontrolled cell proliferation and neoplasia. In this respect, oncogenic viruses have been some of the oncologist's greatest allies for gaining necessary intelligence on the molecular mechanisms of induction of neoplasia.

In the past few decades, studies on the molecular events and interactions in cells infected by different oncogenic viruses have helped us to understand some of the critical pathways of oncogenesis. These include the remarkable demonstration of activation and/or "capturing" of cellular genes by retroviruses to induce cellular transformation, which has also led to the discovery of the first oncogene v-src [54,55]. Several DNA viruses as well, through the molecular interactions of their viral proteins, have contributed to the fundamental insights into oncogenic mechanisms. These include the mechanisms of transformation by viruses such as the simian virus SV40 through its large T antigen, adenoviruses through their E1A/E1B proteins, human papillomaviruses (HPV) through their E5/E6/E7 proteins, and Epstein Barr virus through their nuclear antigens [56,57].

Many of these viral gene products target critical points to disrupt the molecular events in the regulation of cell cycle, transcription, tumor suppressor genes, and apoptosis [58,59]. Investigations into oncogenic viruses can facilitate the distinction between driver and passenger mutations in the cancer genome, as it is becoming clear that the trans-acting viral proteins and cis-acting cancer genome variation converge on common pathways.

17.3.1 Oncogenic mechanisms of retroviruses

The contributions of avian retroviruses to the fundamental understanding of the mechanisms of neoplastic transformation are remarkable. The initial description of transmission of tumors [60], followed by the Nobel Prize-winning discovery of transmissible tumors using filtrates of tumor extracts [55], paved the way for elucidating some of the intricate molecular mechanisms of oncogenesis. Another Nobel Prize-winning discovery was on insertional activation of proto-oncogene by avian retroviruses [61,62].

Based on their oncogenic mechanisms, retroviruses can be broadly grouped into nonacute viruses and acutely transforming viruses that differ markedly in oncogenicity, tumor tropism, and oncogenic mechanisms.

Nonacute retroviruses: Nonacute retroviruses are replication-competent viruses that contain full complement of viral genes that induce tumors by integrating the proviral DNA to induce insertional activation of host proto-oncogenes or genes involved with growth control [63]. Insertional activation of several oncogenes has been reported in ALV-induced tumors [64–69]. Tumors induced by this process are usually monoclonal or oligoclonal and occur after long incubation periods. Insertional activation of c-myc has been observed in LL [64,70] induced by ALV subgroups A/B, as well as ML by subgroup J [44]. ALV integration in c-bic and insertional activation of noncoding gga-miR-155 has been reported in LL tumors and transformed B-cell lines [71–73]. Similarly, insertional activation of Telomerase reverse transcriptase (TERT) expression through hypomethylation in the TERT promoter region has also been reported in ALV-induced B-cell tumors [74].

Acute retroviruses: Compared to the slow onset and mono/oligoclonal nature of tumors induced by nonacute retroviruses, acute retroviruses can cause rapid-onset polyclonal tumors. Many of them transform cells in vitro to produce transformed foci in cell cultures, providing a convenient assay for their identification. This ability of the acute retroviruses to transform cells in vitro and produce tumors in vivo is due to the presence of viral oncogenes in their genomes. These oncogenes are transduced by the viruses from the host genomes by a process of recombination [63]. In nearly all cases, transduction of oncogenes results in deletion of parts of the viral genome making these viruses replication defective. Structure of the defective viral genome differs between viruses. However, all these viruses require helper viruses for their propagation. The potential of acute retroviruses as very potent carcinogens to induce rapid-onset polyclonal tumors is related to the high level of expression of oncoproteins mediated by the viral LTR and continuous selection of mutations that enhance the oncogenic function. A large number of acute retroviruses carrying a wide

range of viral oncogenes, most of which are associated with signal transduction, have been identified. Examples of acute retroviruses include MC29 virus-containing v-myc [43], MH2 virus-containing v-myc and mil [75,76], avian myeloblastosis virus carrying the v-myb oncogene [45], and the acute-transforming ALV-J strain 966 expressing v-myc as a gag-myc fusion protein [44]. Studies on the REV-T strain expressing the v-rel have identified several molecular pathways including the induction of miR-155 [52,53] and TGF- β /Smad signaling [77] involved in the transformation process.

17.3.2 Oncogenic mechanisms of DNA tumor viruses

DNA viruses belonging to different families have been associated with tumors in humans and animals [56,78,79]. Compared to the RNA tumor viruses that activate host genes or use transduced host genes to transform their cell targets, most oncogenic DNA viruses encode their own proteins to induce neoplastic transformation. Many of these viral proteins, despite their diverse origin, act by interfering with cell functions at critical points of the cellular regulatory network. Some examples of such interactions include those of the SV40 large T antigen, the adenovirus E1A/E1B, and the HPV E5/E6/E7 proteins that interfere with tumor suppressors p53 and retinoblastoma [58,59].

Among the oncogenic DNA viruses that induce tumors of the cells of the avian immune system, MDV that induces rapid-onset T-cell lymphomas in poultry is the most important [4,11]. The induction of T-cell tumors in genetically susceptible birds occurs after a robust early cytolytic infection and a period of latent infection in lymphocytes [80,81].

The MDV genome contains more than 100 potential open reading frames, which include both those that are unique to MDV and those that are homologous to other herpesviruses [82]. Genome-wide sequence comparisons between virulent and attenuated strains together with the analysis of viral gene expression in MDV-transformed tumor-derived cell lines have indicated that the genes within the repeat region of the genome are most likely to be associated with oncogenicity of the virus. These include the major oncogenesis-associated genes such as MEQ (MDV *EcoRI*-Q) fragment [83], virus-encoded RNA telomerase subunit [84,85], and a number of microRNAs [4,86].

17.4 Immune responses to oncogenic viruses

The immune system has primarily evolved to eliminate extraneous agents. While the immune responses to oncogenic viruses follow similar patterns used against other

viruses, persistence of oncogenic viruses, usually in a latent state, requires the immune system to adopt a strategy of coexistence with the virus. This would also require steps to contain the multitude of immune evasion strategies of these viruses [87]. Despite its importance and decades of research, the complex nature of the immune responses against oncogenic viruses largely remains unclear [88].

17.4.1 Immune responses to leukosis/sarcoma viruses

Humoral responses mediated through specific neutralizing antibodies are the essential components of immunity against retroviruses. This can clearly be demonstrated in congenital egg-transmitted ALV infections, where the absence of neutralizing antibodies is usually associated with high levels of viremia and virus shedding [5]. Most of these antibodies are directed against the epitopes on the surface of the viral envelope, although responses have also been directed against other retroviral proteins [89–91]. Subgroup-specific neutralization of ALV, however, is associated with the envelope glycoproteins, where most of the diversity and escape mutations also seem to concentrate [92]. Nonneutralizing antibodies that function through antibody-dependent cell cytotoxicity mechanisms have also been reported [93].

Major histocompatibility complex (MHC)-restricted CTL-mediated immune responses [94] have also been identified against retroviral infections. The best example of this is the difference in susceptibility to Rous sarcoma virus (RSV)-induced tumors by different haplotypes of chicken lines [95–97]. Conserved peptide motifs of the RSV proteins that bind to the MHC have been shown to be protective against RSV tumor growth in chickens with the B-F12 haplotype [98,99]. Recent study also showed that expression levels of MHC class I were inversely correlated with promiscuity of peptide binding [100]. In further support of the role of T-cell-mediated immunity against Rous sarcoma, it has been demonstrated that neonatal thymectomy prevented tumor regression [101,102] and that CD8⁺ T cells isolated from birds recovered from v-src-induced sarcomas were able to protect naïve birds [103]. Influence of Bu-1 locus on RSV tumor regression and Th-1 locus on LL is also known [104].

Other cell types such as NK cells and macrophages also play a role in immune responses against RSV tumors. For example, it has been demonstrated that ALV-transformed cell lines (e.g., LSCC-RP9) can be lysed by NK cells [105]. Similarly, macrophages from RSV tumor regressing lines of chicken showed more cytotoxicity on these cell lines demonstrating the role of these cell types in immunity against RSV tumors [106]. Importance of the

interaction between the MHC and CTL in ALV has been demonstrated using RCAS vector expressing MHC chicken class I [107].

17.4.2 Immune responses to reticuloendotheliosis virus

As in the case of ALV, neutralizing antibodies play an important role in immunity to REV [6]. The antibodies against gp90 of the virus are thought to be the most important in protection. This is because recombinant fowlpox virus expressing the glycoprotein [108] or recombinant protein expressed in *Pichia pastoris* [109] protected against viremia after challenge. In addition to humoral immune responses, cell-mediated immunity is considered to be a major element because REV has been used as an important model for studying virus-specific, MHC-restricted CTL responses in chicken [110,111]. CD8⁺ CTL against REV have been identified, although the importance of in vivo responses in REV infection has not been ascertained [112]. However, neonatal thymectomy increased REV-induced mortality in birds challenged with the T strain virus, suggesting that cell-mediated responses are important for protection [113]. Cell-mediated immune responses and NK cells may also influence anti-REV immune responses [114]. Studies have also shown that v-Rel induces expression of class I and II MHC, and interleukin-2 receptor more efficiently than does v-Rel [115].

17.4.3 Immune responses to Marek's disease virus

As a disease controlled almost entirely using live attenuated and antigenically related avian herpesviruses, immune responses are very important in MD. Several studies have looked extensively into the role of immune responses in MD [4,9]. While these studies have undoubtedly identified some of the important cells and pathways of the immune responses to MDV infection and vaccination [116–120], complete picture on interaction of avian immune system with MDV during different stages of the disease is still to emerge.

Vaccine-induced immunity is thought to be primarily an antitumor response, since MDV vaccines do not prevent superinfection with the virus. However, vaccines do reduce cytolytic infection, thereby preventing extensive damage to the immune system through the continued destruction of immune cells. Innate immune responses through Toll-like receptors [121,122] and NK cells [119,123] have been demonstrated in infected and in vaccinated birds. In this context, downregulation of MHC class I by the MDV UL49.5 gene product and its potential

influence on virulence in a haplotype-specific fashion [124] has to be considered, particularly in triggering NK cell responses. Innate immune responses against MDV also include changes in cytokine expression [125]. The Th1-type response induces increased transcription of inducible nitric oxide synthase II, as well as enhanced NK cell and macrophage activity [126]. Interferons also had a positive influence on the antiviral immunity [118,127], and augmented expression of IFN- γ and IL-10 after vaccination enhanced T-cell infiltration into the lungs [128]. Recent studies also demonstrated that MDV can antagonize interferon-mediated innate immune responses by acting downstream of cGAS-STING pathway as well as by antagonizing NF- κ B activation [129,130].

Both humoral and cell-mediated immune responses are initiated after natural infection or vaccination with MDV. However, the importance of humoral immune responses in protection against the disease is believed to be relatively minor because of the highly cell-associated character of the virus. However, the presence of maternal antibodies is thought to confer protection against early cytolytic stages of infection and may also interfere with vaccine-induced immunity, especially when cell-free vaccines are used. Even though the role of antibodies in protection against MDV infection is minimal, B-cells do play an important role as targets for the early cytolytic infection of MDV. However, a recent study using gene-edited chickens lacking mature peripheral B cells [117] confirmed that early B-cell infection is not critical as demonstrated previously using B cell depletion approaches [131].

Cell-mediated responses through specific CTL have been identified as critical components of immunity against MD. The crucial role of CD8⁺ T cells in controlling MDV infection has been confirmed using CD8-deficient chickens [132]. The role of MHC-restricted, antigen-specific CTL in immune responses against MD has been demonstrated using REV-transformed MHC-defined lymphoblastoid chicken cell lines that stably express individual MDV genes [126]. However, the role of MDV-specific CD8⁺ T cell responses in controlling viruses and/or tumor growth in infected or vaccinated birds is still to be demonstrated. Similarly, the role of MDV-specific T-cell responses in genetically resistant versus susceptible chickens in protection against the disease remains unknown. Induction of TGF β + Treg cells is reported to be associated with the pathogenesis of MD, with COX-2/PGE2 pathway involved in immunosuppression induced by MD tumor cells [133].

17.5 Antitumor responses

In addition to its major role in protecting against the multitude of pathogens, a properly functioning immune

system is vital in the fight against neoplastic diseases. Neoplastic transformation is the result of dysregulated proliferation resulting from a multistep process involving mutational events in multiple genes such as tumor suppressor genes, transcriptional regulatory genes, and proto-oncogenes. As neoplastic transformation is a dynamic process, recognition and elimination of tumor cells by host defenses are essential for maintaining homeostasis. One of the recognized outcomes of the antitumor immune responses is the “spontaneous” tumor regression observed in many species, including birds. Antitumor immunity stems from the ability of the immune system to recognize and destroy individual tumor cells. The arms of the innate, as well as the acquired, immune system are thought to participate in antitumor immunity.

For a tumor cell to elicit an immune response, the B and T lymphocytes must recognize specific epitopes from the antigens expressed on them. These tumor antigens, either intra- or extracellular, are presented in an immunological context with MHC class I by the antigen-presenting cells to naïve T cells, which in an appropriate cytokine environment, are activated into various forms of effector cells (see Chapters 9 and 10). Increasing data demonstrate the role of extracellular vesicles (EVs) in cell to cell communication. Comparative analysis of the serum EVs from vaccinated birds and naïve MD tumor-bearing birds showed significant differences in miRNA profiles, which can help in biomarker discovery and insights into vaccine responses [134].

The tumor antigens recognized by the immune system in this manner can be derived from the host itself or can be viral antigens. The host-derived tumor antigens include (1) differentiation antigens (e.g., carcinoembryonic antigen) that are upregulated at an incorrect time in ontogeny; (2) tumor-specific shared antigens (e.g., melanoma-specific MAGE) that are normally expressed only in immunologically privileged sites; (3) tumor-specific mutated antigens (e.g., mutated *ras*); and (4) overexpressed normal antigens (e.g., HER-2/neu).

Compared to the immune responses against avian tumor viruses, very little data are available on the nature and significance of antitumor responses. Regression of RSV tumors in genetically resistant birds is strongly associated with antitumor immune responses directed against src [98,99]. In MD, it is often suggested that vaccine-induced immunity is predominantly an antitumor response, although vaccines do influence the reduction of cytolytic infection. Immunomodulatory processes in chronic persistent infections and tumors are mediated by inhibitory receptors such as CTLA4, programmed cell death-1 (PD-1), and its respective ligand programmed death ligand-1 (PD-L1). While the role of the immune checkpoint molecules in avian oncogenic virus-induced tumors needs further studies, characterization of chicken

PD-1, PD-L1, and the specific antibodies [135] opens the potential to explore the role of PD-1/PD-L1 pathway in the latency mechanisms and immunosuppression in oncogenic viral infections of chickens.

A group of antigens, generally referred to as MD tumor-associated antigens (MATSA), have been identified on MDV-transformed cells [136], the majority of which are markers of T-cell activation. One such MATSA, recognized by the monoclonal antibody AV37, is the chicken homolog of CD30, a member of the tumor necrosis factor receptor II family [137,138]. Increased expression of the CD30 on MDV-transformed tumor cells, demonstrated to be due to hypomethylation [139], and detection of specific anti-CD30 immune responses in infected MD-resistant chickens suggest that immunity to CD30 could be a mechanism of antitumor responses against MD lymphomas [140].

17.6 Conclusion

In summary, the tumors of the immune system caused by the avian oncogenic viruses, constitute a major source of economic loss to the poultry industry. In addition to providing a better understanding of the pathogenesis of these diseases, these tumors present opportunities to gain insights into various facets of the avian immune system. Since a well-functioning immune system is crucial both for responding to challenges from a multitude of pathogens and for generating adequate immune responses to the cocktail of vaccines administered in the lifetime of a chicken, effective control measures against tumors of the immune system are vital for preventing direct losses from these diseases.

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Practical aspects of poultry vaccination

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18.1 Introduction

Commercial poultry is vaccinated routinely to protect the birds against infection or disease caused by several pathogens. For parent flocks, vaccination can also be aimed at inducing a high level of maternally derived antibodies (MDA) for protection of the progeny by prevention of vertical transmission or early challenge. The number of vaccines that are used in a particular flock depends on many factors such as disease pressure, pathogenicity of the pathogen, life span of the chicken, type of chicken, efficacy, availability, and costs of the vaccines, labor, regulations, efficacy of MDA, housing system, and relevance of vertical transmission.

Broilers with a short life span are commonly vaccinated during the first few weeks of life with a limited number of vaccines mostly against viral pathogens and sometimes against coccidia. Usually, the first vaccinations are already applied in the hatchery. Long-living layers, breeders, and grandparents are vaccinated against a list of pathogens, using live and inactivated vaccines. Due to the use of several vaccines in a relatively short period, vaccination programs should be designed considering the potential of interference between vaccines.

Vaccines can be applied individually or by mass application. Mass application by spray, feed, or drinking water is relatively cheap, fast, and safe regarding potential breaks of biosecurity as few people can vaccinate many birds in this way. An individual application is used for inactivated vaccines and for live vaccines that cannot, or cannot in a reliable way, be applied by a mucosal route.

18.2 Vaccine types

To date, there are two main types of vaccines available for poultry: live and inactivated. Many vaccines are lyophilized, several are also available in tablet form, and some vaccines are available deep-frozen. In general, a

vial, tablet, or bottle of live or inactivated vaccine contains at least 1000 doses, making the availability of vaccines for back-yard poultry very limited.

18.2.1 Live vaccines

The immune response induced by a live vaccine is influenced by the type of microorganism, the degree of attenuation, the delivery route, the site of replication, as well as the age and immune status of the bird. Live vaccines in regular use within the poultry industry include those with antiviral, -bacterial, and -parasitic specificity. Due to their ability to replicate in the host, live vaccines induce a variety of innate and adaptive immune responses, the onset, magnitude, duration, and quality of these responses being associated with the unique characteristics of each vaccine. Many live vaccines can be applied by spray of drinking water, an advantage when one needs to vaccinate large numbers of birds.

The wide-spread use of live vaccines in the poultry industry also reflects the requirement for an early onset of immunity preferably by a single application, with the chicks sometimes exposed to field challenge shortly after vaccination. For several diseases, a single vaccination with a live vaccine provides life-long protection, for others, revaccination with live and or inactivated vaccines is needed, especially in long-living birds.

There are three main groups of live vaccines available for poultry: (1) attenuated live vaccines; (2) immune-complex vaccines; (3) genetically modified vector-based live vaccines.

Attenuated live vaccines

In general, the traditional live vaccines in use by the poultry industry have been attenuated by serial passage in cell culture, eggs, or embryo-derived tissues, with the aim of maintaining the immune response induced by the parent organism while attenuating the ability of the microorganism

to cause disease or immunosuppression. Some live bacterial vaccines have been attenuated by removing a gene related with the pathogenicity or decreasing the ability to replicate. Examples are vaccines for *Escherichia coli* [1], *Salmonella typhimurium* [2], and *Salmonella montevideo* [3].

The residual pathogenicity of the vaccine strain varies from very low to mild or moderate, depending heavily on the pathogen involved. The clinical response to the vaccination is called the vaccine reaction. The intensity of the vaccine reaction is only partly related to the residual pathogenicity of the vaccine strain. Other factors, such as quality and application method of the vaccine, general health of the chicks, presence of other vaccines or coinfections, age, and presence of MDA, play an important role as well. For several pathogens the presence of MDA can neutralize the vaccine [e.g., infectious bursal disease virus (IBDV), chicken infectious anemia virus (CIAV), avian encephalitis virus]. In general, vaccines with a mucosal primary site can be applied in the presence of MDA as most of the MDA consist of IgY. The presence of MDA at the time of the vaccination with these mucosal vaccines however may lower the humoral response despite the induction of protection.

Immune-complex vaccines

A mucosal application of a live attenuated IBDV vaccine in the presence of MDA will result in neutralization of the vaccine. Recently, a live vaccine strain for *in ovo* application or subcutaneous injection at day of hatch was introduced in some countries that was able to overcome the MDA under experimental conditions (De Wit, personal communication). A way to avoid this neutralization that is used worldwide is to inject an IBDV immune-complex vaccine *in ovo* or at day of hatch by the subcutaneous route. In an immune-complex vaccine the virions are covered with specific IBDV IgY antibodies. This cover of antibodies prevents the neutralization of the vaccine virus by the MDA. The immune complex will then be retained in the follicular dendritic cells of lymphoid organs from which the virus will be released continuously. These virions will be neutralized by the MDA if the level of MDAs is above a certain level. At a certain age of the chicken, depending on the initial level of MDAs, the level of MDA will be decreased sufficiently and the virus released will not be neutralized anymore, will reach the target organ bursa of Fabricius, and will start to replicate in the bursa and induce an immune response [4].

Genetically modified organisms

Besides live vaccines that have been attenuated by removing a gene related with the pathogenicity or decreasing the ability to replicate, an increasing number of genetically modified live vaccines have been introduced with

one or more inserted genes from other pathogens. Fowl pox virus (FPV) or herpesvirus of turkeys (HVT) are used as vectors for the insertion of genes. Especially HVT-based vectors are used worldwide. Both vectors do not spread horizontally and must be injected individually into the embryo (*in ovo*) or at hatching. Examples of available vaccines are HVT vaccines [5] carrying genes such as the fusion (F) gene of Newcastle disease virus (NDV) [6,7], the glycoproteins (gI and gD) genes of infectious laryngotracheitis virus (ILT) [8,9], the outer capsid (VP2) gene of IBDV [10–12], or the hemagglutinin (H5 and H7) genes of avian influenza virus (AIV) [13–17]. Examples of available FPV vector vaccines are vaccines carrying genes such as the fusion (F) and hemagglutinin-neuraminidase genes of NDV [18], the UL-27 and UL-34 genes of (ILT) [9], the H5 gene of AIV [19,20], and the 40k and mgc genes of *Mycoplasma gallisepticum* [21]. Recently, vector vaccines capable of protecting simultaneously against multiple diseases have become available, for example, HVT vaccines with inserts of the F gene of NDV and the VP2 gene of IBDV.

Vectors other than FP and HVT would be useful as the combination of more than one HVT-vectored vaccine or more than one FP vaccine in the same bird will result in interference causing decreased protection (see Section 18.4.6). Aviadenovirus group A, C, D, and E vectors have been evaluated and shown promise in veterinary and human health [22–25]. Aviadenovirus vectors were able to induce protection against infectious bronchitis virus (IBV) [26,27] and AIV [28–32] in specific pathogen-free (SPF) birds. The level of protection induced by these adenovirus vector vaccines might differ in SPF birds and commercial birds with MDA as high levels of MDA to H5 interfered with vaccine efficacy against challenge by AIV H5 [32]. Attempts have been made to develop live vaccines with ILTV [33], AIV [34,35], or NDV [36] as vector for *in ovo* vaccination that are able to provide improved mucosal immunity over HVT vectors, but none are considered safe and effective for commercial application. Nonreplicating human adenovirus vectors have been successfully tested as experimental AI vaccines in poultry [30,31,37–39].

18.2.2 Inactivated vaccines

Inactivated vaccines are mostly used to vaccinate future laying birds (layers, breeders, grandparents, and pedigree stock) prior to the laying period to provide a long-lasting protection against challenge by inducing high levels of serum IgY. Most inactivated vaccines are based on whole antigens. When compared to replicating live vaccines, nonreplicating antigens, and especially the purified or recombinant subunit versions, lack immunogenicity and are, therefore, mostly formulated with immune-potentiating vaccine adjuvants. For

most common pathogens, inactivated vaccines are used to boost immunity that has been provided by priming with live vaccines [e.g., IBV, NDV, avian metapneumovirus (AMPV), reovirus, or IBDV vaccines]. For some diseases the inactivated vaccines are expected to provide life-long immunity without prior priming [e.g., egg drop syndrome (EDS) virus vaccines]. The use of inactivated vaccines in parent, grandparents, and pedigree birds also confers MDA to progeny (maternal antibody; see [Chapter 6](#)), which is especially important for the control of IBDV and reovirus infections. MDA are also very important for CIAV, but inactivated vaccines are not on the market, only live CIAV vaccines. In regions with a high infection pressure by NDV or AIV at a young age, inactivated vaccines may be administered to broilers usually at 1 day of age simultaneously with a live vaccine in the case of NDV or alone for AIV.

Inactivated vaccines are given either by the intramuscular or subcutaneous route using automatic syringes. The subcutaneous injection is usually in the back of the neck, preferably halfway or 2/3 between head and shoulders to prevent potential limitation of the head movements for eating and drinking due to the transient, local inflammation caused by the adjuvanted vaccine. In general, adjuvants used for bacterial vaccines (bacterins) tend to cause more severe local inflammations, especially if they are injected when the vaccines are still cold.

18.2.3 Poultry vaccine adjuvants

Most vaccine adjuvants used for poultry include empirically selected, classical formulations, such as water-in-oil (W/O), oil-in-water (O/W), saponins, and alum-based formulations. In addition to a major influence on the level of the antibody response, the adjuvant also has a major influence on the level of local and systemic reactions. Appropriate adjuvants also lower the amount of antigen that is needed per dose. The number of published papers comparing several adjuvants is limited. Lone et al. [40] compared ten commercially available or experimental adjuvants in their ability to induce antibodies and protection against H7N3 AIV. Mineral- and vegetable oil-based adjuvants generally induced the highest antibody titers with 100% seroconversion by 3 weeks postvaccination. Chitosan also induced positive antibody titers in 100% of the chickens, but the titers were significantly lower than those of most of the oil-based adjuvants. Antibody levels from calcium phosphate and alginate adjuvanted vaccines were like the titers from nonadjuvanted vaccines.

It is important to realize that using a good adjuvant alone is not a guarantee for a good vaccine as several other factors play a role as well such as quality and amount of antigen, homogeneity of the emulsion, and good stability within the expiration time of the product. The trend to include many distinct antigens in one

formulation may hamper vaccine efficacy as a result of interference between antigens in the final formulation or in the animal [41].

18.3 Vaccine application

The popularity of chicken meat has driven the growth of the poultry industry around the world and effective vaccine application is an everyday challenge to the poultry professional worldwide. Vaccination of broilers has greatly migrated to the hatchery, where the most common administration methods involve mass application including *in ovo* injection of eggs at 18–19 days of embryonation and spray vaccination at the day of hatching. Individual applications such as subcutaneous or intramuscular injection, intraocular, beak, and nasal drop at the day of hatching are used in the hatchery and at the farm. Application methods at the farm include spraying, addition to drinking water, wing-web stabs, and subcutaneous or intramuscular injections. Individual vaccine administration is believed to be more effective because, at least in theory, all birds get vaccinated with the same amount of antigen. However, it is impractical to individually vaccinate the large numbers of chickens grown worldwide for human consumption. It is highly recommended that poultry operations keep written protocols for each type of vaccination to assure consistency of good practices and effectively prevent disease in poultry flocks.

18.3.1 Mass application

To accomplish vaccination of approximately 24 billion chickens annually worldwide, avian vaccines are routinely administered using mass-application methods. Coarse spray in the hatchery at the farm and drinking water at the farm is perhaps the most widely used mass vaccination methods. The success of mass application of avian vaccines depends on a multiplicity of factors, which will be discussed in detail for each method.

Spray vaccination

Coarse spray vaccination is a widely used method in poultry that deposits vaccines in the upper respiratory tract [42–44], where it elicits immune responses detectable as early as 3 days after vaccination. Around 2–3 weeks postvaccination optimal antibody titers are present conferring protection against disease [45]. In the modern poultry operation, coarse spray vaccination against Newcastle disease, infectious bronchitis, AMPV, and coccidiosis are standard procedures in the hatchery and poultry houses [46–51]. Other products such as live *Salmonella* vaccines and probiotics, among others, are also applied by spray in

the hatchery [52]. Spray administration has also proven successful to immunize chickens against IBD [53].

There are many devices available to perform spray vaccination at the hatchery or chicken houses. In the hatchery, equipment normally consists of a pressure pump and nozzles that are set in a vaccination cabinet or as an in-line spray over the chick conveyor belt. These pieces of equipment usually deliver 7–20 mL/100 chicks in droplet sizes ranging between 70–150 μm . For coccidiosis vaccines the volume increases to 20–30 mL/100 chicks and vaccine uptake is proposed to occur by self and hatch mate preening [49]. Vaccines for spray application are usually diluted in distilled water. More recently, gel application has been introduced for hatchery vaccination to prolong preening and improve coccidiosis vaccination efficacy [48,54,55]. Spray equipment used for vaccination in poultry houses delivers droplet sizes ranging from 100 to 150 μm and usually has a longer reach to allow vaccination of birds on the floor (broilers, layers, breeders) or in cages (broilers, layers). It is very important to use only poultry-specialized equipment to ensure proper vaccine delivery and good coverage of the flock. Improper spray practices or inadequate equipment may result in fine spray with droplets of less than 20 μm penetrating deep into the respiratory tract of the bird, producing excessive reactions to respiratory vaccines and compromising vaccine efficacy. Good spray vaccination application in the hatchery usually starts by checking the equipment and doing a test spray using paper in the bottom of the chick box confirming that the droplets cover the whole surface. Problems with pressure, defective nozzles, or obstruction of the spray will cause an irregular pattern and uneven spray vaccination. Spray pattern checks should be conducted several times during the vaccination period. At the same time the total volume sprayed by each nozzle should be measured with a graduated cylinder to ensure that each nozzle is delivering the right vaccine volume. For spray vaccination in the poultry house, mechanic ventilation should be stopped to avoid drafts that may take the spray away from the chickens. Enough vaccine solutions should be prepared to ensure coverage of all birds in the house.

Drinking water vaccination

Drinking water vaccination is a very useful method that allows vaccination of large numbers of birds in a relatively short amount of time. Optimal results require that several basic steps be followed before, during, and after vaccination. (1) Eliminate residues of chemicals and disinfectants from the water lines at least 2 days prior vaccination. Any previous water treatment or medication should be discontinued at least 2 days before and after vaccination. Commercially available stabilizers [56],

skimmed milk powder (2 g/L of vaccine water), or cold sterilized skimmed milk (1 L per 50 L of vaccine water) [57,58] are commonly used. Flushing of water lines and checking of drinker systems is also recommended before vaccination to allow proper flow and distribution of the vaccine. (2) To achieve a successful vaccination, birds need to have some degree of thirst, which is normally created by limiting the access to drinking water typically for 2 hours prior to vaccination. This period may be adjusted depending on the house environment and climate conditions. (3) The vaccine needs to be made available for 2–4 hours to ensure that all birds have a chance to drink, which is important to accommodate the pecking order in the flock and seasonal changes that affect water consumption. (4) After the vaccine is circulating in the drinker system, it is very important to walk along the house along drinker lines to stimulate water consumption.

18.3.2 Individual applications

Eye-drop or intranasal vaccination

Vaccinations by intraocular or intranasal routes are practiced by using a vaccine manufacturer-provided dropper that typically delivers 0.03 mL of vaccine solution in the eyes or nares. A compatible dye is usually added to the vaccine diluent to evaluate vaccination accuracy in the hatchery or poultry house. When using this method special attention needs to be given to the cold chain, since vaccines can warm rapidly in the hand of the vaccinator and negatively affect the vaccine titer. This method is very effective but is labor intensive and requires trained personnel. Vaccination by eye-drop was popular in the early stages of the poultry industry and it has been gradually replaced by drinking water and spray vaccination. In modern poultry operations, eye-drop vaccination is usually limited to the administration of ILTV vaccine [59,60], although water vaccination for ILT is widely used in broilers. Live Mycoplasma vaccines are also commonly applied by eye-drop during rearing of parent and layer flocks.

Subcutaneous and intramuscular injection

Several live vaccines such as Marek's disease (MD), fowl pox, and reovirus vaccines, among others, are administered at the hatchery by the subcutaneous route. Inactivated viral and bacterial oil emulsion vaccines are administered by subcutaneous or intramuscular injection in layer, broiler breeder, and turkey breeder pullets prior to the onset of egg production. The use of inactivated vaccines in breeders is critical for the transfer of antibodies to the progeny for early protection against field challenge, especially with immunosuppressive viruses [61,62]. Subcutaneous injections are usually applied under the

skin of the neck between the head and the shoulder using an 18-gauge, 0.25–0.50-inch needle. In intense challenge conditions, subcutaneous vaccinations are also used in broilers, especially to apply inactivated vaccines against velogenic Newcastle disease and avian influenza. Intramuscular injections are usually applied with an 18-gauge, 0.25–0.50-inch needle to deliver inactivated vaccines in the breast or leg. For breast vaccination the vaccine should be deposited in the superficial pectoral muscle 2–4 cm from the keel bone in the upper half of the breast to avoid accidental abdominal injections. Intramuscular injection in the leg is usually applied in the gastrocnemius muscle of broiler breeder pullets. Both types of injections require periodical changing of the needle during the process to avoid secondary bacterial infections. The response to subcutaneous or intramuscular vaccination depends on the type and quality of the antigen used and the consistency of the injection process across the flock.

Wing-web inoculation

The wing-web stab vaccination is a method requiring individual handling of every bird. It involves extending the wing, locating the wing web, and vaccinating using an individual stabber with a plastic handle and two stainless steel prongs with a sharp bevel on the tip with small holes to hold the vaccine. Vaccination using the wing stabber requires that the prongs be submerged in the vaccine before each application. There is another type of wing-web vaccinator that is a syringe with a barrel that can hold a larger amount of vaccine and two prongs for wing-web stabbing. Using this device avoids having to dip the prongs in the vaccine for each dose like the individual stabber. As mentioned for eye-drop vaccination, maintaining the cold chain is critical to ensure the integrity of the vaccine throughout the vaccination process. Live fowl pox, avian encephalomyelitis, and CIAV vaccines in chickens and live *Pasteurella* vaccines in turkeys are commonly applied using this method [63]. Wing-web vaccination is usually evaluated around 7 days after vaccination by looking for scabs in the form of small nodules that can be palpated on the inoculation site, commonly known as “vaccine takes.” The correlation between the vaccine “takes” and protection may vary depending on the type of bird vaccinated and the vaccine applied [64,65].

In ovo vaccination

Vaccination *in ovo* was developed for vaccination against MD [66,67] and mass-application devices started to be available to the poultry industry in 1992. *In ovo* vaccination is usually applied when eggs are transferred from the setter to the hatcher around embryo incubation day (EID)

18–19 to protect chickens against MD using the HVT vaccine. The main use of *in ovo* vaccination continues to be for delivering MD vaccines and HVT-vectored vaccines to protect against MD and other diseases. The process starts by poking a hole in the eggshell and injecting 0.05–0.1 mL of vaccine by inserting a needle into the broad end of the egg [67]. The site of the deposition of the inoculum in the egg is somewhat variable and depends on the stage of embryonal development at the time of vaccination [68,69], the quality of the vaccination procedure, and the age of the hens that will, in turn, determine the size of the eggs. Vaccination of embryos involves more than simply puncturing eggs. The vaccine may be deposited in several different compartments of the egg and this can negatively impact the efficacy of the vaccine applied. For example, *in ovo* vaccination with MD vaccines serotypes 2 and 3, which will be significantly less efficacious when deposited in the allantoic fluid sac or the air cell as compared to the amniotic fluid or the body of the embryo [70,71].

Commercially available egg injection machines deliver the vaccine into the amniotic fluid or embryo body (right breast area of developing embryo) in over 90% of inoculated eggs. Equipment with multiple-head injector systems (MIS) is commercially available; these are capable of simultaneously injecting vaccine inoculum into an entire tray of eggs, enabling mass vaccine delivery. Under normal circumstances, two persons operating the MIS can vaccinate 35,000–70,000 eggs per hour. The ease of operating the MIS and the efficiency of processing large numbers of eggs account for the substantial savings in labor costs compared to conventional posthatch vaccination procedures. The MIS are continually undergoing improvements, and newer models have incorporated technology that selectively injects vaccine only in eggs that contain viable embryos, thus minimizing vaccine wastage and reducing potential contamination from nonviable embryos. The vaccine viruses injected *in ovo* should not adversely affect hatchability of eggs or the performance of hatched chicks. Chicks hatching from vaccinated eggs show evidence of MD vaccines serotypes 1, 2, and 3 viremias at hatch or at an early age [72,73]. In the specific case of HVT vaccination *in ovo*, viremia and chickens are protected after challenge at one day of age with pathogenic MDV. However, each MD vaccine is unique and age at vaccination, passage level, and vaccine dose greatly affected vaccine efficacy [74,75]. *In ovo* vaccination using live HVT, serotype 2 and 3 MDV, immune-complex vaccines, IBDV, fowl pox, reovirus, and FPV as a vector vaccine has been practiced for several years. In recent years, commercial applications of the *in ovo* vaccination technology have been expanded with the availability of recombinant HVT vaccines mentioned earlier in this chapter [5–21]. Experimental DNA vaccines have

also shown to be able to induce an immune response following *in ovo* administration [76–84]. Furthermore, the use of live coccidia oocysts can be administered *in ovo* to protect against coccidiosis.

Laboratory and field studies have shown that multiple agents may be combined in the same vaccine inoculum, thus reducing the need for manipulating flocks for administering individual vaccines [85,86]. *In ovo* technology has been used experimentally for nonvaccine-related applications (e.g., delivery of growth-promoting substances, probiotics, and *in ovo* feeding of nutrients) to hasten intestinal maturity and improve feed conversion [87–91]. In addition, *in ovo* technology could be used in future for chick sexing before hatch [92–97].

The advantages of the *in ovo* technology are clear, but there are also drawbacks. One of the shortcomings of *in ovo* technology is that it is economically suitable only for large-volume hatcheries. However, recent introduction of semiautomated machines will facilitate *in ovo* technology utilization by smaller operations. For any size of machine a major additional consideration regarding *in ovo* vaccination is the external contamination level of the eggshell after collection from the farm plus the sanitation level of the hatchery during and after injection [69]. High levels of bacterial and/or fungal contamination in the incubated eggs may result in a transfer to clean eggs during the vaccination process despite the disinfection routines that have been incorporated to *in ovo* vaccine administration. This increase in contamination causes an increase in first-week mortality in the farm and negatively affects production parameters.

At this time, the current use of *in ovo* vaccination is largely restricted to broiler flocks because eggs of both sexes need to be vaccinated. This is not the case for layers and breeders making *in ovo* vaccination too expensive for these birds. *In ovo* vaccination of breeder and laying flocks may gain popularity when gender-restricted MIS are developed for commercial use.

18.4 Factors influencing vaccine responses

Avian diseases and vaccination programs are separated in textbooks for teaching purposes. However, they all mix in the poultry house. One of the main roles of the modern poultry professional is to understand and troubleshoot these interactions. Successful poultry farming is based in poultry population health, where flocks of many thousands of birds are vaccinated with several vaccines in a relatively short period of time. The task of implementing a successful vaccination program depends on the following factors: (1) status of the immune system at the time of vaccination; (2) proper vaccine storage, preparation, and

administration; (3) the role of maternal antibodies on vaccine efficacy; (4) age of vaccination; (5) vaccine interactions; (6) genetics; and (7) interval between vaccinations.

18.4.1 Status of the immune system at the time of vaccination

Vaccination of immunosuppressed birds will lead to incomplete or impaired vaccine responses, increased susceptibility to disease, and confound the proper interpretation of vaccination results. Immunosuppression may be assessed by immunological tests or examination of the main immune organs or cells. The following tests are used to ascertain the status of the immune system: Evaluation of antibody responses to specific vaccines or foreign antigens such as sheep red blood cells (SRBC) and Brucella can be used to measure immunocompetence (see Refs. [98,99] and Chapter 14). Comparison of the body and bursa weight is a widely used method to determine if immunosuppression is related to atrophy of the bursa of Fabricius [100,101]. Typically, the body and bursa weights are recorded and the ratio is determined by dividing the bursa weight by the body weight and multiplying by 1000. The ratio is compared to a control that is not challenged or under the same conditions of the test flocks. The need for a control is a shortcoming for the use of this method under field conditions. The visual inspection is usually the first assessment of bursal health. Other comparisons like bursa to spleen weights are also used. Macroscopic evaluation of the thymus should always be part of the visual evaluation of the immune system. This is typically done by cutting the skin in the base of the neck of euthanized birds and pulling the skin toward the head. The thymus should clearly appear as 7–8 lobes on each side of the neck [102]. Difficulties to locate the thymus may be associated with atrophy and therefore, decreased CD4+ T-helper cells and CD8+ cytotoxic T cells (CTL) affecting antibody responses and cell-mediated immunity, respectively [103–108].

Microscopic examination of the bursa, spleen, and thymus within 12–42 days of age will reveal if there is lymphocyte depletion of the main immune tissues of the bird. This evaluation is normally done as a bursal survey, when birds from 12 to 15 farms within a complex are brought to a central location and samples of the immune organs are collected in 10% buffered formalin and submitted for analysis. The observations allow determining if there were lesions in these organs and if there is recovery over time.

18.4.2 Maternally derived antibodies

Passive immunity provided by MDA was first demonstrated in 1893 by the transfer of immunity to tetanus toxin from vaccinated hens to chicks [109]. Originally,

there were doubts with regard to the isotypes that could be transferred to the yolk [110,111], but it is now generally accepted that IgY is exclusively transferred [112] by a receptor-mediated transfer involving specific sequences found on IgY but not on IgM or IgA isotypes [113]. The amount of transfer of IgY is correlated with the amount of IgY present in the maternal circulation. In the embryo-nated egg, IgY is found in the yolk throughout the incubation period; in the egg white, after EID 4; in the allantoic and amniotic fluids; and in the embryo serum from EID 12 onward. IgY in the yolk sac continues to be transferred for at least 48 hours after hatching, so peak levels of MDA are not necessarily at 1 day of age. The half-life of the IgY in the serum of the hatched chick is shorter in faster growing chickens and varies from 3 days (fast-growing broilers) to 5.5–6 days for layer pullets. In line with this and depending on the level of MDA at day of hatch, MDA is catabolized over a period of 2–3 weeks in broilers and 4–6 weeks in layer pullets [114].

Providing high levels of MDA has become an important factor in deciding vaccination policy of parent birds in the poultry industry. Prime boost strategies, using live vaccines followed by inactivated vaccines, are utilized to protect the laying hen or breeder from infection or disease throughout the laying period. The increased level of protection in the parents also reduces the risk of vertical transmission of pathogens to the progeny and results in the transfer of high levels of antibody into the progeny, providing an early protection against infection.

The level of protection provided by MDA depends on several factors such as the level of MDA, the pathogen, and route of infection. MDA can be fully protective against early infection by IBDV and CIAV in chicken and hemorrhagic enteritis virus (HEV) in turkeys. High levels of MDA against reovirus can protect young broilers inoculated orally at day of hatch against developing tenosynovitis lesions at 3 weeks of age. Interestingly, there is no protection against subcutaneous challenge [115].

In general, MDA can modulate but not fully protect against infection of pathogens and their vaccines that replicate in the epithelium of the respiratory or intestinal tract such as IBV, NDV, ILT, and AMPV despite the detection of IgY MDA in the respiratory mucus of hatched chicks [116]. Globally, a high percentage of the day-old chicks with high levels of MDA against IBV and NDV is vaccinated routinely with IBV and NDV vaccines with registered live vaccines that have proven to be effective in presence of MDA. However, presence of MDA at time of vaccination or infection can delay and decrease the humoral response to vaccination or infection [117,118]. Vaccination with these respiratory pathogens at day of hatch of MDA positive birds can result in seronegative birds despite high protection against challenge [50,119], showing the importance of the local protection

for these young birds. MDA also decrease the humoral response to inactivated vaccines that are used for some diseases such as NDV and AI at a very young age [120].

18.4.3 Vaccine storage, preparation, and administration

Directions for storage, preparation, and administration depend on the type of vaccine. Modified live vaccines are usually marketed as freeze-dried (vast majority) or frozen products. MD and HVT-vectored vaccines are usually sold as frozen products in glass ampoules for *in ovo* or subcutaneous administration at the hatchery and stored in liquid nitrogen tanks. These vaccines can only be transported or stored this way or significant titer losses will occur [121]. At the time of vaccination, the amount of vaccine needed is calculated accurately and only the vaccine to be used should be removed from the liquid nitrogen tank. Thawing and re-freezing MD vaccines are not acceptable because it causes up to a 95% drop in titer. After the vaccine is removed from the liquid nitrogen storage, it should be thawed in a water bath filled with distilled water at a temperature around 27°C (80°F) in a time-sensitive manner of not over 1 minute. After the vaccine is thawed, it should be removed from the ampoule with a syringe using an 18-g needle and placed in a diluent bag of the appropriate volume for *in ovo* or subcutaneous vaccination. Once reconstituted, vaccines should be kept on ice bath until vaccination starts and applied within a 1–2-hour time period.

Typically, freeze-dried and inactivated vaccines are stored at temperatures between 2°C and 7°C. Temperatures above or below the indicated range will have a detrimental effect on these products. Inactivated vaccines are bacterial or viral suspensions in oil emulsions, aluminum hydroxide, and other synthetic adjuvants. The level of local reactions and efficacy of inactivated vaccines depend on the quality of the vaccine and administration techniques. Killed products should be brought to room temperature before vaccination. In some cases, warming of oil emulsions, especially W/O products, will improve fluidity, making injection easier, and decreasing the probabilities of cold shock, especially when small birds are vaccinated. Warming is usually done by placing an inactivated vaccine, previously brought to room temperature, in a water bath at 35°C for approximately 30 minutes. This procedure must be conducted following the specific recommendations of the manufacturer since time and temperature applied to oil emulsion vaccines may cause damage to the antigen formulation. Ease of injection is an important factor to ensure consistent vaccine administration. After each application of a killed vaccine, the used amount must be compared to the number of

birds vaccinated and the volume injected to make sure the flock was properly vaccinated. For example, if 10,000 pullets were vaccinated with a killed product and each bottle holds 1000 doses, at the end of the day 10 bottles should have been utilized. Vaccine storage areas should be electronically monitored to detect any temperature fluctuation that may have compromised vaccine quality. For frozen vaccines, liquid nitrogen levels on each tank should be measured and recorded periodically and a detailed log must be permanently available to review in the hatchery. Rooms with large numbers of liquid nitrogen storage cabinets need to be monitored for air prior to entering because a breakdown of equipment may result in an atmosphere filled with nitrogen, which can be deadly. Instructions from the vaccine manufacturer for transport, storage, preparation, and administration of vaccines must be followed closely.

18.4.4 Age at vaccination

The development of the avian immune system begins early in embryogenesis. As described in [Chapter 3](#), bursal precursor cells can be detected from EID 7 onward and a rudimentary bursa, in which B cells differentiate, can be detected from EID 10 onward [\[122,123\]](#). Cells expressing surface IgM, IgY, and IgA can be detected from EID 10, 14, and 16, respectively. Three distinct phases of B-cell development have been observed (see Refs. [\[124–126\]](#) and [Chapter 3](#)). Differentiation of T cells occurs in the thymus following the entry into this tissue of precursor cells during three closely regulated periods at EID 6.5, 12, and 18 (see Refs. [\[122,127\]](#) and [Chapter 3](#)).

In accordance with the appearance of B- and T-cell progenitors in the embryo, adaptive immune responses to antigens may be inducible in chicken embryos from 12 to 14 days onward [\[128,129\]](#), although it should be noted that it is also possible to induce tolerance in embryos and newly hatched chicks depending on the immunogen and the age of administration [\[123,130–132\]](#). Most examples demonstrating a functional embryonic immune system have been with replicating microorganisms. In particular, vaccinations with MDV, fowl pox, IBDV, and AMPV vaccines [\[85,133,134\]](#) at EID 18 have shown that the embryo can respond adequately to these viruses, mounting protective immune responses soon after hatch. Vaccination *in ovo* with MD vaccines has also demonstrated that the innate immune response is functional in embryos [\[66\]](#).

Interestingly, embryo vaccination can result in better immune responses than posthatch vaccination [\[67,134\]](#). This could be linked to the fact that the vaccine can access parts of the embryo through oral and cloacal drinking of amniotic fluid which are inaccessible to vaccines given after hatch. However, as the live vaccines replicate and persist, it is difficult to establish the immunocompetence of

the embryo at the time of vaccination, and in some regards the ability of the embryo to respond to a replicating organism may be linked with its ability to withstand the infection. This is clearly demonstrated by the fact that inoculation of 12–14-day-old embryos with HVT can be lethal [\[132\]](#), although at EID 18 the embryo can withstand and respond to vaccination with multiple microorganisms [\[85\]](#). The clearest evidence of a functional embryonic immune response has been demonstrated with vaccination of EID 16 embryos with inactivated nonadjuvanted *Campylobacter jejuni* [\[135\]](#). Antibody responses with IgY, IgM, and IgA isotypes were detected in serum, bile, and intestinal scrapings of chicks 5 days after hatch. In addition, there was a significant increase in the number of Ig-containing cells in these tissues plus an increase of T cells in the blood. Regardless, it is clear that, although embryos can mount innate and adaptive immune responses to vaccines, the response is suboptimal in comparison to a mature immune system [\[136\]](#) with peak immunological maturity occurring several weeks after hatching [\[74,137–139\]](#).

18.4.5 Duration of immunity

Differences in the duration of immunity are factors to take into consideration according to the type of bird being immunized. Broilers are usually kept for 5–8 weeks, and a single vaccination generally suffices. In contrast, broiler breeders and layers may be kept for up to 2 years, and it is unlikely that live vaccines (with the exception of MDV and IBDV vaccines), especially if given at 1 day of age, can provide immunity for this period of time. For this reason, booster vaccinations are given, usually in the form of inactivated vaccines at the last part of the rearing period. In most countries, a boost to the antibody response that can maintain immunity to IBV, NDV, and AMPV throughout the laying period is essential. Little is known about the effect of boosting with inactivated adjuvanted vaccines following live priming because only the antibody response has been measured. Regardless, this is an effective way to maintain antibody response. It should be noted that vaccination with inactivated, adjuvanted vaccines such as those for EDS and Salmonella, given at 12–18 weeks of age, can protect birds throughout the laying period without the requirement for live priming. The need for knowing how to induce long-lasting protection is increasing as the length of the laying period is increasing due to the genetic improvement of the persistence of egg production.

18.4.6 Interference between vaccines

The design of a vaccination program needs to take multiple factors into account such as field challenge, type of bird, age of vaccination, maternal immunity, application routes, and vaccine interactions. Vaccines that have the same

target tissues will naturally compete and possibly interfere with the immune response to each other. Application of the AMPV vaccine 6 days post-IBV vaccination resulted in a very significant drop in effectivity of the AMPV vaccine [140]. Studies by Vagnozzi et al. [141] showed that IBV and NDV vaccines alone or in combination decreased the replication of tissue culture origin ILT vaccine in the epithelial cells of the trachea. In addition, seroconversion against IBV and NDV was reduced when vaccines were simultaneously applied with chicken embryo origin ILT vaccines. More recently, it has been observed that the combination of more than one HVT-vectored vaccine in the same birds will result in interference causing decreased protection for one or both products applied [142]. Moreover, responses to vaccination with more than one HVT-vectored vaccine can vary according to the genetic background of the chickens [143]. A similar situation occurs with fowl pox vectors where previous immunity against fowl pox prevents the vector from providing consistent protection against disease [19,144].

However, there are examples of successful combinations practiced for a number of years in various types of commercially grown chickens such as the application of commercially available combination vaccines for ND and IBV with good protection results against both diseases [145–147], or the simultaneous administration of NDV, IBV, and AMPV vaccines with satisfactory protective results for all three pathogens [148–151]. Although IBV may interfere with the replication of AMPV, reducing antibody response, there is no detrimental effect to the protective ability of AMPV vaccines when administered simultaneously with IBV H120 [148].

18.4.7 Time intervals between vaccinations

Although it has been demonstrated that a significant proliferation of immune cells takes place after vaccination, it remains unknown whether birds maintain their immune response in a similar manner to mammals. The stimulation of memory and duration of immunity of a vaccine is associated with the type of vaccine (i.e., live or killed—adjuvanted), the delivery route, and the age and immune status of the bird at vaccination. MDV vaccines, like many herpesviruses, establish latency [152]; the recrudescence from latency is likely to provide immune restimulation and concomitant life-long immunity. In contrast, AMPV, IBV, and NDV vaccines that replicate in the respiratory tract for a maximum of 2 weeks have been shown under experimental conditions to provide homologous immunity up to 12–14 weeks [153,154], although immunity can begin to decline after 6 weeks [155]. Under experimental conditions, revaccination with a live vaccine applied only a few weeks after an effective application with a similar live vaccine that can

provide at least 6 weeks of high protection does not increase the circulating antibody level. Even a challenge can occur without a detectable seroconversion due to the very high level of protection [156–158]. However, under field conditions, early revaccination with a live homologous vaccine is used regularly, partly to compensate for potential mistakes or inadequate take of the earlier vaccination.

A short interval between two vaccines that target the same organ(s) may result in a lower or delayed response to the second vaccination [140]. Under field conditions, the IgM response postvaccination with an IBV variant vaccine around 2 weeks of age was decreased when the vaccination was performed within 14 days postvaccination at the hatchery, every day earlier resulted in a 2.5% decrease of birds that seroconverted [159]. A potential cause for the lower response, when vaccines with the same target organ are applied shortly after another, might be the recovery time needed by the tracheal epithelium after the first vaccination [160–163].

18.5 Immunosuppression

A fully functioning immune system is of great importance for poultry health and performance. The ultimate result of poultry health is a combination of proper nutrition, management, and disease prevention practices. The immune system must function under intensive rearing conditions to maintain the health of the flock by preventing or controlling invasion by poultry pathogens such as virus, bacteria, parasites, and any foreign molecule. When vaccines are applied, the expected protective effect expected from response to vaccination depends heavily on an effective immune system. There are multiple factors of infectious or noninfectious nature that can negatively affect the chicken's immune system and thus, diminish proper response to vaccination. There are factors of noninfectious nature like stress and mycotoxins that can also cause immunosuppression in birds. A more comprehensive description of immunosuppression can be found in [Chapter 14](#).

18.5.1 Stress and immunosuppression

Stress, immunosuppression, and lymphoid depletion of bursa, spleen, and thymus in birds have been associated with elevated blood corticosterone levels [164]. Nevertheless, stress-derived immunosuppression can occur without high bloodstream levels of corticosterone [99]. Management-related changes leading to stress, even from the embryonic development during incubation can negatively affect the immune system and the capacity to respond to infectious challenges. Suboptimal incubation temperatures have been shown to negatively affect the response of hens to *Eimeria* and IBV challenge at 106 days of age [165]. Lymphocytes from the bursa go into

the bloodstream and colonize other tissues [166]. Posthatch feed restriction will cause lower immune bursa and spleen weights [167] and delay colonization of immune tissues, especially the gut-associated lymphoid tissue [167,168]. Juul-Madsen et al. [169] showed that 48-hour feed restrictions impair the response of chickens to subsequent vaccination with killed IBD vaccines. Changes associated with the environment of the chicken house such as stock density, nutrition, general conditions of the house and heat/cold stress have a negative effect on the proper development and functioning of the immune system [99,170–173] and therefore increase susceptibility to disease [174–178]. Immunosuppression may be antigen-specific, where birds raised on slats versus litter had decreased cellular and antibody responses to SRBC and tetanus toxoid but not to human serum albumin [99]. Not every study showing the negative effect of the various forms of stress on the avian immune system involves vaccination. However, it is conceivable that they also will negatively affect response to vaccination.

18.5.2 Mycotoxins

From all immunosuppressive elements that can be present in a poultry ration, mycotoxins are perhaps the most immunosuppressive [179,180]. Mycotoxicosis is a general denomination for various clinical syndromes in birds following consumption of contaminated feed containing mycotoxins. Various immune responses of humoral and cellular nature can be affected by the presence of mycotoxins in animal diets. More specifically in poultry production, involution of immune organs such as spleen, thymus, and bursa of Fabricius, decreased antibody response to vaccination, and impaired innate immune responses can occur as a consequence of mycotoxin contamination of chicken feed, even in concentrations lower than those required to produce clinical mycotoxicosis signs [181–183]. Some of the most common mycotoxins found to cause immunosuppression in chickens are aflatoxins, ochratoxin, citrinin, oosporein, fusarium toxins, fumonisins, and thrichothecenes [184], see also Chapter 14. Contamination of poultry feed with Aflatoxins, ochratoxins, deoxynivalenol, and fumonisins actively interfere with immune responses against viral and bacterial vaccines [185–191].

18.5.3 Immunosuppression by vaccines

The licensing of poultry vaccines in developed countries are closely supervised by regulatory bodies with special attention to the safety of the finished product in poultry and target species. In the United States and EU, all safety tests should pass regulatory requirements in SPF birds. Proper vaccine use in accordance with the manufacturer's instructions should not cause adverse effects in vaccinated

birds. Although live vaccines have been shown to be very efficacious against a variety of poultry diseases, inadequately concurrent or inappropriate vaccinations may cause immunosuppression and vaccine interference. Vaccines are normally designed for a given age range and route of administration. Improper vaccine use or unlicensed administration methods for any vaccine may result in a certain level of immunosuppression. Special attention is always given on this matter to live IBDV vaccines with different levels of attenuation leading to a variable degree of the bursal lesions and thus, variable levels of immunosuppression [192–194]. Impaired responses to Newcastle disease vaccines and decreased performance have been reported after the use of IBD “hot” vaccines [194,195] and immunosuppression assessed after *in ovo* reovirus vaccination [196]. Other vaccines that replicate in immune organs such as live CIAV and HEV vaccines have been suggested to cause variable levels of immunosuppression [197,198]. Atrophy of bursa and thymus has been also observed with experimental MDV serotype 1 vaccines [199]. Placement of vaccines in a vaccination program, especially if these vaccines replicate in immune tissues of the bird, must be done carefully since the ultimate impact of the vaccine on the immune system depends on multiple factors besides the vaccine itself such as health status of the flock, presence of other immunosuppressive factors, quality of the vaccination process, and existing active or passive immunity.

18.5.4 Influence of immunosuppression on vaccination

Even the most comprehensive vaccination program may fail if immunosuppression is present. There are general indicators such as increased mortality, decreased body weight, increased feed conversion, and condemnations at processing that suggest immunosuppression. Increased incidence of diseases such as gangrenous dermatitis, inclusion body hepatitis, and aplastic anemia are also non-specific indicators of immunosuppression. Decreased resistance to field challenge, atrophy of the main immune organs (bursa of Fabricius and thymus), and failure to respond to vaccinations or to clear bacteria from the bloodstream are indicators specifically pointing to immunosuppression. Additional factors like the introduction of new pathogens (e.g., variant IBV) or exotic respiratory agents may confuse the recognition of the signs of immunosuppression. The presence of immunosuppression of any sort will prevent proper response to vaccination and will become a significant obstacle in disease prevention in poultry operations. Faragher et al. [200] showed that early challenge with IBDV reduces the protective efficacy of Newcastle disease vaccines and the severity of

immunosuppression was inversely proportional to the age of the bird. Early IBDV challenge in susceptible birds decreases responses to vaccinations against ILT, IB, and ND and increases the persistence of the NDV and IBV in the trachea [201]. The deleterious effect of IBDV effect on the efficacy of MD vaccines has also been described [202,203]. The size of the bursal follicles after recovery from IBDV challenge is directly correlated to the ability to respond to killed *Salmonella enteritidis* vaccination [204]. The detrimental effect of challenge with CIAV has been described [204,205]. Simultaneous challenge with CIAV and Newcastle disease (ND) vaccination caused increased vaccine reactions compared with CIAV-unchallenged hatch mates [206]. Decreased antibody response after killed ND vaccination has also been observed in chickens infected with CIAV [207]. The immunosuppressive effect on humoral and cell-mediated responses, more specifically responses to vaccination were observed with birds challenged with CIAV and IBDV [208]. Infection with CIAV in antibody-negative birds results in an impairment in cytotoxic T cells responses toward specific pathogens [209]. More comprehensive reviews on the immunosuppressive effects of CIAV have been published [197,210]. MD challenge causes well-described damages to the immune system [211,212] and more specifically interferes with immunity conferred by ILT vaccines [213,214]. Other viruses such as reovirus, reticuloendotheliosis virus, avian leukosis subtype J can also interfere with response to vaccination [215–220]. Additional examples of infectious and non-infectious immunosuppression examples are described in Chapter 14 and Section 18.5.

18.6 Quality control of response to vaccination

Numerous papers have reported the high levels of efficacy that can be obtained with the registered vaccines for a number of poultry diseases [221–241]. Also, under field conditions, these vaccines have proven to be able to provide high levels of protection when applied by a mass-application route. However, it is well recognized that the mass applications offer less guarantees for a successful vaccination than individual applications. Even individual applications can be compromised when vaccinators are more focused on speed than on a careful application. This might result in lower levels of protection, a delayed protection, more rolling reactions due to circulation of the vaccine with more bacterial secondary infections and condemnations, increased risk of reversion to virulence, and more interference with other vaccinations. Therefore monitoring the take of the vaccine, certainly when it is applied by mass application or individually at a large

scale, is essential to be able to manage the quality of the vaccinations.

Two major methods are used to monitor the success rate of the vaccination and vaccination programs: detection of the humoral antibody responses after live or inactivated vaccines or detection of the live vaccine strain several days post application.

18.6.1 Serology

Humoral responses to live vaccines have been well characterized. The type and duration of antibody responses are dependent on the microorganisms used and the route of vaccine delivery. Systemic vaccination with MD vaccines provides a broad-spectrum serum antibody response, although the relevance of this response in vaccine-induced immunity is unclear. In contrast, vaccinations against viral respiratory diseases via a mucosal route, for instance, IBV and NDV live vaccines, induce local IgA in the Harderian gland [242,243], lacrimal IgA and IgM [242,244], or IgY [242] and respiratory tract IgY [245,246], in addition to IgA, IgM, and IgY responses in serum [247]. The role of local antibodies in protection from respiratory disease is clearly important [242,245], while the role of systemic antibody responses is potentially more important for the duration of immunity [248] and protection against drops in egg production caused by infection of the oviduct [249–251]. In contrast, live IBDV vaccines, delivered in drinking water or *in ovo*, induce strong IgY virus-neutralizing responses in serum and, although this response is thought to correlate with protection against disease, an important role for cell mediated immunity (CMI) has also been postulated [252].

Serum antibody responses are also induced with live *Salmonella* and coccidia vaccines, although local antibody and T-cell responses are more likely to play a role in protection [253,254]. In contrast, no, or very weak, serum antibody responses have been detected following vaccination with mild live *Mycoplasma* vaccines; again, the local antibody response is more likely to be playing a role in protection [255].

The role of T-helper cells in the antibody responses induced by live vaccines has yet to be elucidated, but it is known that the production of IgY antibodies normally requires help from antigen-specific CD4⁺ T-helper cells [256]. Despite the evidence for other correlates of protection, the systemic antibody response is used as a measure of immune status for many poultry vaccines because simple methods to study alternative potential immunological correlates of protection are unavailable. Seroconversion derived from vaccination indicates the birds received the vaccine and mounted a successful antibody response. Nevertheless, protection against challenge will depend on the quality of those antibodies in terms of how specific

they are to neutralize the challenge virus strain the chickens are exposed to. In some cases, it may be necessary to correlate antibody titers with challenge studies. Antibodies measured with usual methods like ELISA in serum or tears were unrelated to protection against IBV challenge [257] and protection was observed without detectable circulating antibodies [258]. Protection against respiratory viruses such as NDV or IBV may be observed a few days after vaccination before an antibody response can be detected by routine methods [47,259,260].

18.6.2 PCR/culture

The most common surveillance tool used in poultry is without a doubt serology. Other laboratory techniques such as virus or bacterial culture and PCR are also used. Virus re-isolation is the gold standard to assess the efficacy of most respiratory vaccines such as IBV, NDV, and ILTV. Usually around 5–7 days after challenge with IBV in vaccinated and control birds, tracheal swabs, or tracheal tissue samples are collected and virus isolation conducted. In vaccinated birds, the lack of virus re-isolation in laboratory systems or the ciliostasis test results in tracheal organ culture in vaccinated birds will indicate protection [221,261,262]. In recent years, culture techniques have largely been replaced by PCR and sequencing for everyday diagnostic evaluations [263]. Real-time quantitative (RT)-PCR is the most sensitive test for IBDV [264,265] and is used for the evaluation of vaccination programs and to monitor the emergence of new field strains and reovirus diagnostics. An RT-PCR technique has been developed to evaluate vaccination against MD using feather pulp samples and to estimate the protective ability of the vaccination program applied. This technique uses the MDV present in the feather pulp used as a fresh sample or in FTA cards. The ratio between the field and vaccine virus loads is predictive of the expected MD protection of the flock. The test allows for differentiating serotype 1 vaccine and field virus strains [266–271]. Cultures from hatchery and field samples are routinely used for evaluating the progress of control program for bacterial diseases such as *Salmonella* [272], although molecular techniques and combinations of PCR/sequencing and culture have started to be used [273,274]. Although a rapid development of PCR and sequencing techniques have made them widely used for poultry diagnostic and evaluation of vaccination programs, culture is still essential to conduct a full characterization of the various virus and bacterial strains found to be relevant for poultry.

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Comparative immunology of agricultural birds

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19.1 Introduction

Knowledge of the immune system has advanced at a much faster pace for chickens than for other avian species due to the availability of the chicken genome sequence. However, now that genome sequences and transcriptomes are available for turkey [1], duck [2], goose [3], and quail [4], the cloning of immune genes with low homology to chicken genes is no longer a handicap. Among agricultural species, the immune system of the duck has been studied most extensively due to the biomedical relevance of the duck hepatitis model and avian influenza. Here, we will focus on available functional work for genes involved in immunity.

A strong interest in the phenotypic characterization of duck immune cells and immunoregulatory mediators such as cytokines and chemokines arose from its role as a surrogate infection model for the hepatitis B virus (HBV), the causative agent of acute and chronic hepatitis B in man. Pekin ducks (*Anas platyrhynchos*) are the natural host of a related hepadnavirus, duck hepatitis B virus (DHBV) [5]. Like HBV, DHBV has a narrow host range, such that it does not infect Muscovy ducks (*Cairina moschata*), although they belong to the same order as Pekin ducks [6,7]. Noteworthy, it is not the virus per se that causes the death of infected hepatocytes in man but rather HBV-specific cytotoxic T cells (CTL) are involved in the destruction of the liver. Although infection in the duck exhibits the same age-related outcomes [8,9], there is no histological evidence that DHBV is associated with hepatitis in ducks.

The recent outbreaks of highly pathogenic influenza A viruses (HPAIV) spurred further interest in the regulation of immune responses in ducks and other domesticated agricultural avian species. Ducks play a key role in the maintenance and dispersal of influenza A viruses, serving

as the natural reservoir. All strains of influenza viruses have been isolated from waterfowl [10]. Ducks are usually asymptomatic carriers of influenza viruses, and these can be transmitted to other avian and mammalian hosts, including humans. H5N1 avian influenza outbreaks have caused mortality in ducks, although a range of pathogenicity is seen with different isolates [11]. Ducks are implicated in the propagation and endemnicity of this virus, contributing to its spread throughout Asia and Europe [12]. Efforts to understand the difference in susceptibility of ducks and chickens to avian influenza have led to extensive comparison of innate immune responses between ducks and chickens and have been recently reviewed [13,14]. Development of vaccines for poultry (including ducks) is ongoing and their evaluation depends on having measurable parameters for avian immune responses [15].

In Europe and many other Western countries, the consumption of bird meat, other than chicken meat, is increasing annually. In view of the growing number of commercially held turkeys (*Meleagris gallopavo*), ducks (*Anas platyrhynchos* and *Cairina moschata*), geese (*Anser anser*), and ostrich (*Struthio camelus*) and their economic importance, these birds need to be protected against pathogens by improving their natural and specific defense mechanisms through vaccination. Furthermore, calls for the reduction or elimination of growth-promoting antibiotics from poultry feed also inspire research into improving immune competence through diet, therefore increasing our understanding of the immune systems of agricultural species is of value.

Here, we review the immune system of ducks, geese, turkeys, quail, and ostriches with an emphasis on recent studies concerning the molecular characterization of cytokines, chemokines, pattern recognition receptors, and cell surface markers.

19.2 Innate immunity

19.2.1 Toll-like receptors

Innate immunity provides a first line of host defense against infection through microbial recognition and killing while simultaneously activating a definitive adaptive immune response (see Chapter 8). Pathogen recognition by avian pattern recognition receptors and downstream signaling has been the subject of recent reviews [13,14,16]. Toll-like receptors (TLRs) belong to a multi-gene family that help to detect foreign invaders by sensing various pathogen-associated molecular patterns that have been conserved in both the invertebrate and vertebrate lineages. Binding of ligands to TLR activates signal transduction pathways and induces the expression of a wide variety of host defense genes, such as antimicrobial peptides, cytokines, chemokines, and nitric oxide synthase [17]. There are six major families of vertebrate TLRs that are each characterized by the recognition of a general class of pathogen-associated molecular patterns. Members of the TLR families are described in detail in Chapter 8, and avian TLRs have been the subject of several excellent reviews [18–21].

Birds possess genes for TLR1 (type-a and b), TLR2 (type-a and b), TLR3, TLR4, TLR5, TLR7, TLR15, and TLR21 [18,20,22–26]. All 10 orthologous genes have been identified in turkeys [27]. With the availability of avian genomes, recent phylogenetic analyses of TLRs from 50 representative species show a complicated history of duplications of TLR7 in some species, deletions of TLR8 and TLR9, and pseudogenization of TLR5 in some birds [21]. Phylogenetic relationships of TLR1 and TLR2 to mammalian orthologues were inferred [28]; however, the picture is complicated by gene conversion. A recent analysis of the nonconverted parts of the TLR proteins shows that TLR1B should be renamed TLR10, while TLR1A is the TLR1 orthologous gene [21].

TLR15 is unique to birds and reptiles [29] and has specificity for a protein component of yeast [30] and diacylated lipopeptide from mycoplasma [31]. Sequence alignment of duck and chicken TLR15 shows differences within the coding sequence, although functional consequences of these polymorphisms are unknown [30]. TLR15 along with TLR2 and TLR4 were upregulated by infection with avian pathogenic *Escherichia coli* [32]. A TLR21, also found in fish and amphibians, was identified in birds. TLR21 is an innate sensor for CpG, thus is a functional analog of mammalian TLR9 [19,33]. This made sense of the previous observations that chickens are capable of responding to unmethylated bacterial CpG DNA, the ligand of mammalian TLR9 [34,35]. duTLR21 is highly expressed in the blood, spleen, bursa of Fabricius, and cecum, and stimulation with CpG initiated

the activation of NF- κ B signaling. Further, infection with duck plaque virus (DPV) induced expression of TLR21 in the spleen and bursa, and overexpression in duck embryonic fibroblasts (DEFs) decreased DPV copy number, while knockdown of TLR21 increased it [36]. In goose, TLR21 is upregulated by gosling viral enteritis virus [37].

Characterization of the chicken TLR7/8 loci revealed an intact TLR7 gene and fragments of a TLR8-like gene with a 6-kb insertion containing a chicken retroviral-like insertion element (CR1) [24]. Sequencing of a genomic clone encoding the duck TLR7 locus also revealed only small fragments of the TLR8 gene [38]. TLR8 is also absent from the turkey genome sequence [27] and appears to be absent from avian genomes entirely [21]. TLR7 duplications are seen in some passerines [20] and shorebirds [39], but not in the Galloanseres [21].

Duck TLR7 is highly expressed in the spleen, bursa, and lung [38]. Chicken TLR7 is expressed in B cells and other leukocytes [40]. Duck splenocytes responded to imiquimod, a TLR7 agonist, with increased expression of genes encoding interferon- α (IFN- α) and proinflammatory cytokines (IL-1 β and IL-6) [38]. Expression of TLR7 has been examined in influenza-infected duck peripheral blood mononuclear cells (PBMC) and is variable, but effectively not altered by infection [41]. TLR7 expression is increased in HPAI infected ducks, but not low pathogenic avian influenza (LPAI) infected ducks, but is increased in LPAI-infected chickens [42,43]. Intriguingly, rare TLR7 positive cells detectable by in situ hybridization in duck ileum increase in numbers in response to R848 agonist, or infection with avian influenza [44]. These cells resemble intraepithelial round hematopoietic cells and have the same morphology and location as IFN- α positive cells, suggesting a cell population which is positive for both, analogous to the TLR7 responsive professional IFN-producing cells in mammals, plasmacytoid dendritic cells (DCs) [44]. Pigeon TLR7 responds to R848 stimulation producing proinflammatory cytokines from pigeon PBMCs [45]. TLR7 agonists (poly C and loxoribine) showed more promise as antivirals against influenza than TLR3 agonists (poly I:C) in chicken HD11 cells and embryonic fibroblasts [46]. A variety of TLR7 agonists may prove useful as antivirals in poultry species.

TLR3 is highly expressed in the trachea of Pekin ducks and is upregulated in the lung and spleen by duck reovirus infection [47]. In Muscovy ducks, TLR3 expression is widespread and is upregulated by HPAIV [48], as is also true for H5N1 influenza-infected chickens [49]. Goose TLR3 is weakly expressed in most tissues but is highly induced along with proinflammatory cytokines in isolated leukocytes by infection with H5N1, and in the lung and spleen of infected geese [50]. In turkeys, TLR3 expression is highest in the bone marrow and spleen [27].

Duck TLR4 has the highest tissue expression in the liver, spleen, intestine, and brain [51]. Turkey TLR5 is expressed in a wide variety of tissues and stimulation with TLR5 agonist flagellin resulted in the production of inflammatory cytokines and nitric oxide (NO) [52].

19.2.2 Retinoic acid induced gene-I (R)-like receptors

RIG-I-like receptors (RLRs) comprise a family of intracellular pathogen sensing antiviral pattern recognition receptors (reviewed in Ref. [53]). To date, three receptors have been identified including retinoic acid-inducible receptor (RIG-I), melanoma differentiation-associated factor 5 (MDA5), and laboratory of genetics and physiology 2. The RLRs detect viral or self RNA in the cytoplasm to initiate a signaling cascade involving type I IFN and downstream innate immune genes ultimately controlling an infection. In mammals, RLRs are involved in the detection of a large number of RNA viruses (reviewed in Ref. [53]). RIG-I specifically detects RNA having a 5' triphosphate overhang and panhandle structure [54,55] and can detect Sendai virus, Influenza A, hepatitis C, Lassa virus, Ebola, and Rift Valley Fever virus. MDA5, a sensor for double-stranded RNA, is involved in the detection of picornaviruses and vaccinia virus, while both receptors are involved in the detection of Japanese Encephalitis virus, Dengue virus, and West Nile Virus (WNV). Thus RLRs are involved in detecting viruses relevant to birds including influenza virus, Newcastle disease virus (NDV), and WNV.

Chicken MDA5 was the first avian RLR identified [56]. When overexpressed in chicken DF-1 cells, MDA5 could transactivate an IFN β luciferase reporter. MDA5 is targeted for direct interference by the V protein of all avian and human paramyxoviruses [56]. MDA5 is upregulated by IFN and influenza infection of chicken cells, but knockdown of MDA5 does not alter influenza titers [57]. Silencing of chMDA5 decreases the chIFN- β level in response to influenza infection, suggesting that it is involved in antiviral sensing of influenza [58]. Chicken MDA5 can detect *in vitro* transcribed RNA with or without 5'-triphosphate moieties. Chicken LGP2 has inhibitory effects on activation of the IFN- β promoter but silencing reduced IFN production by influenza infection. Duck MDA5 is also upregulated in HPAI infected tissues, and overexpression of MDA5 CARD domains decreases viral titers [59]. MDA5 is also involved in the detection of duck viral enteritis, with LGP2 enhancing MDA5 function at low concentration [60].

A RIG-I receptor was identified in ducks and shares only 54% identity with human RIG-I [61]. Remarkably, RIG-I could not be identified in chickens [61]. Although always difficult to prove that a gene is absent, several

pieces of evidence suggest this gene has been lost. These include the absence from the syntenic region of the Z chromosome, absence from the extensive chicken expressed sequence tag (EST) database and transcriptomes, and absence of a cross-hybridizing band by low stringency Southern blot using the conserved helicase of duck RIG-I as probe. Using the same approaches, a gene could be identified in zebra finch, which shares 78% identity to duck RIG-I. Chicken cells also lack functional evidence of RIG-I by the absence of detection of 5'-triphosphate agonists by chicken DF-1 cells [57,61]. Duck RIG-I can compensate for RIG-I in chicken cells and restore the detection of RIG-I ligands [61]. Zheng and Satta searched for RLR genes in 62 bird genomes, and RIG-I was found in all species except in chicken, Japanese quail, and turkey [62] supporting their loss or significant sequence divergence. However, the assembly of avian genomes is not complete and high GC content regions are notoriously difficult to sequence by high throughput sequencing [63].

The crystal structure of duck RIG-I gives insight into the function of this receptor in ligand recognition [64]. Notably, duck RIG-I lacks the critical lysine residue K172 upon which polyubiquitin is covalently attached by TRIM25 for activation. While ubiquitin is attached at K167 and K193 residues (conserved in many avian species), mutation of both residues did not prevent activation by TRIM25, presumably through unanchored ubiquitin chains. Further, the duRIG-I crystal structure allowed prediction and mutation of residues interacting with MAVS, which alongside parallel experiments with the human proteins, contributed to a general model of signaling through MAVS [65]. The RIG-I CARD domains form a tetrameric lock-washer structure offset by one caspase activation and recruitment domain (CARD) unit, upon which MAVS oligomerizes and assembles into a helical filament which initiates signal transmission [65]. This mechanism is remarkably conserved, despite poor sequence conservation of RIG-I and MAVS between these species. Indeed, duck MAVS is only 28% conserved with human MAVS [66], and this incompatibility likely accounts for the observation that avian RIG-I induces IFN and interferon induced genes (ISGs) stronger than mammalian RIG-I in chicken DF-1 cells, while mammalian RIG-I and CARD domains induce IFN stronger in HEK293T cells and human A549 cells [67].

Duck RIG-I is rapidly upregulated by 200-fold at 1 day postinfection (dpi) with highly pathogenic AIV strain A/Vietnam1203/04, while upregulated only slightly by the low pathogenic strain A/duck/British Columbia 500/2005 [61,68]. RIG-I gene expression returned to normal levels just as quickly and is down by 3 dpi. RIG-I transfected cells had reduced titers of HPAIV or LPAIV compared to vector-transfected cells. To identify the genes turned on downstream of duck RIG-I, chicken cells transfected with RIG-I

or empty vector infected with high or low pathogenic AIV were compared by microarray analysis using the chicken 44k Agilent microarrays [69]. These microarrays revealed a set of avian RIG-I responsive genes including IFIT, OASL, MDA5, ISG12-2, similar to the genes identified by comparison of wild type to RIG-I knockout mouse embryonic fibroblasts [70]. These same genes were identified by subtractive hybridization following HPAIV infection of ducks and shown upregulated by qPCR [68,71], and analysis of differentially expressed genes in duck transcriptomes [2,72]. A comparison of the duck, quail, and chicken response to H5N1 [4] revealed a low response in chicken and quail within a cluster of 189 genes strongly upregulated in ducks. These genes were in pathways dominated by the RIG-I pathway and IFN-stimulated genes. Finally, the susceptibility of ducks to HPAIV correlated with age, with younger ducks showing much greater sensitivity to infection. This susceptibility correlated with RIG-I expression during ontogeny, with older ducks showing increased RIG-I expression [73].

Subsequently, goose RIG-I has been identified, and expression of RIG-I was increased in NDV-infected geese [74]. Transfected DF-1 cells could recognize the RIG-I agonist and upregulate the activity of the chicken IFN- β reporter. Goose RIG-I transfected 293T/17 cells challenged by infection with NDV showed reduced viral titers. RIG-I was also upregulated in geese infected with NDV, but the strain with the lowest virulence (La Sota) induced the highest RIG-I expression compared to ZJ1 or Herts/33 despite their reaching higher titers. This may reflect NDV interference in the signaling pathway. Transfected pigeon RIG-I can initiate an antiviral response against influenza virus and Infectious Bursal Disease Virus (IBDV) in DF-1 cells knocked down for chMDA-5, but not when MAVS is knocked down [75]. When transfected into DF-1 cells, goose and duck RIG-I induced more IFN- β upon infection with AI or IBDV than pigeon RIG-I. Interestingly, goose RIG-I has a glycine at residue 8 [75], which is a serine subject to phosphorylation in mouse and man for autorepression of RIG-I [76]. The G8S mutant of goose CARDs induces significantly less IFN than wild type [75], suggesting that repression by phosphorylation at this site in avian RIG-I is conserved, and goose RIG-I without serine 8 may be more easily activated.

19.2.3 Antimicrobial peptides

Antimicrobial peptides are considered endogenous antibiotics that play a multifunctional role in innate immunity of mammalian and avian species. A review of the evolution of β -defensins and cathelicidins explored their conservation and selection across 54 avian species [77]. The beta-defensins contribute to mucosal and epithelial defense, also acting as signal molecules for cellular

components of innate and adaptive immunity (see chapter 8). Avian β -defensins have been found in the blood of chickens, turkeys, and ostriches, epithelial cells of chickens and turkeys and stomach contents of king penguins. Fourteen different members of this family have been identified in chickens (AvBD1-14) and no other groups of defensins could be identified in the chicken genome in a comprehensive screen [78]. An analysis of the duck genome and transcriptome identified 18 β -defensins, showing lineage-specific duplications of AvBD3 [2]. In addition, three β -defensins have been isolated from turkey heterophils (turkey heterophil peptide, THP-1 to 3, renamed AvBD) so far. AvBD1 (THP-1) killed *E. coli* and *Staphylococcus aureus* in vitro, whereas AvBD2 (THP-2) and AvBD3 (THP-3) killed only *Staph* [79]. Five β -defensin homologs (AvBD1, 2, 4, 7, and -8), originally named ostricacins, have been purified from ostrich heterophils and characterized [80,81]. Comparison with the known β -defensins from mammals and chickens revealed that the four ostricacins share the six conserved cysteine residues known as the “ β -defensin motif.” The ostricacins were effective in inhibiting the growth of Gram-positive and Gram-negative bacteria, but as chicken, turkey, and king penguin β -defensins, ostricacins displayed only moderate activity against yeast. So far, an antimicrobial activity against filamentous fungi has been described only for king penguin β -defensin [82].

Several duck beta-defensins have been characterized in recent years. Genes orthologous to AvBD2, 4, 7, 9, 10, and 12 were identified in ducks [83], and expression of AvBD4, 7, and 12 was enhanced by duck hepatitis virus (DHV) infection which also showed antiviral activity against DHV [83]. Five others were identified in a subsequent study (AvBD 1, 3, 5, 6, and 16) and recombinant GST-tagged proteins showed antimicrobial activity against a variety of bacteria [84]. Two groups characterized duck AvBD2 as antibacterial [85,86] and as having immunomodulatory properties [85]. Characterization of hundreds of wild mallards and other related waterfowl show limited genetic diversity of most AvBD genes, except AvBD10 and AvBD3b (including radical replacements in one of the duplicated genes) [87]. Further work is required to study other roles of avian β -defensins in innate and adaptive immunity such as the chemotactic function shown by their mammalian counterparts.

19.3 Cytokines

19.3.1 Interferons

Type-I interferon

IFN was the name given the “interfering factor” during an influenza A viral infection of the allantoic membranes of embryonated chicken eggs by Isaacs and Lindenmann in

1957 [88]. Despite recognition of the antiviral activities of IFN first in chicken, cloning of the first avian type-I IFN [89] was delayed due to low identity to mammalian IFN- α and IFN- β . Shortly thereafter, a second intronless chicken type-I IFN gene was identified (IFN2) that was only 57% identical to the first one [90]. Functional studies support that the avian type-I IFNs are analogous to mammalian IFN- α and IFN- β [90,91], key antiviral effectors. Previous phylogenetic analyses suggest that the type I IFNs independently duplicated after the separation of mammals and birds and are not direct orthologues [92]. More recent phylogenetic analyses show that type I and type II IFNs are distantly related to their mammalian counterparts [93] and are divided by the receptors to which they bind.

Homology screening approaches to identify IFN- α genes of other birds were also slow due to low amino acid identity to chicken IFN- α : 82% in turkey [94], 51% in duck [95], and 48% in goose [96]. In functional comparisons, duIFN- α exhibited antiviral activity on quail CEC-32 cells; however, chIFN- α exhibited no detectable activity on duck cells [97]. Turkey IFN- α exhibited a 16-fold lower activity on chicken cells than turkey cells [94]. Goose IFN also shows cross-species activity [96]. Like chIFN- α , duIFN is strongly expressed in response to virus infection and oral treatment with imidazoquinoline S-28463 (ligand for TLR7). Recombinant duIFN- α effectively inhibits DHBV replication in primary hepatocyte cultures and in ducklings [95,98–100]. Single-cell analysis revealed the existence of 10 copies of closely related intronless IFN- α in ducks ([101]; Schultz U., unpublished). As in chickens, they form a cluster on the short arm of the duck Z chromosome [102]. These results support the view that the cloned duIFN represents the functional homolog of mammalian IFN- α [100].

Type I IFNs are strongly upregulated upon viral infection, engage their receptors, and trigger the production of IFN-stimulated genes. In Pekin ducks challenged with H5N1 viruses, duIFN- α transcripts were highly upregulated in the spleen, while duIFN- β transcripts were upregulated in the lung [103] as quantified by qPCR. This may correlate with different cell types contributing to each tissue; IFN- α is expressed by leukocytes, while IFN- β is primarily expressed by epithelial cells. Two duIFN- β cDNA sequences have been deposited in Genbank (Table 19.1), but functional analyses have yet not been done. The receptors, IFNAR1 and 2, have been identified in chickens [104], annotated in ducks, and their expression shown to be very different in goose tissues [105]. Despite binding to the same receptor, chIFN- α and chIFN- β induce different sets of IFN-stimulated genes downstream of activation in chicken DF-1 cells [106]. ChIFN- α turned on more antiviral IFN-stimulated genes while chIFN- β was more potent at turning on genes involved in signaling pathways.

Type-II interferon

Type II IFN is secreted by activated leukocytes and is critical in coordinating innate and adaptive immune responses. Due to its potential application in agricultural birds to improve their immune responses, it has been characterized in most agricultural birds. Initially, functional approaches were employed to identify cDNA encoding chIFN- γ [134,135]. Subsequently, cDNA from several other galliform species, including Japanese quail and turkey, were isolated [127,136]. Both turkey IFN- γ and chicken IFN- γ could induce production of NO by both turkey and chicken macrophages. This activity could be neutralized by an MAb raised against chicken IFN- γ indicating a close antigenic relationship [136,137]. Using the chIFN- γ cDNA as probe, duIFN- γ was identified in lambda phage cDNA libraries of mitogen-stimulated splenocytes [97,108]. Goose IFN- γ shares 93.3% identity with duck [124], and both show lower sequence identity to chIFN- γ (67%–68%). Showing cross-species activity, duIFN- γ showed antiviral activity on quail CEC-32 cells, though the effect was $\sim$ 16-fold less than on duck fibroblasts. Recombinant duIFN- γ has the capacity to induce chicken macrophages to produce NO, albeit the specific activity was approximately 16-fold lower than with chIFN- γ . Further, a rabbit serum raised against duIFN- γ could neutralize the activity of chIFN- γ [108]. In contrast, chIFN- γ , like chIFN- α , exhibited species-specific activity in that it showed undetectable levels of reactivity with duck fibroblasts. Importantly, duIFN- γ blocked the replication of DHBV in primary duck hepatocytes [97]. Recombinant goose IFN- γ showed biological activity in inhibiting goose paramyxovirus replication and stimulating isolated goose abdominal macrophages to make NO [124]. Recombinant duIFN- γ had antiviral activity against avian influenza virus and vesicular stomatitis virus in DEFs, and pretreatment decreased viral replication and mortality of H5N1 in ducklings [138].

Type III interferons

Type III IFN (also called IFN- λ) is a single copy gene in ducks [110]. Recombinant duck IFN- λ harvested in supernatant can stimulate activation of duck IFN-stimulated genes, Mx and OASL in cultured primary duck hepatocytes [110]. Expression of duck IFN- λ transcripts are induced by infection with influenza virus in DEFs [139] and upregulated in H5N1 infected ducks [103], although transcript levels are low compared to other IFN transcripts.

19.3.2 Interleukins

Interleukin-1

Interleukin-1 is a proinflammatory cytokine and plays a central role in immune responses to injury and infection. Chicken interleukin (IL)-1 β was identified by expression cloning using a cDNA library generated from

TABLE 19.1 List of cloned duck, goose, turkey, and quail cytokine cDNA.

Cytokine	Accession number	References
Duck ^a		
• Interferon		
IFN- α	X84764; AB128861; AY879230	[95,107]; Chen B., Cheng A., Wang M., and Tang Q., unpublished
IFN- β	KM035791; KT428159	Li H., Song S., Jin H., and Luo R. unpublished; Chen S. and Lin S., unpublished
IFN- γ	AF087134; AJ012254; AY166850	[97,108,109]
IFN- λ	NM001310792; KT950257	[110]; Shilong C. and Shuncheng L., unpublished
• Interleukin		
IL-1 β	AY426338 ^b , DQ383268.1	[111,112]
IL-2	AF294322, AF294323 AY232490, AY707747 AY173028AY193713 ^c AY821656	Schmohl K. and Schultz U., unpublished [113] [114]
IL-4	ATP76495	Wang J.Y., Zhou J.Y., and Chen J.G., unpublished [115]
IL-6	AB191038 ^b , JQ728554	Ruan X.F. and Xia C., unpublished; Elfeil W.M. and colleagues, unpublished.
IL-10	JN786939, JN786940, JN786941	[116]
IL-15	DQ490136	Schultz U., unpublished
IL-16	AF294320, AF294321	Schmohl K. and Schultz U., unpublished
IL-17A	EU366165	[117]
IL-18	AF336122; DQ490137 DQ522948	Chan W.-S., Warr G.W., Middleton D.L., Lundqvist M.L., and Higgins, D.A., unpublished; Mannes N.K. and Schultz U., unpublished [118]
• Chemokine		
CC chemokine "MIP-1 β "	AY641437	[119]
CC chemokine "RANTES"	AY641435	[119]
CC chemokine	AY641436	[119]
CCL19	AY682098	[120]
CCL21	HQ174214	[120]
CXC chemokine "chCXCLi2/IL-8-like"	AB205199 ^b	Chen W.H. and Xia C., unpublished [121]; Chen W.H. and Xia C., unpublished
• Other cytokines		
CD154	DQ267671	[122]
BAFF	DQ445092	[123]
Goose		
• Interferon		
IFN- α	AY524422 ^d	[96]
IFN- γ	AY524421 ^d	[124]
• Interleukin		
IL-1 β	DQ393271	[112]
IL-2	AY392557 ^e	[125]
• Chemokine		
CXC chemokine "chCXCLi2/IL-8-like"	DQ393274; AB213393 ^e	[121]; Guan R.L., Chen W. H., and Xia C., unpublished
• Other cytokines		
BAFF	DQ874394 ^e	[126]
Turkey		
• Interferon		
IFN- α	U28140	[94]
IFN- γ	AJ000725	[127]

(Continued)

TABLE 19.1 (Continued)

Cytokine	Accession number	References
• Interleukin		
IL-1 β	DQ393271	[112]
IL-2	AJ007463; AF209705	[128]; Romero C.H. and Cai X.Z., unpublished
IL-10	AM493432	[129]
IL-12 β -chain	AJ564203 ^b	[130]
IL-13	AM493431	[129]
IL-18	AJ312000	[131]
• Chemokine		
CXC chemokine	DQ393276	[121]
“chCXCLi2/IL-8-like”		
Quail		
• Interferon		
IFN- α	AB154298	Koyama, K. and Takahashi, T., unpublished
IFN- γ	AJ001678	[127]
• Interleukin		
IL-1 β	AB559570 ^b	[132]
IL-2	AY707748	[113]
IL-4	AB559571 ^b	[132]
IL-6	AB559572 ^b	[132]
IL-10	AB559574 ^b	[132]
IL-12	AB727361 ^b , AB559575 ^b	[132]
IL-13	AB559576 ^b	[132]
IL-17	FJ788630 ^b	Zhang, X., Zhang, C., Li, Y., Cheng, X., and Wu, T. unpublished
IL-18	GU140074; AB559577 ^b	Zhang, X., Zhang, C., Cheng, X., Wu, T. and Li, Y., unpublished; [132]
• Chemokine		
CXC chemokine	AB559573 ^b	[132]
“chCXCLi2/IL-8-like”		
BAFF	EU599084	[133]

^aPekin duck (*Anas platyrhynchos*) except sequences indicated.^bPartial coding sequence.^cMuscovy duck (*Cairina moschata*).^d“Gray” goose (*Anser anser*).^eSwan goose (*Anser cygnoides*).

lipopolysaccharide (LPS)-stimulated HD-11 cells [140]. Subsequently, a partial cDNA was amplified by PCR from Pekin duck liver using primers designed to the chIL-1 β cDNA (Table 19.1) [111]. Genes encoding IL-1 β have subsequently been cloned from duck, goose, turkey and pigeon [112], and quail [132]. IL-1 β is synthesized as a precursor that is cleaved by caspase-1 upon assembly of the inflammasome. Resembling chIL-1 β , the predicted amino acid sequence of duIL-1 β lacked the highly conserved aspartic acid residue necessary for recognition and cleavage by the mammalian protease. However, IL-1 β from duck, goose, or turkey is cleaved by mammalian (or fish) caspase-1 at a different conserved aspartate [141]. Thus it appears that cleavage of IL-1 β is conserved throughout phylogeny, but this remains to be confirmed with avian caspase-1.

IL-1 β transcripts are upregulated upon inflammation. IL-1 β transcripts are expressed in the spleen and liver of

ducks in response to an intravenous injection of *E. coli* complexed to various ratios of immunoglobulin (Ig)Y:IgY (Δ Fc) [111]. As expected of a proinflammatory cytokine, stimulation of isolated duck splenocytes with TLR7 agonists induced IL-1 β transcripts [38]. Recombinant IL-1 β proteins from duck, goose, turkey, and pigeon were able to induce cytokine K60/chCXCLi1 mRNA expression in chicken DF-1 cells, albeit at reduced levels to recombinant chIL-1 β [112].

A cDNA for the IL-1 receptor chain 1 (IL-1R1) of chicken was cloned [142]. Homologs in other avian species have not been characterized to date. Furthermore, cDNA for the avian homolog of IL-1 α or IL-1R α await identification.

Interleukin-2

IL-2 is mainly made by helper T cells, and its signals are necessary for differentiation and proliferation of effector

and memory T cells [143]. Chicken IL-2 was recovered by expression cloning, testing supernatants of cells transfected with pools of cDNA for their proliferation-inducing activity [144]. In earlier studies, duck lymphocytes stimulated with phytohemagglutinin (PHA) released cytokines, which could induce the proliferation of duck lymphoblasts [145,146]. Using the chIL-2 cDNA as probe, two Pekin duIL-2 cDNA differing in the 3'UTR were identified by screening a lambda phage cDNA library from PHA-stimulated duck splenocytes. Subsequently, IL-2 cDNA of Muscovy duck [114], goose [125], turkey [128] and Japanese quail [119] were identified by RT-PCR using sequence information on the previously identified chIL-2 and duIL-2 (Table 19.1). Comparative analysis of avian IL-2 amino acid sequences revealed identities varying from 59.3% (chicken:Muscovy duck) to 95.7% (Pekin duck:Muscovy duck). Phylogenetic analysis of avian IL-2 amino acid sequences shows that the similarity of avian IL-2 sequences parallels the presumed evolutionary relationships between the species: the galliform birds (chicken, turkey, quail) forming one clade and the anseriform birds (Pekin duck, Muscovy duck, goose) forming a distinct clade. Analysis of the gene organization revealed remarkable conservation between avian and mammalian IL-2 genes, with four exons and three introns and a very short 5' UTR. Transcription factor-binding sites and the predicted transcription start site with respect to the chIL-2 sequence showed high conservation in Pekin duck, quail, and turkey IL-2 promoters [113,128].

Recombinant IL-2 and antibodies raised against the proteins are valuable tools for modulating and assessing immune function. Recombinant duIL-2 was expressed in HEK293T cells (Schmohl K. and Schultz U., unpublished) and *E. coli* [114] and shown to be biologically active in lymphocyte proliferation assays. Interestingly, duIL-2 and chIL-2 cross-react in lymphocyte proliferation assays, although they share only limited sequence identity [114]. TuIL-2 and chIL-2 (~70% identity) also cross-react in functional assays [128]. Recombinant goose IL-2 induces proliferation of goose, duck, and chicken lymphocytes, though the observed effect on duck and chicken lymphocytes is relatively weak (~83% and 63% amino acid sequence identity, respectively) [125]. Bioactivity of recombinant duIL-2 and goose IL-2 was assessed in vaccination studies [114,125]. Recombinant IL-2 enhances duck and goose immune responses when inoculated with an inactivated vaccine against avian influenza virus (rise in virus-specific antibody titers). Polyclonal antibodies and mouse MAb raised against *E. coli*-produced duIL-2 and goose IL-2 neutralize the biological activity of both recombinant and endogenous duIL-2 and goose IL-2, respectively [114,125]. Further, the functional domains of duck IL-2 for binding to the IL-2 receptor α -chain were mapped using MAb [147].

Co-delivery of IFN- γ and duck IL2 encoding plasmids induces a better neutralizing antibody response to a DNA-based vaccine encoding DHBV preS/S [148]. No functional data are available for quail IL-2 at present.

The receptor of both IL-2 and IL-15 cytokines is composed of three subunits (IL-2R α /CD25 or IL-15R α /CD215, IL-2R β , and IL-2R γ). While both cytokines have their own α -chain, they share the β - and the common γ -chain. Information on avian homologs is emerging. Chicken CD25 was cloned and used for generation of MAb for flow cytometry. Characterization of chicken CD25 surface expression showed it is expressed on chicken macrophages, thrombocytes, CD4 and CD8 positive T cells, and is a marker of T cell activation [149]. Among the CD4 + CD25 + T cells in chickens, regulatory activities were found, and rIL-2 reversed the suppressive properties [150]. The duck IL-2R α -chain (CD25) was cloned and the recombinant protein used as immunogen in mice to make an MAb [151]. The MAb can neutralize duIL-2 induced lymphocyte proliferation and also label duck lymphocytes for flow cytometry. Regulatory T-cell properties have also been ascribed to the CD4 + CD25 + T cells in ducks [152] and turkeys [153]. Common cytokine receptor γ chain (CD132) was cloned from ducks and quail and two transcripts are produced by alternate splicing in each species (Table 19.3) [154]. In ducks, an alternate splicing variant produces a common γ chain lacking a transmembrane region, although the functional significance is unknown.

Interleukin-6

Proinflammatory cytokine IL-6 is rapidly produced locally in response to infection or injury and stimulates the systemic acute phase response [155]. In the duck, an IL-6-like biological activity was first demonstrated in supernatants of LPS-stimulated duck monocytes [145]. Not surprisingly, no transcripts hybridizing with human IL-6 could be isolated. Subsequently, a partial cDNA for duIL-6 shows high amino acid identity with chIL-6 (89.9%) and only 45.7% identity with human IL-6. Duck splenocytes increase expression of duIL-6 transcripts upon induction with TLR7 agonists [38], and duck PBMCs express IL-6 transcripts following infection with LPAIV [41,73,156]. While IL-6 expression is normally transient, prolonged or dysregulated IL-6 expression is associated with increased pathology in viral infections, as seen in infections with highly pathogenic avian influenza especially in chickens [157,158], but also in ducks [103,159].

Interleukin-8

In mammals, IL-8 is a CXC chemokine named for the two N-terminal cysteines separated by one amino acid,

and has therefore been renamed CXCL8. Molecules that may represent the avian homolog of mammalian IL-8/CXCL8 are discussed in the section on chemokines (Table 19.1).

Interleukin-10

Mammalian IL-10 is a key immunoregulator during infection with viruses, bacteria, or parasites, which has a central role in preventing inflammatory pathology [160]. Three transcripts encoding IL-10 were cloned from duck splenocytes [116], two with alternate 3' UTRs, and a splice variant lacking exon 5. The duIL-10 protein has 79% homology with chIL-10 and shares exon structure with other vertebrates but has an additional terminal exon. DuIL-10 transcripts were most abundant in primary and secondary immune organs and lung. Recombinant duIL-10 suppressed IL-2 mRNA expression from PHA-stimulated PBMCs in a modified functional bioassay demonstrating that anti-inflammatory properties are conserved [116]. Thymic CD4⁺CD25⁺T cells isolated with cross-reactive anti-chicken CD25 MAb, putative T regulatory cells from ducks, showed elevated IL-10 transcript expression in comparison to CD4⁺CD25⁻ cells [152].

IL-10 mediates anti-inflammatory effects through two cell surface receptors, IL-10R1 and IL10-R2. Polymorphisms in these receptors have been implicated in HBV persistence in humans, prompting their analysis in the duck [161,162]. The cDNA and genomic structure of duck IL-10R2 suggest that several features of the mammalian IL-10 receptor are conserved, but the low-affinity interaction site in loop 2 is unique to mammals [162].

TuIL-10 and IL-13 have 92% and 79% amino acid identity with chIL-10 or IL-13, respectively. Recombinant tuIL-10 showed bioactivity by inhibition of IFN- γ synthesis from activated splenocytes [129]. Chicken and tuIL-10 are conserved enough to cross-react in functional assays.

Interleukin-12

In mammals, IL-12 is a heterodimeric 70 kDa cytokine composed of two components, a 40-kDa heavy chain (IL-12 β or p40) and a 35 kDa light chain (IL-12 α or p35). Both chains from the chicken have been cloned taking advantage of partial chicken expressed sequence tag (EST) sequences with homologies to mammalian IL-12 subunits [130,163]. Antibodies to chicken IL-12 have been produced and used for the development of a capture ELISA for detecting the proteins [164].

Using primers designed on the chIL-12p40 a partial turkey IL-12p40 cDNA was obtained by RT-PCR from turkey splenocyte RNA. The turkey cDNA encodes a predicted protein with 95% identity to chIL12p40 (Table 19.1) [130]. To develop a method for analyzing immune responses in the quail, the nucleotide sequences

of various interleukins, including both IL-12 chains, were determined based on the sequence of the chicken genes and quantitative real-time PCR assays have been developed (Table 19.1) [132]. To our knowledge, no further IL-12 genes have been characterized for domesticated agricultural birds. Primers based on the chicken IL-12 sequence have been used to assess immunological responses to vaccines in ducks [165,166], which could be improved with the predicted sequence from the duck genome.

Interleukin-15

IL-15 is a pleiotropic cytokine with similarity to IL-2 and regulates the activation and proliferation of T and NK cells [167]. IL-15 has been cloned in two avian species, chicken and duck. The cDNA for duIL-15 was identified by screening a lambda phage cDNA library from PHA-stimulated duck spleen cells with the chIL-15 cDNA probe (Table 19.1). The predicted mature duIL-15 showed ~76% identity with chIL-15 and only ~30% identity with human IL-15. No functional data are yet available for duck IL-15. ChIL-15 has been explored for potential as an adjuvant to enhance the immunogenicity of DNA vaccines. ChIL15 as adjuvant along with the *Eimeria* 3-1E gene as vaccine provided protection against *Eimeria acervulina* in homologous challenge. IL-15 as a genetic adjuvant coadministered with an H5 DNA AIV vaccine showed some improved immunogenicity compared to the H5 DNA vaccine alone [168].

Interleukin-16

IL-16 is a chemoattractant and activator for CD4 T cells [169]. Cloning of duIL-16 cDNA was achieved by screening a lambda phage cDNA library from PHA-stimulated duck spleen cells using a chIL-16 C-terminal probe (Table 19.1). Two different cDNA gave rise to proteins differing only in three amino acid residues with molecular weights of 65 kDa. Comparison of the duIL-16 propeptide with the propeptide of chIL-16 and human IL-16 indicated that duIL-16 shares 86% identity with chIL-16 and 51% with human IL-16. The duck sequence has four aspartate residues (477, 486, 495, 499) in a region in which cleavage of mammalian propeptides by caspase 3 occurs. Aspartate 486 of the duIL-16 propeptide is closest to the residue used for cleavage of human IL-16; however, this aspartate residue is not conserved in chIL-16. DuIL-16 transcripts were expressed abundantly in lymphoid tissues (Schmohl K. and Schultz U., unpublished). No functional data are available at present.

Interleukin-17

The IL-17 family is closely related to regulatory cytokines [170] produced mainly by activated CD4⁺ T cells [171],

epithelial cells, and other innate lymphocytes [170]. Chicken IL-17 was initially identified from an EST from *Eimeria*-infected intraepithelial leukocytes of chickens [172]. Primers based on the chicken sequence allowed identification of a duck IL-17 cDNA with 84% similarity to chicken IL-17 [117]. Transcripts for duIL-17A were detected in Con A-activated splenic lymphocytes. Two chicken MABs (IG8 and 2A2) [173] cross-reacted with epitopes from recombinant duck IL-17 in ELISA and on Western blots and may prove useful tools for the characterization of the Th17 subpopulation in ducks [117]. Two more duck IL-17 cytokine family members have been subsequently studied, IL17A and IL-17D. IL17A was upregulated [174], while IL-17D was downregulated in the spleen and liver of *Riemerella anatipestifer* infected ducks [175]. In addition, a partial cDNA of quail (qu)IL-17 has been cloned (Table 19.1) [132].

Interleukin-18

IL-18 has been cloned from chicken [176], turkey, and duck. The tuIL-18 was cloned by RT-PCR from PMA-stimulated turkey splenocytes using primers based on the chIL-18 cDNA and 5' and 3' RACE (Table 19.1) [131]. While the 3' UTR of chIL-18 has only one copy of the instability motif ATTTA the tuIL-18 3' UTR contains two copies of this motif, which is involved in rapid mRNA degradation. Overall, the deduced tuIL-18 amino acid sequence has 97.4% identity with the chIL-18 sequence. No information is available to date on the biological activity of tuIL-18. Two database entries provide sequence information on duIL-18 (Table 19.1). Comparison of the two deduced amino acid sequences revealed four amino acid differences (V11E, L19P, F21Y, Q54R). They showed ~86% identity to chicken and tuIL-18, 26.4% to human IL-18, and 20% or less to fish IL-18. Like chicken, turkey, and mammalian IL-18, bioactive duIL-18 seems to be generated from an inactive precursor by cleavage at a conserved aspartate residue after amino acid residue 30 to give rise to a mature protein with a calculated molecular mass of 19.7 kDa. Recombinant duIL-18 produced in *E. coli* induces the proliferation of duck splenocytes and the secretion of IFN- γ from the chicken B cell line B19-2D8 indicating that duIL-18 is able to bind to and activate the chicken IL-18 receptor [118]. The IFN- γ -inducing activity could be neutralized by preincubation of duIL-18 with a polyclonal rabbit antiserum raised against duIL-18. IL-18 gene expression has been observed in peripheral blood lymphocytes (PBLs), spleen, thymus, and bursa of Fabricius but not in the liver (Mannes N.K. and Schultz U., unpublished).

cDNA encoding the IL-18 gene from Gushi layer ducks was cloned and used to generate recombinant

protein and polyclonal and monoclonal antibodies. Recombinant duIL-18 showed bioactivity in the lymphocyte proliferation assay, and both MAB and polyclonal Ab antibodies were able to block the proliferation of ConA stimulated splenic mononuclear cells [118]. Goose IL-18 is upregulated in the lung following NDV infection [177].

19.3.3 Tumor necrosis factor family

The tumor necrosis factor (TNF) superfamily consists of members that are distantly related to TNF and have important functions in the regulation of immune responses, inflammation, and tissue homeostasis. Although various biological activities have been described for supernatants of stimulated chicken macrophages believed to contain the chicken homolog of TNF- α , efforts to clone it failed and this gene was presumed missing in birds. Only recently, was a bona fide TNF- α identified in chickens [178]. This gene eluded identification because it was highly GC-rich with only 45% homology to the mammalian homolog. Using the chTNF- α sequence, Rhode and colleagues identified homologs in several avian species including turkey, duck, goose, and crow. In the crow, they identified the gene in a region of synteny with mammalian major histocompatibility complex (MHC), but on a different chromosome, suggesting this gene was relocated during evolution. Chicken TNF- α transcripts are upregulated by monocyte-derived and splenic macrophages upon stimulation with LPS (100 ng/mL). Examination of other leukocytes revealed that TNF- α transcripts were upregulated in stimulated CD4⁺ T cells, not CD8⁺ T cells. Finally, they showed that codon-optimized TNF- α expressed in HEK293 cell supernatant was functional in activating an NF- κ B promoter in quail CEC-NF- κ B luciferase reporter cells. Chicken TNF- α receptor genes had been previously identified [179]. It has been pointed out [63] that all studies previously describing TNF- α in birds were examining an unrelated cytokine, LPS-induced TNF- α factor (LITAF) [63], leading to some confusion in the literature.

B-cell activating factor of the TNF family (BAFF) is the master regulator of B cell development [180]. Chicken BAFF was cloned after identification in a chicken bursa EST database. Soluble recombinant chicken BAFF binds to B cells thereby promoting the selective expansion and survival of chicken B cells [181]. In addition, it selectively binds to duck B cells but not to other duck lymphocytes (Fig. 19.1) [182]. Duck BAFF has been cloned by RT-PCR and RACE [123]. The amino acid identities between biologically active duck and chicken BAFF and human BAFF is 97% and 78%, respectively. RT-PCR analysis showed that the duck BAFF gene is strongly expressed in the bursa of Fabricius. Purified recombinant duck BAFF is able to promote bursal cell

	mAb/Ligand							
	CD4	CD8	CD3-12	2-4	14A3	K1	Ch BAFF	BA3
B cells	-	low	-	-	+	-	+	-
T helper cells	+	-	+	+	-	-	-	-
Cytotoxic T cells	-	high	+	+	-	-	-	-
Thrombocytes	-	-	-	-	-	+	-	+
Monocytes/ Macrophages	-	-	-	-	-	+	-	-

FIGURE 19.1 Range of monoclonal antibodies (MAb) suitable for phenotyping the major leukocyte populations in ducks. MAb specificity: CD4 (CD4 antigen), CD8 (α -chain of CD8), 3–12 (conserved peptide of the ϵ -chain of CD3), 2–4 (CD28), chBAFF (BAFF receptor on B cells), 14A3 (λ chain), K1, chBAFF (common antigen on thrombocytes and monocytes/macrophages), BA3 (antigen on thrombocytes) [184].

survival [123]. Quail BAFF has been cloned and is expressed in lymphoid tissues including the spleen, liver, brain, bursa, kidney and thymus, and muscle. Recombinant quBAFF promotes survival of quail bursal B cells *in vitro* [133]. Goose BAFF was also cloned and shares 98% identity with duck and 92% identity with chicken BAFF. Recombinant goose BAFF promotes B cell survival [126]. Similarly, ostrich BAFF was cloned and a recombinant ostrich BAFF promotes survival of ostrich B cells in culture [183].

TNF superfamily 15, the homolog of human TL1A [185,186], LITAF [187,188], and CD154/CD40 ligand [189] have been cloned from chicken. Duck CD154/CD40L (duCD154) was isolated by RT-PCR from RNA from duck splenocytes cultured in the presence of concanavalin A and LPS (Table 19.1) [122]. Amino acid sequence identity to chicken CD154 is 84% and to mammalian CD154 ~40%. When only the extracellular domain was expressed, it was secreted, formed dimers, and enhanced the proliferation of duck splenocytes. In mammals, CD154 has been used as an adjuvant or in immunotargeting approaches to enhance vaccine responses. When ducks were immunized with constructs expressing the functional domain of duCD154 fused to the truncated DHBV core protein, they developed accelerated and enhanced core-specific antibody responses and showed enhanced proliferative responses of duck PBMC compared to ducks immunized with truncated core alone [190]. Similarly, administration of a DNA vaccine comprised avian influenza virus subtype H5 fused to CD154 followed by challenge with a highly pathogenic H5N1 strain, resulted in lower viral titers and reduced disease

signs; however, oropharyngeal shedding rebounded in the second-week postchallenge [191]. These results indicate that targeting of antigens to antigen-presenting cells (APCs) with CD154 is an effective strategy to enhance immunological responses not only in mammals but also in birds.

19.3.4 Th2 cytokines

Using a genomics approach based on conservation of synteny, the chicken Th2 cytokines IL-3, IL-4, IL-5, and IL-13 were isolated [192]. The Th2 response-promoting IL-10 was initially isolated from an EST library derived from RNA from cecal tonsils of *Eimeria*-infected chickens [193]. Of the Th2 cytokines, duIL-10 [116], tuIL-10 and tuIL-13 [129], quIL-10 and quIL-13 [132] were described previously. Recently, duck IL-4 was cloned and shares 49%–50% identity with chicken and quail IL-4. Recombinant IL-4 can inhibit inflammatory responses due to IL-17A in ducks following infection with *R. anatipestifer* [115].

19.4 Chemokines

19.4.1 CXC chemokines

The first anseriform chemokine identified was duck IL-8 (Chen W.H. and Xia C., unpublished.) and goose IL-8 (Guan R.L. and Xia C., unpublished). Amino acid sequence comparison of the duck protein revealed 92% identity with chCXCLi2. Subsequently, primers based on the chCXCLi2 (IL-8) sequences were used to clone IL-8

from three additional avian species including goose, pigeon, and turkey [121]. Each IL-8 encoding region was 103 amino acids long and amino acid similarity ranged from 87% to 100%. Subsequently, eight CXC chemokines were identified in the duck genome [2], four had expression in lung tissue that was markedly increased (CXC3L1, CXCL13, and two IL8 genes), and three had expression that was greatly decreased (CXCL12, CXCL13L2, and CXCL14) upon infection with highly pathogenic avian influenza.

19.4.2 CC chemokines

A comprehensive survey of the duck genome listed 13 CC chemokines in duck, compared to 14 in chickens [2], without indicating which chemokine was not identified in ducks. Of these, transcripts for nine CC chemokine genes were significantly upregulated in lung tissues of ducks following infection with HPAIV H5N1, including CCL4L2, CCL5, CCL6, CCL17, CCL19, CCL20, CCL21, CCL23, and CCL24.

C chemokine related to mammalian lymphotactin has been described in chickens [194]. One C chemokine was identified in the duck genome and upregulated during influenza viral infection [2].

Using subtractive hybridization, Sreekumar et al. [119] identified three mRNAs in mitogen-stimulated splenic mononuclear cells of a duck with similarity to CCL chemokine sequences of chicken (Table 19.1). The predicted protein of one mRNA (AB163) consists of 90 amino acids with 71% identity at the amino acid level with chCCL chemokine ah294 and about 52% identity with mammalian RANTES. The second mRNA (AB330) coded for a 90 amino acid protein that showed 76.6% identity to chicken MIP1- β . The third mRNA (AB187) has similarity to chCCL1 at ~41% identity. Basal level expression of all three molecules could be demonstrated in the bursa of Fabricius, trachea, kidney, and bone marrow. AB163 and AB187 could be detected in the liver and lung, while the spleen showed the presence of AB163 and AB330. All three chemokine mRNAs are upregulated upon mitogen stimulation of splenic mononuclear cells. Subsequent functional analyses using recombinant duck RANTES expressed from HEK293T cells showed it mediated chemotaxis of DT-40 chicken B cells and primary duck splenocytes [195]. Staining of these migrated duck splenocytes with anti-duck CD4 or CD8 showed enhanced chemotaxis of CD8⁺ cells.

The mammalian CCL19 and CCL21 chemokines are involved in trafficking of lymphocytes to the secondary lymphoid tissue, and further, induce a proinflammatory cytokine profile in licensed DCs [196]. These two CC chemokines were initially identified in a duck spleen EST project [120]. Like the chicken counterparts, duck CCL19

and CCL21 and their corresponding receptor CCR7 (Table 19.3), appear to be well conserved across phylogeny [197]. These chemokines may have potential as vaccine adjuvants. CCL19 transcripts were abundant in the lung, kidney, spleen, duodenum, bursa, while CCL21 was amplified from each of these tissues and heart. CCL19 and CCL21 transcripts were rare in tissues isolated from the lymphatic network. These tissues previously described to contain lymph nodes [198] do not contribute significantly to lymphocyte recruitment [197]. Because of the apparent lack of lymph nodes, tertiary lymphoid tissues showing significant leukocyte infiltration at the site of infection were examined in ducks. CCL19 and CCL21 transcripts are highly upregulated duck lung tissues following infection with HPAIV [197].

19.5 CCR7

The chemokine receptor CCR7 is a member of the G protein-coupled receptor family (syn. CD197). It is activated by two different ligands, CCL19 and CCL21, and is responsible for the proper recruitment of lymphocytes and mature DCs to lymphoid tissues. Several chemokine receptors including CCR7 have been identified in chicken [186], and their expression in isolated CD4⁺ T cells from blood and various tissues were investigated [199]. Together with its ligands, CCR7 from ducks has also been cloned [197]. Lung, kidney, and spleen show the highest expression of CCR7. The amino acid sequence of duCCR7 is 90.1% identical to the chCCR7, and 67% identical to human CCR7. Lysines important for chemokine binding are conserved.

19.6 Cell surface antigens

19.6.1 Anti-chicken monoclonal antibodies cross-reacting with turkey, quail, and duck leukocytes

Intensive studies of poultry immune systems depend on the availability of MAb against leukocyte surface antigens. Most studies of avian leukocyte phenotypic markers are on chicken. From the panel of anti-chicken MAb used to study the tissue distribution, ontogeny, and function of lymphocyte populations in the chicken, most react with turkey cells, some with quail cells but very few bind to cells of duck lymphoid organs (Table 19.2) [200]. Flow cytometric analysis of PBMC from individuals in five turkey lines revealed that certain anti-chicken CD8 α MAb (3–298, 11–39) showed good cross-reactivity with the corresponding molecule on turkey cells. However, the MAb CT8 failed to detect the CD8 α molecule in some turkeys, and there were large line differences in the ability to detect the epitope, suggesting, as is the case with

TABLE 19.2 List of monoclonal anti-chicken antibodies cross-reacting with turkey, quail, and duck cells.

Antibody	Antigen	Specificity	Turkey	Quail	Duck	References
HIS-C7	CD45	All leukocytes	—	—	—	[200,204]
LT40	CD45	All leukocytes	nd	nd	(+) ^a	[205]; Bean A., Reaiche G., Bertram E., Lowenthal J., Jilbert A., unpublished ^b
HIS-C1	Bu-1	B cells	+	—	—	[200,204]
AV20	Bu-1	B cells, subsets of monocytes/macrophages	+ / —	nd	—	[206,207]; Kaiser P., unpublished ^c ; Southern Biotechnology, unpublished; Kothlow S., Kaspers B., Schultz U., unpublished ^d ; Bean A., Reaiche G., Bertram E., Lowenthal J., Jilbert A., unpublished ^b
HIS-C12	IgM	IgM-positive B cells	+	+	—	[200,204]
M1	IgM	IgM-positive B cells	+	nd	nd	[137,208,209]
CVI-ChIgG-47.3	IgG	IgG-positive B cells	+	+	—	[200,204]
AV-G3	IgG	IgG-positive B cells	+	nd	nd	[208]
CVI-ChIgA-46.5	IgA	IgA-positive B cells	+	—	—	[200,204]
CVI-ChIgL-47.5	Ig λ chain	All Ig-positive B cells	+	—	—	[200,210]
CT1	Common thymocyte antigen	Thymocytes	nd	+	—	[211,212]; Bean A., Reaiche G., Bertram E., Lowenthal J., Jilbert A., unpublished ^b
CT3	CD3	T cells	—	—	—	[200,211–213]; Bean A., Reaiche G., Bertram E., Lowenthal J., Jilbert A., unpublished ^b
AV29	CD4	Helper T cells	+	+	—	Davison T.F., unpublished; [137,200]
CT-4	CD4	Helper T cells	+	—	—	[201,211,213,214]; Kaiser P., unpublished ^c ; Southern Biotechnology, unpublished; Kothlow S., Kaspers B., Schultz U., unpublished ^d ; Bean A., Reaiche G., Bertram E., Lowenthal J., Jilbert A., unpublished ^b
2–35	CD4	Helper T cells	+	nd	nd	[201,215]
7–125	CD4	Helper T cells	+	nd	nd	[201,215]
2–6	CD4	Helper T cells	+	nd	nd	[201,215]
EP96	CD4	Helper T cells	nd	nd	—	Bean A., Reaiche G., Bertram E., Lowenthal J., Jilbert A., unpublished ^b
CT-8	CD8	Cytotoxic T cells	+/-	—	nd	[201,211,213,214,216,217]
CVI-ChT-74.1	CD8 α chain	Cytotoxic T cells	—	—	—	[200,218]
11–39	CD8 α chain	Cytotoxic T cells	+	nd	nd	[201,219]; Kaiser P., unpublished ^c
3–298	CD8 α chain	Cytotoxic T cells	+	nd	—	[137,201,220]; Southern Biotechnology, unpublished; Schultz U., unpublished ^d
EP42	CD8 β chain		+	nd	nd	[201,205]
AV-7	CD28	αβ T cells	+	nd	nd	[137,221]
2–4	CD28	αβ T cells	nd	nd	+	[182]
AV-6	CD44	Most cells incl. B and T cells, monocytes, subsets of epithelial cells	+	nd	(+) ^a	[137]; Southern Biotechnology, unpublished; Bean A., Reaiche G., Bertram E., Lowenthal J., Jilbert A., unpublished ^b
TCR1	γδ TCR	γδ T cells	—	—	—	[200,211,213,222]; Schultz U., unpublished ^d ; Bean A., Reaiche G., Bertram E., Lowenthal J., Jilbert A., unpublished ^b
TCR2	αβ ₁ TCR	Subpopulation of αβ T cells	+	—	—	[137,211,213,223,224]; Schultz U., unpublished ^d ; Bean A., Reaiche G.,

(Continued)

TABLE 19.2 (Continued)

Antibody	Antigen	Specificity	Turkey	Quail	Duck	References
TCR3	$\alpha\beta_2$ TCR	Subpopulation of $\alpha\beta$ T cells	—	—	—	Bertram E., Lowenthal J., Jilbert A., unpublished ^b [211,213]; Schultz U., unpublished ^d ; Bean A., Reaiche G., Bertram E., Lowenthal J., Jilbert A., unpublished ^b
AV33	$\alpha\beta_{1/2}$ TCR	$\alpha\beta$ T cells	—	+	—	Davison T.F., unpublished; [200]
CVI-ChNL-68.1		Mononuclear phagocytes	+	+	+	[200,204]
CVI-ChNL-74.2		Subpopulation of tissue macrophages	—	—	—	[200,225]
K1		Monocytes/macrophages, thrombocytes	+	nd	+	[137,182,226]
CVI-ChNL-68.3	MHCII	Antigen-presenting cells, B cells	—	—	—	[200,227]
2G11	MHCII	Antigen-presenting cells, B cells	+	nd	nd	[137,228]
F21–2	MHCI	Most nucleated cells	+	nd	+	[229]; Kaiser P., unpublished ^c ; Southern Biotechnology, unpublished; Schultz U., unpublished ^d
F21–21	β_2 m	Most nucleated cells	+	nd	—	[137,230]; Southern Biotechnology, unpublished; Bean A., Reaiche G., Bertram E., Lowenthal J., Jilbert A., unpublished ^b

nd, Not determined; PBMC, peripheral blood mononuclear cell.

^aFlow cytometric analysis of Pekin duck PBMC, $\leq 7\%$ positive cells.

^bFlow cytometric analysis of Pekin duck PBMC.

^cFlow cytometric analysis of turkey PBMC.

^dFlow cytometric analysis of Pekin duck thymocytes and splenocytes.

chicken, polymorphism of the turkey molecule (Table 19.2). This suggestion is fostered by the observation that MAb 3–298 distinguishes two different populations in single-color staining: CD8 α^{bright} and CD8 α^{dim} [201]. In chicken, the CD8 α^{dim} population of PBL expressed CD8 α as a homodimer in addition to CD4 [202,203], whereas all CD8 α^{bright} cells were CD4 negative and expressed CD8 β as a CD8 $\alpha\beta$ heterodimer [203]. Further experiments are needed to extend these observations to other avian species. In the same setting, most of the anti-chicken CD4 MAb reacted with a determinant on turkey PBMC (Table 19.2). It should be pointed out that these MAbs may not be recognizing the same molecule on turkey cells or that these molecules occur on a different cell population in turkeys. However, certain anti-chicken MAbs that were found to bind to epitopes on turkey PBMC and splenocytes, recognized molecules that might represent the turkey homologs of chicken Bu-1, CD4, CD8, CD28, and CD44 as these molecules show high sequence identities at the amino acid level to their

chicken counterparts (84%–95%) [137]. In addition, immunoprecipitation and western blotting showed that MAb 3–298 precipitated a 33–35 kDa polypeptide similar to the relative molecular mass of the chicken CD8 molecule [201]. With the MAb T-cell receptor (TCR)2, (which recognizes TCR $\alpha\beta$), M1 (IgM), K1 (an antigen on monocyte/macrophages and thrombocytes), F21-21 (β_2 microglobulin), and 2G11 (MHC class II antigen) five additional MAbs have been made that bind to epitopes on turkey PBMC and splenocytes (Table 19.2) [137]. With respect to the cross-reactivity of MAb Bu-, which recognizes chB1, conflicting observations have been reported. This might be explained by the differences in the genetic backgrounds of the birds resulting in minor modifications to the epitope (Table 19.1) (Kaiser P., unpublished data; Southern Biotechnology, unpublished data).

The fact that certain MAb specific for chicken T-cell markers: CT3 (recognizing CD3), CT4 (CD4), CT8 (CD8 α), TCR1 (TCR δ), TCR2 (TCR $\alpha\beta_1$), and TCR3 (TCR $\alpha\beta_2$) do not cross-react with quail cells has been

used to confirm the identity of chicken cells in chicken-quail chimeras (Table 19.1) [211,213]. However, an MAb detecting a common chicken thymocyte antigen did cross-react with quail thymocytes [211]. Interestingly, an MAb which does not discriminate between TCR2($\alpha\beta_1$) and TCR3($\alpha\beta_2$) does show cross-reactivity with quail cells. One possible explanation is that this MAb recognizes a less polymorphic epitope. In addition, the anti-chicken CD4, MAb AV29, reacts with a subset of quail lymphocytes. Furthermore, MAbs have been identified that bind to IgM⁺ and IgG⁺ B cells and also mononuclear phagocytes, respectively [200].

Unfortunately, of the group of anti-chicken antibodies specific for B and T cell determinants, none cross-reacted with duck cells (Table 19.2) [200]. It was only after MAbs specific for duck CD4 and CD8, respectively, had been generated that the MAb which recognized chicken CD28 was shown to react with the vast majority of CD4⁺ and a subset of CD8⁺ splenocytes (Table 19.3; Fig. 19.1) [182]. However, two MAbs were found that allowed the identification of duck mononuclear phagocytes [182,200]. One of them (K1) detects a common antigen on chicken monocytes/macrophages and thrombocytes and might therefore prove to be a valuable tool for depleting PBMC preparations of myeloid cells (Fig. 19.1). In addition, it is possible to detect duck Ig with mammalian antisera raised against chicken Ig [231].

19.6.2 Evidence for T and B cell populations in ducks

Until recently, studies of the immune system of ducks have not been successful in classifying lymphocytes phenotypically as cells resembling mammalian/chicken T and B lymphocytes (Table 19.2) [200,231,238]. However, duck lymphocytes have some of the functional characteristics of T cells as they respond to a range of mitogens, with PHA being the best studied [239]. Resembling the requirements for activation of mammalian lymphocytes, the mitogenic response of duck PBMC is dependent on adherent cells [240]. In addition, a polyclonal rabbit antiserum specific for a conserved cytoplasmic domain in the CD3 ϵ molecule binds to a population of duck lymphocytes with a distribution expected for T cells [241]. This anti-human CD3 rabbit antiserum immunoprecipitated a 23 kDa protein from duck lymphoblast lysates. The size is consistent with the molecular mass described for human CD ϵ . Furthermore, B cells have been identified in duck lymphatic tissues using goat anti-duck Ig antisera [242]. Goat antibodies hold the advantage that they are not bound via Fc receptors, thus occurrence of nonspecific binding can be excluded.

19.6.3 Antigens expressed on duck lymphocyte subsets

CD4 and CD8 antigen

CD4 is a single-chain transmembrane glycoprotein expressed on most thymocytes and on a subset of T cells [243]. In addition, in humans CD4 is also expressed on macrophages and granulocytes. CD4 acts as a cellular adhesion molecule binding MHC class II molecules. It stabilizes the interaction of MHC class II-restricted T cells [244]. Interactions of CD4 with MHC class II molecules are crucial during thymic development and subsequently for the function of single-positive CD4 T cells. CD8 occurs as a homodimer of two CD8 α -chains or as a heterodimer consisting of a CD8 α - and CD8 β -chain. CD8 is required for the development of CTL and functions as an adhesion molecule that binds to MHC class I molecules. In addition, in the chicken, the CD8 molecule is also found on some splenic and gut natural killer cells which both express the CD8 $\alpha\alpha$ homodimer [245]. The genes coding for chicken CD4 and CD8 were the first CD antigens identified in an avian species [246,247]. Later on, genes for duck CD4 and CD8, and turkey CD4 and CD8 were identified [248]. cDNA for Pekin and Muscovy duck CD4 and the α -chain of CD8 were amplified by RT-PCR using sequence information deposited by Higgins and coworkers (Table 19.3). Overall, the amino acid sequence of the Pekin duck CD4 precursor showed a higher degree of identity to avian (Muscovy duck 91.3%, chicken 60.2%) than to mammalian sequences (human 19.2%). Similar levels of identity were observed for Pekin duck CD8: Muscovy duck 86.5%, chicken 59.6%, and human 25.1%. Duck CD4- and CD8-transfected 293T cells proved to be suitable to generate MAbs reacting with subsets of duck splenocytes, thymocytes, and PBL preparations (designated duCD4-1, duCD4-2, duCD8-1, duCD8-2) (Fig. 19.1) [182]. Earlier attempts using duck thymocytes or mitogen-stimulated duck PBMC for immunization had produced numerous MAb which reacted with duck lymphocytes; however, it had not been possible to align the reactivities to functionally defined subpopulations [249]. Using two-color immunofluorescence staining, duck thymocyte preparations showed the presence of double-negative, double-positive, and single-positive CD4 or CD8 cell populations, which represent the typical maturation stages during thymic T-cell development. In contrast, peripheral lymphocytes were either CD4⁺ or CD8⁺. Nonetheless, a very small percentage of cells were shown to be double positive. While CD4⁺ cells are represented by a homogenous population, CD8⁺ cells are recognized as two populations with different staining patterns: CD8^{high} and CD8^{low}. This resembles data obtained with young chicks, where CD8^{bright} cells express the CD8 $\alpha\beta$

TABLE 19.3 List of cloned duck cell surface molecules.^a

Molecule	Accession number	References
CCR7	EU418503	[197]
CD3ε-chain	AF378704; AY738731, AY738734 ^b	Chan S.W.S., Middleton D.L., Lundqvist M., Warr G.W., and Higgins D.A., unpublished; [182]
CD4	AF378701; AY738732, AY738736 ^b	Chan S.W.S., Middleton D.L., Lundqvist M., Warr G.W., and Higgins D.A., unpublished; [182]
CD8α-chain	AF378737; AY738733, AY738735 ^b	Chan S.W.S., Middleton D.L., Lundqvist M., Warr G.W., and Higgins D.A., unpublished; [182]
CD25	DQ299949	[151]
CD44 isoform a	AY029553	Chan S.W.S., Middleton D.L., Warr G.W., and Higgins D.A., unpublished
CD44 isoform b	AF332869	Chan S.W.S., Warr G.W., Middleton D.L., and Higgins D.A., unpublished
CD44 isoform c	AY032667	Chan S.W.S., Middleton D.L., Lundqvist M., Warr G.W., and Higgins D.A., unpublished
CD58	AY032731	Chan S.W.S., Middleton D.L., Lundqvist M., Warr G.W., and Higgins D.A., unpublished
CD132	HM579917, HM579919, HM579921	[154]
CTLA-4/CD152	GQ995929, GQ995930, GQ995931, GQ995932	[232]
DCAR	DQ916297, DQ916298, DQ916300	[233]
DCIR	DQ916296	[233]
DRA	AY905539 ^c , HQ317493 ^c	Chong C.S.W., Samuel J., and Magor K.E., unpublished [234]
MHC class I	AY294416 ^c , AY885227	[235,236]
TCRα-chain	AF323922 ^d ; AF542183 ^d	Chan S.W.S., Warr G.W., Middleton D.L., Lundquist M.L., and Higgins D.A., unpublished; Chan S.W.S. and Higgins D.A., unpublished
TCRβ-chain	AF068228 ^d ; AY039002	[237]; Chan S.W.S. and Higgins D.A., unpublished
TCRγ-chain	AF378702	Chan S.W.S., Ko O.K.H. and Higgins D.A., unpublished
TCRδ-chain	AF415216 [236]	Chan S.W.S. and Higgins D.A., unpublished

^aPekin duck (*Anas platyrhynchos*) except for sequences indicated.^bMuscovy duck (*Cairina moschata*).^cOne of several alleles.^dPartial coding sequence.

heterodimer, while CD8^{dim} express the CD8αα homodimer. In older chickens, this phenotype is lost and all CD8⁺ cells express the heterodimer [246]. The analogous situation does not apply to duck lymphocytes, as shown by the unexpected observation that the vast majority of duck bursal cells react with duCD8-1 and duCD8-2. Confirming the flow cytometric data, CD8α gene expression can be demonstrated in the bursa, while CD4 and CD3ε transcripts are not detected [182]. In addition, more than 90% of bursal cells were double-positive using two-color immunofluorescence staining involving duCD8-1 in combination with 14A3, a duck Ig light chain specific MAb (see below and Fig. 19.1). Furthermore, three distinct cell populations could be identified in spleen cell preparations under these conditions: double-negative cells

representing CD4⁺ lymphocytes, CD8^{high}/14A3⁻ cells which could be CTL and, finally, CD8^{low}/14A3⁺ cells representing B lymphocytes. By CD3ε staining only, CD8^{high} cells can be identified as T cells, while the CD8^{low} cells were CD3⁻ but express surface Ig. Therefore it should be emphasized that the CD8^{low} cell population in ducks is not identical to the CD8^{dim} cell population that has been described in young chickens [246]. To our knowledge, CD8 antigen expression on B cells has not been reported in other species. It is tempting to speculate about the role of CD8 expression in duck B-cell development and B-cell function. Preliminary results, at least, suggest that the CD8 molecule is functional as tyrosine phosphorylation can be observed upon binding of duCD8-1 to bursal cells (Schultz U., unpublished). It is

further intriguing to know whether this phenomenon is restricted to ducks and maybe other anseriforms or can be found outside this order. However, it should be noted that the duck-specific MAb for CD4 and CD8 failed to stain lymphocytes from Muscovy ducks suggesting that the epitopes recognized are not conserved among these two species.

Using a cross-reactive anti-chicken CD25 MAb [150] and anti-duck CD4 MAb DuCD4-2 (Serotec), CD4⁺CD25⁺ cells could be detected in duck thymus, potentially the duck counterpart of mammalian T regulatory cells (Tregs) [152]. CD4⁺CD25⁺ cells had higher transcript levels of IL-10, TGF- β , CTLA-4, and LAG-3 than thymic CD4⁺CD25⁻ cells. However, a FoxP3 ortholog, a marker for Tregs in mammals, has yet to be identified in ducks and chickens.

The CD3/TCR complex

As in mammals, the chicken CD3 complex (γ , δ , ϵ , and ζ chains) is found on the surface of T cells in association with the TCR complex (either $\alpha\beta$ or $\gamma\delta$) and together are required for antigen recognition [250]. MAb reacting with CD3 permits the identification of all peripheral T cells, regardless of their TCR and are therefore used to distinguish T cells from B cells. In addition, some chicken lymphoid cells express cytoplasmic CD3 molecules but not TCR molecules. These cells, which have been identified in the gut and embryonic spleen, are considered to be avian NK cells [245]. The cDNA for Pekin duck CD3 ϵ was deposited in NCBI by Higgins and colleagues (Table 19.3). Subsequently, cDNA clones comprising the entire open reading frame of Pekin and Muscovy duck CD3 ϵ were obtained (Table 19.3) [182]. The precursor proteins of Pekin and Muscovy duck CD3 ϵ reveal 86.5% amino acid identity that decreased to 61.7% and 39.3%, respectively, when compared to chicken and human CD3 ϵ polypeptides. CD3 ϵ gene expression was observed in PBMC, spleen, and thymus, whereas transcripts were not found in the bursa of Fabricius and liver. Duck CD3 ϵ can be detected by intracellular staining using MAb CD3-12 that recognizes the highly conserved epitope PPVNPDPYEP of CD3 ϵ , which is also present in the duck molecule. Double staining with MAb CD3-12 and MAb 2-4 revealed that the majority of CD3⁺ cells are 2-4 positive confirming that the chicken CD28 specific antibody is also a valuable pan-T cell marker in ducks: [182] (Fig. 19.1). T-cell subdivision is not well studied in other birds than chicken because none of the chicken-specific TCR mAbs cross-reacted with the respective molecules on duck cells (Table 19.3).

Early efforts were made by Higgins and colleagues to clone the genes for TCR α , β , γ , and δ (Table 19.3). Using sequence data from these cDNA, Zhao and colleagues

isolated bacterial artificial chromosomes (BACs) corresponding to these genes. They determined the germline organization of duck TCR genes on BAC clones [251]. The organization of TCR α , γ , and TCR δ and TCR δ 2 is conserved, but the TCR β locus has a unique organization of two tandem genes of D β -(J β)₂-C β 1-(J β)₄-C β 2. They identified 33 V region segments in their BAC clones, and 46 more within the expressed repertoire by 5' RACE, and most can be aligned with their respective genes. Most duck V region subgroups have orthologues in chicken. All duck J β segments were found in their expressed repertoire.

The CD28 antigen and CTLA-4 and their ligands CD80 and CD86

The chicken CD28 is a glycoprotein which, in contrast to mammalian CD28, does not form disulfide linked homodimers due to the lack of cysteine residues [221]. Its presence has been demonstrated on all peripheral T cells carrying the TCR $\alpha\beta$ and on subpopulations of TCR $\gamma\delta$ -bearing T cells. MAb2-4, which was shown to react with the chicken CD28 molecule, was found to cross-react with subsets of duck PBMC. Two-color staining of duck splenocytes revealed that virtually all CD4⁺ cells are MAb2-4⁺, in addition to the vast majority of CD8^{high} cells, while the CD8^{low} population was found to be MAb2-4⁻. It is worth noting that when using MAb2-4 in combination with recombinant fluorescent-labeled chBAFF, mutually exclusive subsets of cells are stained. In conclusion, when used in combination with a CD8-specific MAb, the MAb 2-4 is able to discriminate between CD8^{high} CTL and CD8^{low} B cells (Fig. 19.1) [182].

The CTLA-4 (CTL-associated antigen-4; syn. CD152) is a member of the immunoglobulin superfamily. It is expressed on the surface of T helper cells and transmits an inhibitory signal to T cells [252,253], whereas CD28 transmits a stimulatory signal. Both molecules CTLA4 and CD28 bind to CD80 (syn. B7-1) and CD86 (B7-2) on APCs. T-cell activation through the T-cell receptor and the costimulatory receptor CD28 leads to increased expression of CTLA-4. The duck CTLA-4 (duCTLA-4) cDNA and a transcript lacking the predicted transmembrane encoding region were isolated from splenocytes using RT-PCR [232]. The predicted duCTLA-4 protein was 92%, 49%, and 47% identical to chicken (Young J. R., Zoorob R., Mwangi W.N., Wright K.E., Staines K.A., and Singh A.K., unpublished), human, and mouse homologs, respectively, and revealed conservation of residues implicated in ligand binding and intracellular signaling. DuCTLA-4 was constitutively expressed on freshly isolated duck PBMCs and showed a modest increase upon mitogen stimulation. The ligands for CD28 and CTLA-4,

CD80 and CD86, have not been identified in other domesticated agricultural birds besides chicken (Acc. no. AM050146 and AM050135; Mwangi W.N., Wright K.E., Staines K.A., Tregaskes C.A., and Young J.R., unpublished).

19.6.4 C-type lectin immune receptors

DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN/CD209) belongs to the family of type II membrane-associated, C-type lectins that function as cell adhesion molecules and pathogen receptors. CD209 plays an important role in immune responses and immune escape. Phylogenetic analysis of a total of 25 CD209 family members from 11 species including chicken (Acc. no. BU125096) [254] revealed a 28.7% identity with human and a 34.0% identity with mouse CD209 [255]. To our knowledge, no further CD209 genes have been characterized for other agricultural birds, although predicted sequences are available. The availability of CD209 sequences may prove useful for the generation of an antibody that will eventually allow the identification of DCs in chickens and other birds.

Two other C-type lectin immune receptor genes encoding DC inhibitory and activating receptors (DCIR and DCAR) were identified in a duck spleen EST library [120] and subsequently cloned from a cDNA library and genomic library [233]. Both transcripts are preferentially expressed in the spleen, bursa of Fabricius, intestine, and lung. DCIR, with an immunoreceptor tyrosine-based inhibitory motif, shares 45% or 41% amino acid identity within the carbohydrate recognition domain with human and mouse DCIR. The amino acid sequence of the duDCAR transcript shares 39% homology to human BDCA-2 and 35% similarity to mouse DCAR. In mouse, DCIR2 is an endocytic receptor expressed on CD8⁺ splenic DCs, and targeting antigens with anti-DCIR2 antibody results in internalization and stimulation of MHC class II-restricted T-cell responses [256].

19.6.5 Surface immunoglobulin

Surface membrane-bound Ig is a characteristic feature for B cells in most species. Polyclonal antibodies raised to duck Ig, however, seemed to bind not only B cells but also react with a large proportion of duck lymphocytes from the blood, spleen, thymus, and bursa of Fabricius as well as erythrocytes impeding the identification of B cells [257]. However, more recently an MAb to the duck Ig light-chain (14A3) reacts with the IgM and IgY of both Pekin and Muscovy ducks, and also with goose, though not with chicken IgY. In addition, the western blot analysis revealed that 14A3 reacted with IgA purified from Pekin duck bile (Fig. 19.1) [182]. MAb 14A3 stained

>90% of bursal cells but only minor populations in spleen and blood cell preparations. Two-color immunofluorescence analysis with MAb and fluorescent-labeled chBAFF revealed a distinct double-positive cell population in the spleen and PBMC with only few single-positive cells. The lack of double-positive cells in the thymus reflects the virtual absence of B cells in this organ. Taken together, these studies confirm that MAb 14A3 is a valuable tool to identify B cells in ducks and geese (Fig. 19.1).

MAb to purified duck bile Ig, recognizing two different epitopes of IgA heavy chain, DuIgA1 and DuIgA2 were made by Sonja Härtle and colleagues and are commercially available through Serotec (MCA5689 and MCA5690). The MCA5689 works in ELISAs and western blotting and immunofluorescence. The MCA5690 antibody works in ELISA and western blots. It is not known whether they bind surface Ig, as neither MAb have yet been tested for flow cytometry. MAb 16C7 directed at duck IgY heavy chain, allowing analysis of duck Ig from sera, was recently made commercially available (MCA2481 Serotec). It is suitable for flow cytometry, ELISA, and western blotting.

19.6.6 Major histocompatibility complex

Comparison of vertebrate MHC genomic regions shows that it is the most dynamic part of the genome [258]. Polygeny and polymorphism contribute to the breadth of the immune response. The number of functional and defunct MHC loci can differ greatly in each species, and no orthologous genes can be identified between orders. The MHC of the chicken has been referred to as the “minimal MHC” [259]. There are two clusters of MHC genes, the B locus and the Rfp-Y locus, both located on the same microchromosome [260]. The B locus contributes to all the hallmarks of the MHC. It contains 19 genes within 92 kb and therefore is very compact (discussed in Chapter 7). Within the B locus, there are two class I genes that flank either side of the transporters for antigen processing (TAP) genes. Accepted nomenclature refers to these as the major (BF2) and minor (BF1) MHC class I loci [261]. Kaufman argues that in this organization, the MHC class I and transporter proteins have an opportunity to coevolve to function together, and evolutionary forces select for inactivation of the redundant loci [259]. Using a functional assay for peptide translocation, they showed that the polymorphic chicken TAP genes confer specificity, which was determined by the dominant allele in each haplotype [262]. The limitation to one major MHC class I locus will have significant implications for the immune responses of the chicken. This is clearly demonstrated in the response of chickens to the Rous sarcoma virus (RSV), with the MHC genotype conferring either

protection or none [263]. The crystal structure of one BF allele (BF2*0401) from the B4 haplotype associated with susceptibility to Marek's disease demonstrates how this binding cleft excludes most peptides. Nonetheless, even fewer are bound than possible, implicating also TAP restriction of available peptides [262]. TAP genes of chicken, turkey, pheasant, and guinea fowl are polymorphic [264].

The MHC of other birds appears to have undergone extensive duplications and the minimal MHC of the domestic chicken is not the norm for all birds. The red jungle fowl has 14 copies of MHC class I like genes, 3 of which are pseudogenes, suggesting the minimal MHC of the chicken is a derived characteristic [265]. The MHC of the quail has been completely sequenced and has the same overall organization as that of chicken [266]. However, it has undergone duplication in several regions. There are 7 MHC class I, 10 MHC class II β , 4 NK receptors, 6 lectin, and 8 B-G genes. MHC class I genes flank the TAP loci; however, there are four MHC class I genes and three pseudogenes. Of these, one gene seems most likely to be the orthologue of the chicken BF2 gene, and two loci are weakly expressed. The turkey MHC was characterized in detail. It has a similarly constricted locus with 34 genes within a 0.2 Mb region, in perfect syntenic accordance with the chicken genes [267]. The turkey MHC is divided into MHC-B and MHC-Y as in the chicken and expressed MHC-Y genes have been identified [268]. The size and similarity of the turkey MHC to that of the chicken suggests that MHC-linked disease association will also be found in the turkey.

The duck MHC class I region encodes five class I genes that lie adjacent to the TAP2 gene [235]. Using northern blotting and allele-specific oligonucleotide probes, we showed that the duck predominantly expresses the gene adjacent to the polymorphic TAP2 gene Anpl UAA [236]. Consistent with dominant expression of one gene, we amplified 61 sequences by reverse transcription PCR from 38 wild mallards [269]. Fleming amplified TAP2-UAA fragments from these mallards, which enabled assignment of most expressed alleles to the UAA gene. A second locus, UDA is expressed at 10-fold lower level, due to *let-7* microRNA downregulation involving the 3' UTR [270]. The predominant expression of a single MHC class I locus is expected to have significant functional consequences, influencing the selection of the T-cell receptor repertoire, as a consequence of the peptide repertoire that can be presented. Nonetheless, high allelic diversity exists within the population.

19.7 Secreted antibodies

Birds have four classes of immunoglobulins, IgM, IgD, IgA, and IgY [271] (see Chapter 5). IgM and IgY are

present in serum, while IgA is expressed in a variety of secretions. The IgY is an avian version of IgG, which is sufficiently different from IgG to warrant that name [272]. In addition, ducks and geese have a smaller version of IgY, called IgY Δ Fc, a secreted antibody composed of two C region domains. Ducks have a single light chain of the lambda type [273]. The majority of birds (including ducks, turkey, pigeon, and quail) such as chickens generate their antibody repertoire through limited functional rearrangement of the variable region and generate diversity through gene conversion from a pool of pseudogenes in the bursa of Fabricius [274]. Using southern blots, Lundqvist and colleagues showed that heavy and light chains of mallard ducks undergo limited genomic rearrangements [275]. Despite this mechanism of generating diversity, examination of heavy and light chain immunoglobulin sequences from White Pekin duck indicates that extensive diversity is achieved [275].

Ig heavy chain genes have been characterized in ducks, and recently in ostrich, and surprisingly not yet done in chickens. In ducks, the Ig heavy chain locus has been sequenced on overlapping clones from a D gene segment through the epsilon gene [276,277]. There are three clusters of heavy chain gene exons, but their organization is unusual. The mu (μ) gene is followed by an inverted alpha (α) gene, and epsilon (ϵ) lies furthest downstream in the locus. This organization will make splicing for Ig class switching cumbersome. Switching to ϵ involves normal excision of the switch circle, but switching to α must involve the inversion of this fragment in the locus [276]. In ostrich, there are two μ genes, two ϵ genes, an inverted α gene, and remarkably a delta δ gene thought to be missing in birds [278]. Comparison with other avian genomes suggests the presence of a δ gene in the last common avian ancestor.

In ducks, the organization of the locus has implications for expression and function of antibodies. Switching to α is delayed in ontogeny, and transcripts are barely detectable until ducks are about 2 weeks old. Ducklings are likely to encounter influenza A virus before IgA can play a role in the defense. The ϵ gene encodes three different forms of IgY heavy chain through alternate splicing [279]. Typically, the ratio of full length and truncated IgY in sera varies between individuals, but the truncated IgY predominates later in an immune response. After repeated injections of antigen, IgY Δ Fc made up 85% of the specific antibody to BSA [280] or *E. coli* [111]. Since this antibody lacks the Fc portion, it cannot participate in secondary effector functions, such as complement fixation, opsonization, and Fc-mediated macrophage clearance of viruses. Presumably, it functions primarily in neutralization. In 2011 Magor reviewed duck immunoglobulin genetics in the context of antibody responses to influenza and vaccines in ducks [281]. Higgins and colleagues

examined contributions of different isotypes to protection of ducks against influenza. IgM, isolated from sera early in the response (3–5 days postinfection), could inhibit hemagglutination and neutralize virus, and later in the response (16–29 days) this function is attributed to full-length IgY. The contribution of the IgY Δ Fc is less clear, as it was shown to not inhibit hemagglutination [282]. It is presumed to function in virus neutralization, but this was not tested. Isolated bile Ig, corresponding to IgA, had hemagglutination inhibiting activity.

Sequencing of a BAC clone encompassing the entire duck λ gene showed that there is a single J λ and C λ , and 9 V λ regions that appeared intact for recombination, and 79 pseudogenes for further diversification through gene conversion [283]. Sequencing of λ chain locus in the pigeon [284] reveals a single functional V region, like the chicken.

The cross-reactivity of many MAb against chicken antibodies was tested in ducks, turkeys, and quails; none reacted with duck Ig, two were found to react with quail antibodies, and several were found to react with antibodies of turkeys [200]. Serum antibodies of turkeys showed cross-reactivity with commercially available MAb; the chicken-specific MAb AV-G3 detects turkey IgG, while the MAb M1 reacts with IgM [208]. Serum antibodies of the ostrich were not cross-reactive with antibodies against Ig of many species including chicken [285]. Biochemical analyses of Ig of quail and pheasants and antigenic relationships with chicken Ig were determined [286–289]. MAb 3C10 and CH31 that detect chicken λ chain were tested against sera from 34 avian species [290]. In western blots, both mAbs reacted with turkey and pheasants, but not partridge. These MAbs also failed to detect light chains from six orders of birds in immunoassays, including ducks and geese. L chain sequences from pheasants, turkeys, and partridge were deposited in Genbank (accession no. FJ649651, 652, 653).

19.8 Cell lines

Numerous lines of chicken B and T cells and various lines of nonlymphoid cells have been established using Marek's disease virus, RSV, avian leukosis viruses (ALV), and reticuloendotheliosis virus (REV). Depending on the age of the bird, the origin of cells, such as, bone marrow cells versus splenocytes, the activation status, and the strain of virus employed, infection resulted in transformed populations containing predominantly T cells, B cells, or monocytes/ macrophages [291,292]. REV which is the most versatile transforming virus is not only pathogenic in chicken, turkey, and quail but also in ducks [293] and geese [294].

Higgins and coworkers reported the establishment of lymphoblastoid cell lines from organs of ducks infected

with REV-T, a replication-defective strain [237]. In uncloned lines, the presence of T and B cells was suggested by the expression of the β -chain of the TCR and the expression of Ig polypeptides. However, the cloned lines that were obtained appeared to be of the $\alpha\beta$ T cell lineage. The failure to generate B cells lines was explained by stringent growth requirements for B cells, for example, cytokines derived from T cells [237].

Kaiser and coworkers succeeded in establishing a turkey macrophage cell line (LSTC-IAH30) after challenge of adherent PBMC with acutely transforming ALV [137]. By flow cytometric analysis, it was shown that LSTC-IAH30 cells are positive for markers recognized by M, K1 (detects a common antigen on chicken thrombocytes and macrophages), F21-21 (chicken β 2 microglobulins), and possibly M1 (detects chicken IgM) but not KUL01 (chicken macrophage marker) (Table 19.2) [295]. Furthermore, production of NO has been demonstrated for LSTC-IAH30 cells and they have, therefore, been used to measure turkey IFN- γ activity as a function of nitrite accumulation [137].

Some chicken cell lines have proven useful for the analysis of avian immune responses. HD-11 cells, a well-known chicken macrophage-like cell line, proved to be suitable to measure duck IFN- γ activity; however, duck IFN- γ had a specific activity that was about 16-fold lower than chicken IFN- γ that served as control [108]. Magor and colleagues have also used chicken DF-1 cells, a spontaneously immortalized embryonic fibroblast cell line [296], to analyze innate signaling [61], using the IFN- β gene reporter based on the chicken Mx gene promoter [56,91]. A novel chicken lung epithelial cell line CLEC213 made from lung explants of white leghorn chickens is also a valuable tool for the analysis of respiratory tract defense against viruses [297].

CEC-32 cells were reported to have been established from chicken embryo fibroblasts [298]. Due to their virus susceptibility and sensitivity to IFN, they have been successfully used to study the functions of the chicken and duck IFN systems. However, comparison of the karyotype of CEC-32 cells with chicken and quail karyotypes revealed that the CEC-32 cell line might not have originated from chicken but have come from quail [299]. Seven quail myogenic cell lines were generated from the quail fibrosarcoma cell line QT6 [300], and one of these, QM7 ATCC CRL-1962, has been used recently for targeted genome editing using CRISPR/Cas9 [301].

The duck cell line (ATCC CCL-141) is an embryo fibroblast cell line deposited by M. Marcovici, J. Prier, and M. Allen. Careful subculturing protocols are listed for this cell line, or it dies out. A duck embryo fibroblast line is available from the cell bank at the Friedrich-Loeffler-Institute (Insel Riems, Germany). It has been used to

analyze the growth kinetics of influenza strains [302]. Unfortunately, this is a finite nontransformed line.

EB66 a duck embryonic stem cell line has been generated and explored as an alternative production system to chicken eggs for vaccines and industrial protein production, including antibodies [303,304]. These cells, obtained from embryonic tissues of ducks, retain the biological characteristics of stem cells, have the ability to renew indefinitely, and show long-term genetic stability. Furthermore, they are not virally transformed. These cells achieve high densities (30 million/mL) and have short culture kinetics (doubling time of 15 hours).

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Evolutionary and ecological immunology

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20.1 Introduction

One of the central tenets of evolutionary biology is that favorable heritable traits increase in abundance as a result of natural selection. Infectious diseases interfere with survival and reproductive success and, as a consequence, evolutionary pressure will favor individuals that are effective at combatting attacks from parasites and pathogens. Although all animals exhibit such defensive systems, these vary greatly across individuals, populations, and species. Ecological and evolutionary immunology (also known as ecoimmunology or immunoecology) address why this variation arises and how it is maintained. Two main lines of the research can be recognized which principally follow the Gene-for-gene and Matching-alleles hypotheses of host–parasite interactions [1]. An ecoimmunological approach consistent with the Gene-for-gene model hypothesizes that immunity is costly (e.g., energetically expensive) and thus subject to resource-allocation trade-offs. Under certain conditions animals may maximize fitness if they invest less in immunity and more in reproduction [2]. Conversely, if selective pressure from pathogens is high, they may need to increase investment in immunity at the expense of reproduction. In contrast, the Matching-alleles model does not necessarily involve differential costs, but predicts specific, mutually exclusive immunological adaptations in defense to different pathogen lineages. In this scenario, the trade-off emerges from the impossibility to adequately adapt to all diverse pathogen pressures at the same time. Both mechanisms likely contribute to the variation in immunological strategies observable in free-living birds.

Examining immunology from an ecological and evolutionary perspective places immune activity within the framework of how organisms interact with their environment. For example, the ecology and evolution of immune function and disease are influenced by population-level phenomena and social environments. In addition, in many

clinical and laboratory studies variation among individuals is considered statistical noise. However, an ecoimmunological approach is interested in explicitly determining the causes and consequences of individual variation. This is because differential survival and reproduction among individuals are key mechanisms of evolutionary change and variation is assumed to represent, in part, the outcome of natural selection as well as the variation upon which selection can act in a population.

Generally, ecoimmunology addresses biological questions related to life history theory, a subfield of evolutionary biology that examines the strategies that have evolved over time to govern investment in reproduction, growth, and longevity [3]. Because maintenance of the immune system evolved in the context of the resource and adaptational trade-offs and constraints, optimization of immune defense strategies is needed [4]. For example, maintaining a functional immune system requires energy and scarce nutrients [5]. Therefore resources invested in immune system self-maintenance are not available for investment in reproduction. In addition, responding inappropriately (i.e., too strongly or too weakly) to perceived parasitic threats can have a strong influence on reproduction [6,7]. Adaptive regulation of this balance can be predicted to involve both genetic and ecological components.

In contrast to domesticated birds such as chickens, and to a lesser degree turkeys and ducks, there is a paucity of information on immune responses in any given free-living bird species. Usually, studies on immune responses are performed using birds with unknown histories as long-term studies are challenging. As a consequence, information on immune responses in a given species is often incomplete. Moreover, major differences, such as whether a species is altricial (developmentally immature at hatch, lacking feathers and eyesight and remaining in the nest for a period of time) versus precocial (developmentally mature at hatch with feathers and eyesight and the ability

to leave the nest shortly after hatching) can have profound impacts on immunological needs.

The studies that apply immunology to test life history theory in free-living bird species tend to be fragmented, with no clear model species. For this reason, interpretations of many studies should be still considered preliminary as fundamental assumptions in the field are tested and reevaluated. This is true also for the evolving terminology in ecoimmunology, where for example the originally pivotal term “immunocompetence,” shifted its meaning [8]. In this chapter, we discuss the challenges of investigating immune defenses in ecological and evolutionary contexts, evaluate differences and similarities in immune responses among free-living bird species, and make comparisons with domestic species where appropriate. We also discuss how immunological responsiveness may reflect various ecological factors, differential selection pressures and evolutionary outcomes.

20.2 Assessing immune function in free-living birds

Selecting the proper techniques can be difficult when studying immune responses in free-living bird species. Many of the tools available for model organisms and domesticated species are not practical to use in the wild because of issues like the species-specificity of molecular tools, requirements for rapid or sterile processing, and high costs. Thus when compared to immunological studies in poultry, the list of methods available for field evolutionary and ecological immunology is relatively limited [9,10]. As prior reviews have provided extensive discussions of such techniques [9,10], an exhaustive list of such techniques is beyond the scope of this chapter. Rather, we provide an overview of techniques used in free-living birds with a special focus on those with the highest promise in the near future.

For many precocial and altricial species, single-point measures remain the most practical approach. However, if birds can be followed and recaptured over time, multiple-time-point assays can provide additional, more-tightly controlled information. Inducing immune responses in free-living birds typically utilizes noninfectious agents, which can include antigens unrelated to pathogens [e.g., sheep red blood cells (SRBC), keyhole limpet hemocyanin (KLH)], inactivated pathogen-derived antigens [e.g., inactivated Newcastle disease virus (NDV), lipopolysaccharides (LPS), or other Toll-like receptor agonists], and lectins [e.g., phytohemagglutinin (PHA), concanavalin A (ConA)]. Further, when multiple captures are feasible, medication experiments can lend insight into immune responses to natural levels of parasitism.

20.2.1 Single-time-point assays

Single-time-point assessments only provide a snapshot of immune defenses at the time of assessment, but can still lend insight into differences in immune investments across species, sexes, seasons, etc. Among the most common techniques are counts of leukocytes or ratios of heterophils to lymphocytes (H/L ratio) [11]. For example, a study of 19 species of shorebirds revealed that longer-lived species and those with higher annual survival showed lower H/L ratios, consistent with an interaction between life history strategy and immune strategy at the macroevolutionary level [12]. Although intraspecific repeatability was relatively low in that study, others have found that H/L ratios appear highly heritable [13], consistent with their potential to evolve in concert with life history traits. Further, other studies have found differences in H/L ratios with other factors like sociality and breeding latitude [14,15]. Because H/L ratios change rapidly with stress and infection status, it may be important to control for such factors when interpreting this metric [14]. Similarly, Vinkler et al. [16] found a positive association between levels of blood peripheral basophils and *Haemoproteus* infection in common rosefinches (*Carpodacus erythrinus*: Passeriformes), confirming that such cell counts not only reflect standing defenses, but also current levels of parasitism.

The relative weight or volume of lymphoid organs, such as the spleen or (in juvenile birds) the bursa of Fabricius and thymus, are often used if birds can be euthanized at the time of capture [17,18]. These measures are also sensitive to individual variation, overall health status or past activity, and enlarged spleens may reflect pathological processes and/or robust standing defenses. A study by Ardia [18] reflects some of these problems. Nestling European starlings (*Sturnus vulgaris*: Passeriformes) were inoculated with saline or PHA by subcutaneous injection in the wing web. Spleen sizes were highly variable among individuals after inoculation with saline, indicating that the use of spleen size in comparisons within and across species is likely to be confounded by high variability. PHA stimulation did not cause spleen enlargement relative to saline-injected controls [18]. Assessing the bursa of Fabricius or thymus is relatively uncommon in field studies because of their small sizes. Moreover, in most species the bursa of Fabricius and the thymus recrudescence during sexual maturity [19].

A commonly used single-time-point measure is the examination of levels of constitutive innate or natural antibodies (NAbs). These antibodies, which are mostly IgM but also include IgY and IgA, are likely the result of responses to cross-reactive epitopes present in the natural environment of the host (e.g., intestinal tract) and do not

require experimental antigen exposure. NAb react to a diversity of epitopes, making them useful to assess levels of constitutive defenses among individuals [20]. This technique is highly repeatable and is effective in a wide range of avian species [20].

The levels of NAb may reflect differences in genetic potential for acquired immune responses based on studies in chickens. Levels of NAb to rabbit red blood cells increased in chickens selected for acquired responses to SRBC, but only after 14 generations [21] and higher NAb titers to other antigens were associated with selection for increased acquired responses to SRBC [22]. However, body condition needs to be carefully monitored in free-living birds because this may influence NAb titers. In nestling Leach's storm-petrels (*Oceanodroma leucorhoa*: Procellariiformes), NAb titers increased during nestling development but were inversely proportional to wing growth, suggesting an energetic trade-off in developing nestlings [23].

Natural antibodies often react with SRBC or other red blood cells and can confound results when birds are experimentally immunized with SRBC. Testing of sera for antibodies at the time of first immunization is essential, but is not always included in experimental designs, and will reduce or eliminate the potential confounding factor of NAb. Another excellent single-time-point assay uses ELISA to determine general serum immunoglobulin levels, as well as specific antibodies, in free-living birds [24], but species-specific antisera may need to be generated for these assays [25].

Another informative single-time-point metric is the microbicidal capacity of whole blood or plasma. In addition to its relatively straightforward execution, this assay can be applied to a wide range of species, assesses the ability to neutralize a potential pathogen (rather than an inert antigen), and integrates several different types of immunological defenses at once (natural antibodies, complement, other antimicrobial peptides, phagocytosis, etc.). A known amount of live microbes, most commonly *Escherichia coli*, *Staphylococcus aureus*, or *Candida albicans*, is incubated for 15–60 minutes with a fresh or frozen plasma sample or with a fresh whole-blood sample [26–28]. This mixture is then streaked onto agar plates, incubated 24–48 hours, and the number of microbial colonies is compared against control cultures containing no blood or plasma samples [27,28]. Alternatively, mixtures can be incubated with liquid growth media for 12–24 hours, and the amount of microbial growth is determined via spectroscopy [26]. While spectroscopy data were more consistent than colony-counting, spectroscopy still showed significant variability and had reduced resolution when samples showed low levels of bacterial killing [26]. Performing this technique on whole-blood samples requires rapid access to laboratory equipment,

but plasma samples can be frozen and assayed at a later date, although longer storage times can reduce a sample's microbicidal capacity [26]. Additionally, while most studies use common, commercially available microbes, different microbes could be used to incorporate true pathogens.

Several assays of acute phase proteins have also proven valuable across a range of wild species. Some are based on commercially available kits (e.g., haptoglobin-like activity) [29], while others require compiling several separate reagents (e.g., lysozyme) [27], but most rely on some form of a spectrophotometric readout (e.g., ovotransferrin) [30]. These assays seem reasonably repeatable within individuals, a critical consideration when used with single-time-point samples [30,31]. Moreover, Matson and colleagues [31] found that baseline levels of haptoglobin-like activity predicted levels after challenge with LPS. This suggests that such baseline levels of acute phase proteins may provide insight into overall inflammatory phenotypes.

Research into oxidative burst also shows that single-time-point samples can lend insight into inflammatory processes among wild birds. For example, Sild and Hõrak [32] used a Phorasin-based chemiluminescence kit to measure the production of extracellular reactive oxygen species in whole-blood treated with LPS. Their results showed reasonable repeatability within individual greenfinches (*Carduelis chloris*: Passeriformes) and functioned well with small blood samples (20 µL). Samples stored at 5°C for 32 hours showed slightly reduced measurements, but such patterns could be accounted for statistically. Papp and Smits [33] tested a similar assay in American kestrels (*Falco sparverius*: Falconiformes) and five other wild species, finding similar patterns and high repeatability after three days of sample storage at 4°C. Hahn et al. [34] performed similar assays of oxidative burst, but also measured degranulation responses in brown-headed cowbirds (*Molothrus ater*: Passeriformes) and red-winged blackbirds (*Agelaius phoeniceus*: Passeriformes), though for these assays samples from multiple birds were pooled for testing. Taken together, these results highlight a promising path forward for single-time-point sampling: collection and stimulation of leukocytes (or whole-blood) in vitro to study immune responsiveness without the need for multiple captures.

Recently mRNA expression has been used to compare immune strategies across evolutionary or ecological scales [35,36]. For example, Martin and colleagues [37] found that *TLR4* expression in blood was lower among house sparrows (*Passer domesticus*: Passeriformes) at the edge of their regional range than at the core of this range. While such techniques hold great promise, some (e.g., quantitative (q)RT-PCR) require species-specific primers or confirmation that cross-species primers are functional. Moreover, because relatively few genes associated with

immune responses have been characterized in free-living birds, researchers cannot draw strong conclusions about protein levels or protein function based on mRNA expression alone.

Finally, when using these and other single-time-point metrics, researchers should be cognizant of sample collection protocols. Factors such as duration of animal manipulation, time of day, and storage times can affect assay outcomes [26,38,39]. However, such impacts can vary across species and assay types, with little to no effect in some [27,29,39–41]. Given this variability, authors should consider normalizing collection and handling times and testing for differences based on these factors statistically.

20.2.2 Multiple-time-point assays

Since levels of various immunological traits measured in ecological research may depend on disease status at the time of sampling [42] and environmental variation, multiple-time-point assays are used to differentiate between preactivation effects and the actual immune responsiveness to stimulation (e.g., by a novel antigen or mitogen). These assays allow the use of standardized experimental designs eliciting specific immunological pathways (typically type 1 or type 2-oriented) considered relevant in responses to certain types of pathogen. Understanding differences between different immunological mechanisms allows determining ecologically-meaningful costs of the responses, their evolutionary significance, and constraints [8].

Assays measuring humoral adaptive antibody responses (type 2) were among the first multiple-time-point methods adopted in ecological immunology, which yielded many important and interesting results [43]. Typically, antibody production is evaluated using enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition, and agglutination tests. Novel antigens, such as mammalian erythrocytes (often SRBC) [44] or inactivated vaccines (e.g., diphtheria-tetanus toxoid or inactivated NDV) [45] serve to trigger the immune responses. The response index is obtained by comparison of primary or secondary responses to pretreatment antibody titers. Being highly conserved across vertebrates, the molecular background of this immunological mechanism is well-understood in birds (see Chapter 4). Certain methodological considerations, however, should be accounted for when interpreting research results. Most importantly, not all antigens are equally immunogenic and based on their molecular features some may stimulate cellular rather than humoral immune responses. For example, Råberg and colleagues [46] found different patterns of natural selection on antibody response to diphtheria versus tetanus in blue tits (*Parus caeruleus*; Passeriformes;

see Fig. 20.1), a result also found in red-winged blackbirds [47]. In an ideal case, antigens with immunogenic features resembling species-specific, naturally occurring pathogen-derived antigens should be used. A validation study comparing correlations among a variety of common antigens used in ecological research would be beneficial to reveal the patterns of resemblance. Finally, it also has to be stressed that different antibodies used in ELISA (e.g., antipasserine IgYs vs antichickens IgYs) may provide different results due to differences in their specificity and affinity [48].

Unlike antibody-based mechanisms (type 2), the immunological mechanisms of cell-mediated immunity (CMI; typically considered as type 1) that are commonly measured in free-living birds remain controversial. Almost universally, CMI is assessed by inducing a hypersensitivity response, usually via subcutaneous injection of PHA. Although formerly interpreted as a measurement of T-cell responsiveness, later research has shown a complex histological composition of the cellular infiltrate into the tissue [49] as well as a nonspecific nature of the mechanism triggering and regulating in vivo responses [50,51]. Since the time interval used in ecological studies is typically too short (6–24 hours) for PHA to trigger any T-cell proliferation, the primary testing in skin may be adopted to measure general innate-immune inflammatory processes. This is of particular interest because inflammation acts as a “double-edged sword” in demanding energetic and redox trade-offs [52].

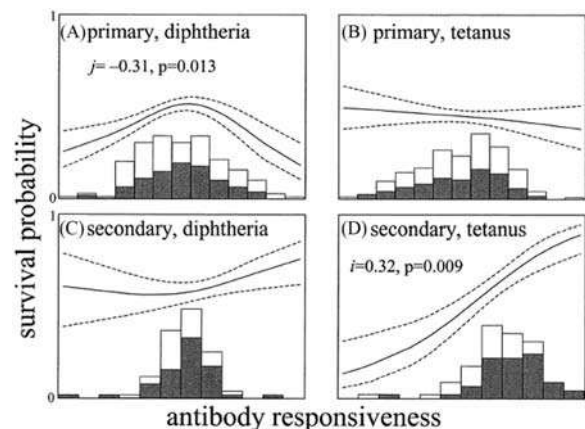


FIGURE 20.1 Survival probability of wintering blue tits (*Parus caeruleus*) as a function of antibody responsiveness for primary and secondary responses to diphtheria-tetanus toxoid. The solid lines are fitness functions as estimated by the cubic spline technique; the dashed lines are standard errors. j is the quadratic selection differential; i , the linear selection differential. P -values were determined by logistic regression. The histograms represent the frequency distribution before (open) and after selection (shaded) [46]. Reprinted from Råberg L., Stjernman M. 2003. Natural selection on immune responsiveness in blue tits *Parus caeruleus*. *Evolution* 57:1670–1678, with permission.

The PHA technique is simple and thus commonly used in field studies [53]. Empirical data suggest little to no negative effect of the experimental manipulation on the individual survival rate [54]. However, because the actual measurements are subjective, the test is prone to interobserver variation, which greatly limits the comparison of PHA responses among studies. This issue may be resolved by standardized quantification of either cellular infiltration [42,49] or RT-qPCR-based expression changes in selected pro-inflammatory cytokines, such as interleukin 1- β (*IL1B*) or *IL6* [51]. Being size-dependent, body size needs to be included as a covariate in the analysis. Finally, since intensity of the skin inflammatory response is positively related to both lymphocyte (typically higher in blood of healthy individuals) and basophil (higher in blood of diseased individuals) counts [42], pretreatment hematological screening is recommended to disentangle the relationship between intensity of the response and health.

The most important set of criticisms against the use of mitogens and other isolated novel antigens is that disease resistance is more complex than a response to a single purified stimulant. Despite this, evidence exists linking strength of the immune response to PHA to survival and longevity in birds [55]. Comparative studies of responses against purified antigens versus pathogenic challenges are still critically needed to validate the PHA skin-swelling test [56]. Recently, the intensity of the inflammatory response to PHA was linked in barn swallows (*Hirundo rustica*: Passeriformes) to specific differences in gut microbiota composition (mainly abundances of genera *Lactococcus*, *Enterococcus* and *Rickettsia* vs genera *Bacillus*, *Staphylococcus*, *Dysgonomonas* and *Streptococcus* [57]). Although not predictive, this evidence confirms biological relevance of the immune response. Similar to PHA, other stimulants such as bacterial LPS or KLH can be used in skin-swelling tests, but these are less frequently used in ecological immunology, probably because they trigger less-intensive responses in birds. On the other hand, especially LPS may be advantageous, because the immunological activation pathway is more clearly understood in this case, which allows more precise interpretation of the results. Further improvement may be achieved by combination of the skin test with haptoglobin, an acute-phase protein, response measurement [58] or tissue transcriptomics [59,60]. A similar protocol, with additional antigen injections performed several weeks apart, can be used to measure the adaptive delayed-type hypersensitivity (DTH), a memory component of CMI [61,62]. While some studies have successfully employed this protocol in captive flocks [63], the utility of this technique in field studies is limited to settings in which sufficient numbers of individuals can be reliably recaptured at the appropriate times.

Recently, interest has grown in understanding in other components of the acute-phase response (APR) in an ecological context, which allows assessing integrative, systemic responses. Apart from the production of inflammatory cytokines [51] and antimicrobial peptides (such as haptoglobin or ovotransferrin [30]), the APR is linked to metabolic changes and behavioral responses such as fever or hypothermia, and sickness behaviors (e.g., lethargy and anorexia) [64]. Because these responses can carry heavy resource costs, researchers have been particularly interested in how the APR may trade off with other life history traits [65–68]. Even though lethargy can make animals more difficult to observe in the wild, several field studies have capitalized on sickness behavior as a proxy for the strength of the APR, recording decreases in behaviors like nestling provisioning and territorial defense after immunological treatment [69–72]. In species with predictable home ranges, radio transmitters can be affixed to individuals at the time of the treatment and then used to monitor both locomotion (lethargy) and fever [71]. Additionally, in species that utilize nest boxes, multiple blood samples can be collected, and microbicidal capacity, or concentrations of specific antimicrobial peptides can be measured [72]. These plasma-based traits are typically convenient from the perspective of methodological feasibility given their lower requirements on sample manipulation and storage [41].

With multiple-time-point immune challenges, results may change based upon several methodological differences, including stimulant dosage levels [53], differences in the stimulant type selected (e.g., PHA-P vs PHA-L) [42,49], time lag between antigen administration and response measurement [73,74] or time of day [75]. Since all these factors may affect the results, it is essential to report these parameters and appropriately adjust interpretations of the results obtained. It has also been demonstrated that microbial environmental contamination may differentially affect different immune responses [76]. Therefore it appears fruitful to merge field research with studies of wild-caught birds housed in standardized conditions and experimentally treated with living pathogens [77–79]. Cytokine mRNA expression in blood [80] and bioptic samples [51] may supplement tissue necropsy sampling [81] as a means of avoiding euthanasia in the experimental animals. As tools for quantifying mRNA gene expression become more readily available for non-model species [81], we anticipate their increasingly important role in assessing immune responses in free-living birds [82]. Yet, in wild nonmodel bird species ecological and comparative immunological research is still highly limited by the insufficient availability of molecular tools necessary for more specialized cellular assays, posing strong pressure on their development and validation [83]. Since higher immune responsiveness does not

necessarily contribute to improved health by either causing immunopathology [81] or nonprotective responses [84], novel combinations of mRNA and protein-level approaches may help to clarify the precise immunological background of the relationships observed in wildlife.

20.3 Development of the immune system in free-living birds

20.3.1 Ontogeny

Two themes are central in an ecological understanding of immunological development: (1) considering the causes and consequences of immune system polarization across arms of immunity [4–7] and (2) patterns and processes involved in the development of immunity in a life history and ecological context. As with the ontogeny of any complex physiological system, patterns of immune system development should reflect the integration of disease and other selective pressures within developmental constraints. As noted, one potential paradigm that integrates competing selective pressures may be differing investment in innate versus adaptive immunity throughout development [85,86]. Although more costly energetically, innate immune responses are broadly protective and do not require long production times; thus early immune responses are predicted to be skewed toward nonspecific responses [66,87].

As evidence of differences in how arms of the immune system might develop differently, young birds can produce robust CMI responses to PHA [18,88–90] and show a bias toward higher levels of heterophils [87]. In free-living tree swallows (*Tachycineta bicolor*: Passeriformes), some aspects of innate immunity (PHA and leukocyte counts) were at levels similar to adults, while others (e.g., natural antibodies and complement) were lower [86]. However, whether levels are similar between adults and nestlings is not necessarily the most robust test of whether innate immunity develops earlier than acquired immunity. Rather, multiple assessments within individuals would provide more robust tests. For example, levels of bacteria-killing ability were present in young nestling tree swallows and steadily increased, but were still below adult levels at fledging [91]. More work is needed to investigate biases in immune system development in an ecological context. It will be important to elucidate whether differences in rates of development reflect development constraints, such as increased timing required for B-cell differentiation or different selection pressures, whereby generalized inflammatory responses are favored early in development. Experimental and comparative studies together could provide the inference to help illuminate this central question in ontogeny. In addition to such

longitudinal studies, life history variation can give insight into broader developmental evolution.

In birds, development to sexual maturity varies along a gradient from precocial to altricial growth. Altricial growth differs significantly from precocial growth in the mode and rate of development, particularly in the allometry of morphological development and the timing of thermoregulation [85]. While most bird species show altricial or semialtricial growth, our understanding of the ontogeny of the avian immune system comes mainly from domesticated birds, which are precocial. Yet, recently we have also increased our understanding of the ontogeny of immune function in altricial birds [85,92,93]. Extrapolating knowledge from domesticated birds to free-living altricial species should be done with caution because of these marked developmental differences and because of differences in body size and evolutionary history. Differences in immunological development between altricial and precocial birds may be due to differing developmental constraints as well as selection pressures associated with different levels of disease exposure. One significant difference in early immune exposure between altricial and precocial birds is the role of the nest environment. Altricial nestlings undergo significant immune development without significant movement from the nest and thus may suffer more from parasites that take advantage of their low mobility, such as hen fleas (*Ceratophyllus gallinae*) and blood-sucking fly larvae. Furthermore, they may also experience increased exposure to bacteria and other antigens during development [76]. The role of nest conditions within and across species in stimulating or constraining immune development is an area of future study.

On the other hand, clear parallels between precocial and altricial species can be observed in some immunological traits, where additional insight into immune ontogeny can come from domesticated species. For example, increases in serum IgY concentrations in the semialtricial Leach's storm petrel and the semialtricial American kestrel are similar to those reported in the precocial domesticated chicken and duck [85]. Serum IgY levels did not differ between captive and free-living kestrels, suggesting that work on some wild-caught species kept in captivity may provide insight into processes occurring in nature. A field study on American kestrels supported these insights, revealing levels of circulating endogenous IgY and specific antibodies to KLH in nestlings as young as 3 days of age [25]. Serum IgY concentrations in 30–55-day-old storm petrel chicks (60-day nestling period) averaged 2.3 mg/mL, a range also reported in poultry [85].

Similar to domesticated birds, nestling free-living birds demonstrate a limited ability to produce an effective humoral immune response. In work done with the altricial rock dove (pigeon) (*Columba livia*: Columbiformes),

B-cell differentiation occurred in a time frame similar to that reported for precocial species [85]. For example, 2–6-day-old squabs are unable to produce a humoral immune response in contrast to 7–10-day-old chicks; the switch from IgM to IgY over this development period supports the hypothesis that individuals were actually developing an antibody population rather than using parentally derived antibodies, [85] so more work is needed to investigate how B-cell differentiation occurs in nondomesticated species.

The most interesting finding on comparative ontogeny of avian immune systems is that surgical bursectomy within 24 hours of hatching in pigeons does not limit future antibody production. This contrasts with findings in poultry, where bursectomy at similar ages in young birds leads to limited humoral immune responses as adults. Retained antibody production occurs, even though pigeons show development of the bursa of Fabricius similar to that found in chickens; the bursa increases in size from hatching and reaches maximal size at 3–4 months of age [94]. This important difference suggests more rapid B-cell differentiation in the altricial pigeon and may indicate an important difference in life history strategies (and thus natural selection pressures) between species. Still, this is a single study performed with captive birds and needs to be replicated in the wild and across a range of species.

Development conditions can have very strong effects on immune system characteristics, especially under variable conditions [95]. For instance, free-living western bluebird juveniles (*Sialia mexicana*: Passeriformes) were challenged with SRBC or inactivated NDV, orally dosed with different amounts of lead shot and examined for PHA responses [96]. Lead, an important environmental immunotoxicant, can cause a shift from Th1 to Th2 responses, especially during early developmental stages, supporting the important role of early life in driving later-life phenotypes [97]. There was no effect on the immune parameters except that the PHA response was reduced in the high-lead treatment group. Interestingly, the chicks challenged with NDV or SRBC had a significantly higher survival rate while committing resources to antibody development [96].

Our understanding of the influence of early life experience in driving a range of physiological traits is expanding rapidly. Experimental work manipulating temperature has revealed the critical effects of temperature on perinatal programming of later life immunity [76,98,99]. This supports previous work showing a link between nest temperature and immunity [66]. These studies, conducted in an organismal context, support work done in poultry that found important links between temperature conditions during development and in immunity [100–102]. Taken broadly, temperature plays a critical role as a

physiological integrator influencing life history evolution [103], perinatal programming for adaptive environment matching [104], and driving suboptimal responses [105]. Understanding the switching point between adaptive versus maladaptive early life conditions in view of rapid environmental change should be a core research priority in ecological immunology.

20.3.2 Parental transmission of antibodies

Mothers may influence the immune system of their offspring in many ways beyond direct genetic contributions. In birds, hens directly transfer antibodies to their naïve offspring, usually in the egg, but in some species also to nestlings. These maternal contributions to offspring immunity are important because (1) they may provide the primary form of humoral immune defense early in development, (2) they may influence offspring growth rates by directly stimulating cell surface receptors involved in growth and/or by reducing the energetic cost of immune activity, and (3) they may stimulate antibody production [106,107], and (4) regulating the timing of antibody production in offspring [45]. In most cases, females transfer immunoglobulin as IgY in the yolk; however, in species that provide crop milk (e.g., pigeons: Columbiformes) parents transfer serum IgA to their offspring [108]. In nonpoultry species examined in captivity, the duration of the period of passive immunity varies based on the antibody titers against relevant antigens in the female at the time of egg production. Initial studies suggest that the half-life of antibodies in altricial birds may be much shorter, with a reported range of 2–3 days in house sparrows [109].

Maternal transmission of antibodies is beneficial for protection against pathogens early after hatching, but maternal antibodies also may prevent stimulation of the neonatal immune system, especially when high levels are transferred [110]. It is clear that maternal antibodies may provide significant benefits in short-term protection. For example, in Japanese quail (*Coturnix japonica*; Galliformes), offspring that received specific antibodies showed lower depression of growth following immune stimulation than control offspring [107]. This may occur not only through neonates having maternal antibodies participating in immune response but also through educating neonatal immune systems through memory [111]. However, maternal antibodies may also be suppressive by blocking neonatal antigen presentation, suggesting a complex dynamic [111].

These potential trade-offs may lead to wide variation in the extent and timing of antibody transmission. Within a species, differences among individuals in exposure, antibody production and transfer may be caused by a variety of factors, including social environment, prior disease

exposure, food resources, and parental condition [106,112]. Great tits (*Parus major*: Passeriformes) exposed to hen fleas have high levels of antiflea antibodies, which are thus passed to their offspring through eggs, increasing nestling resistance to fleas [113], while unexposed females do not. This context-dependent transmission of IgY antibodies functions well because of the high cost of antibody production [106]. As limited data are currently available, future research should examine species-level differences in maternal antibody transmission in view of differences in selection pressure from parasites. For example, ground-foraging birds are hypothesized to face greater exposure to blood parasites than do seabirds, while seabirds suffer more from helminths encountered while eating fish [85]. Thus the suite of specific antibodies transferred in each species should address likely threats. Antibodies in long-lived species may also persist for longer time periods than previously thought. A longitudinal study in Cory's shearwater (*Calonectris borealis*: Procellariiformes) reported persistence of maternal antibodies that were transferred to embryos even after five years [114].

Along with immune system development, transmission of maternal antibodies is an area of ecoimmunology in great need of future study. Specifically, more research is needed on the costs and benefits of maternal transmission, on whether species-level differences reflect adaptive responses to selection pressures, and on how exposure to maternal antibodies affects offspring immune system development. A recent review assessed the effectiveness of an antipasserine IgY, which suggests that new developed methods can aid in increasing the breadth of studies in this area [48]. In addition, there is growing evidence that maternal effects through antimicrobial compounds can be context dependent and increase offspring survival [115,116].

20.4 Factors causing variation in immune responses

20.4.1 Age-related variation

One important source of variation among individuals is age. Differences in immune performance among age groups may occur because of differences in past parasite exposure, body condition, and foraging performance. Accordingly, all field studies should, at a minimum, control for age statistically and experimentally when examining responses. In addition, age differences may represent important life history differences among individuals within a species. For example, senescence, the progressive loss of function with age [117], may be due to the constraints of aging as well to as an adaptive strategy of downregulating investment in factors affecting long-term

survival (e.g., the immune system) to increase investment in reproduction. The complicated interplay amongst life experience, senescence, and individual quality (e.g., a nonrandom subset of individuals that survive longer) suggests that simple patterns of age-related variation should not be expected. Rather, detailed longitudinal studies are the best approach to break apart the interactive effects of multiple factors.

However, some broader patterns emerge. Studies of several bird species (zebra finch, *Taeniopygia guttata*: Passeriformes, tree swallow, Leach's storm petrel) have documented declines in the magnitude of CMI responses with age [72,90,118] (Fig. 20.2). Additionally, in vitro proliferation of lymphocytes from tree swallows showed patterns of senescence when stimulated with PHA and concanavalin A, but not in response to LPS [118]. In terms of absolute age in years, cutaneous responses to PHA decreased more rapidly for short-lived species (zebra finches and tree swallows, with maximum lifespans in the wild of 5 and 12 years, respectively) than in Leach's storm petrels (maximum lifespan in the wild of 36 years) [90]. This suggests that the rate of immunosenescence (decrease per year) may scale with other factors related to overall lifespan, such as metabolic rate, body size and resistance to oxidative stress. Unraveling the relationships among these factors represents an ongoing challenge and speaks to the need for more comparative studies across species with widely ranging lifespans. Additionally, patterns of immunosenescence in cutaneous CMI differed across years in a multiyear study of breeding female tree swallows [72], suggesting that age-related changes in immune responses in the wild are substantially influenced by external environmental factors (see Section 3–5). These studies should be taken as potentially idiosyncratic. The complexity of different rates of age-related change is revealed in a longitudinal study of great tits, which found marked differences in response curves, with natural antibodies peaking in mid-life, while complement decreased and haptoglobin increased with age [119].

Age-related changes in humoral immune responses appear less consistent across species than changes in CMI responses. Older (4–6 years) collared flycatchers (*Ficedula albicollis*: Passeriformes) and barn swallows showed lower antibody responses to the novel antigens SRBC and NDV, respectively, than did younger birds (1–2 years) [120,121]. Common terns (*Sterna hirundo*: Charadriiformes), on the other hand, did not show age-related differences in total IgY [122]. Additionally, tree swallows showed no age-related differences in antibody responses to SRBC in the wild or lymphocyte proliferation in response to LPS in vitro [118]. Production of antibodies in response to LPS injection in the field, however, was lower in older tree swallows, but, as with

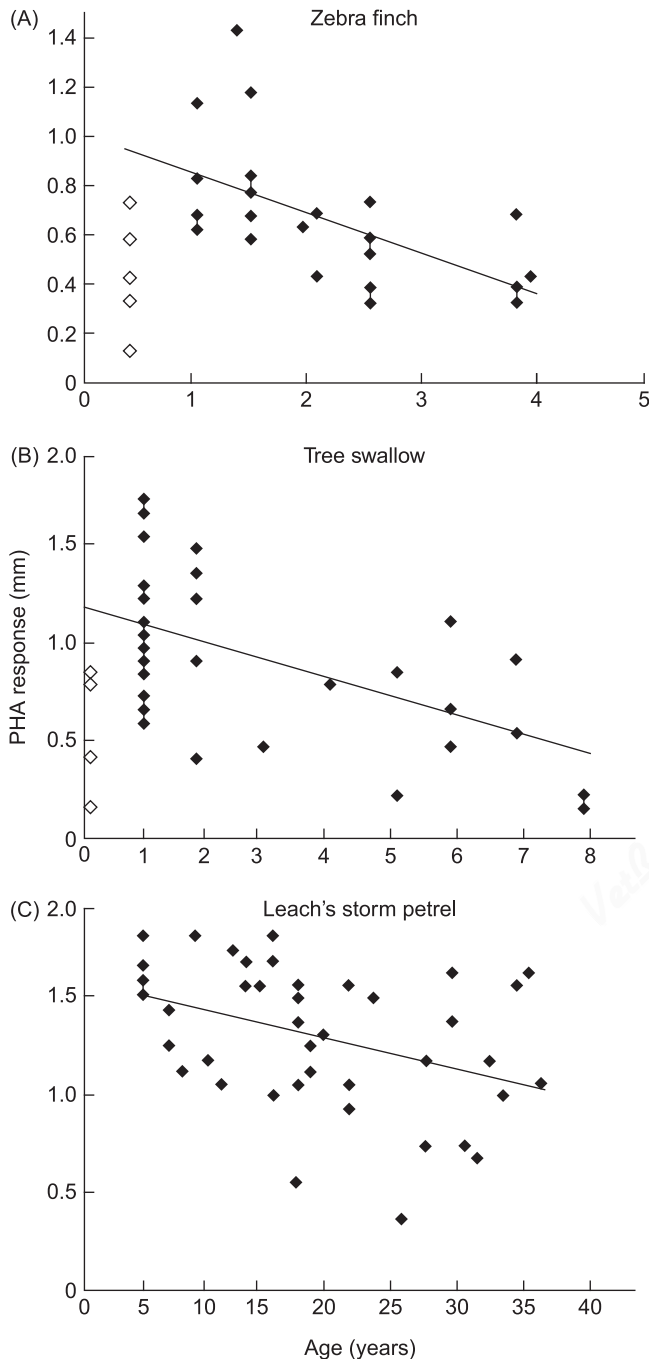


FIGURE 20.2 CMI response to PHA as a function of age for three species of birds. The lines are the least-squares regressions through the data in (A) zebra finches: slope = -0.153 ± 0.04 (SE); (B) tree swallows—slope = -0.107 ± 0.03 (SE); and (C) Leach's storm petrels: slope = -0.016 ± 0.01 (SE). Data are for breeding adults (filled circle) and immature birds (open circle); data from immature birds were not included in the regression analysis [90]. Reprinted from Haussmann M.F., Winkler D.W., Huntington C.E., Vleck D., Sanneman C.E., Hanley D., et al., 2005. Cell-mediated immunosenescence in birds. *Oecologia* 145:269–274, with permission.

CMI, this result differed with the year of sampling [72]. Immunosenescence is important to understand ecology and evolution of senescence and lifespan: if older age classes are more susceptible to infectious diseases, an increase in disease prevalence would disproportionately increase mortality of older age classes.

A broad *meta* analysis found much stronger patterns of senescence in adaptive immunity compared to innate immunity, suggesting processes underlying cellular aging vary between arms of the immune system [123]. However, limited data are available on innate immune responses, so few clear patterns emerged with age. Older barn swallows showed lower levels of natural antibodies but no differences in complement-mediated lysis when compared with younger individuals [124]. Additionally, in tree swallows, constitutive levels of natural antibodies and complement-mediated lysis, as well as LPS-induced microbicidal capacity and lysozyme levels, showed no patterns with age [72,118]. Interestingly, mass loss and reductions in nestling provisioning were more pronounced after LPS injection in older tree swallows, suggesting that older individuals may pay increased costs of innate immune activation [72]. Again, this result depended on sampling year, implying a strong role for environmental heterogeneity in these patterns.

Most studies specifically addressing the influence of age on immune functions in free-living birds have been conducted on relatively short-lived species, utilizing different individuals across age classes. Future studies could significantly improve our understanding of age effects by comparing species with highly divergent life expectancies and following known individuals over time. For example, in captive Steller's eiders (*Polysticta stelleri*: Anseriformes) the oldest animals (14+ years old) had increased IgY levels but lower lymphocyte proliferation [125]. While repeated injections with the same antigen could induce memory effects, confounding the contribution of age, longitudinal studies could randomize the order of several different antigens over the course of several years. Parrots (Psittaciformes) may be an excellent model group for asking these questions because they are generally long-lived yet show considerable variation in longevity among species.

20.4.2 Social environment

Group living carries several advantages, such as enhanced predator detection and more efficient foraging. However, sociality can also increase disease transmission and promote behavioral stressors, which alter individual physiology, including immune defenses [126]. Consistent with such links, social avian species show higher heterophil/lymphocyte ratios than solitary species, although this effect is relatively small when controlling for phylogeny [15].

Within species, however, several captive studies have shown pronounced effects of social environment on immune responses, often in complex ways. For instance, dominance appears to play an important role in determining the magnitude of several immune responses in various species. In single-sex flocks of house finches (*Haemorrhous mexicanus*: Passeriformes), dominant individuals tended to have higher antibody responses to SRBC [127]. However, in mixed-sex flocks, antibody responses to SRBC decreased while DTH responses to PHA increased with increasing dominance [128]. Similarly, in Red Jungle fowl (*Gallus gallus*: Galliformes), dominant individuals displayed higher DTH but showed no difference in antibody responses [129]. In addition, mass lost during an APR to LPS injection was most pronounced among the least dominant individuals [130]. Taken together, these results show that although dominance status appears to alter avian immune systems, effects are not consistent across species or the type of defense measured.

Along these lines, the varied effects of dominance on immune function seem to involve more than just differences in individual quality, which could correlate with natural dominance rank (see Section 20.4.3). Rather, changes in immune function with dominance seem to involve an interaction between individual quality (or prior experience) and current dominance status. For instance, among dominant house finches, experimental reductions in rank yielded decreases in SRBC responses, whereas among subordinate individuals, increases in rank yielded no changes in response [131]. Additionally, experimentally altered dominance in house sparrows resulted in changes in PHA responses that depended on body size—large birds showed higher PHA responses when dominant than when subordinate, but small birds showed lower PHA responses when dominant than when subordinate [132].

Dominance is not the only social factor that affects immune responses in wild birds. For example, Minias and colleagues [133] showed that nestling common terns in larger breeding colonies exhibited lower CMI responses to PHA and elevated H/L ratios. Similarly, when housed alone, zebra finches expressed more pronounced sickness behaviors in response to LPS treatment than when housed in colonies of 30 individuals [134]. In that study, however, the physiological components of the APR (mass loss and cytokine signaling) were similar between social treatments [134]. The relative abundance of potential mates or competitors may also contribute to socially-mediated differences in immune responses. For instance, male-biased sex ratios decreased DTH responses to PHA in both male and female dark-eyed juncos (*Junco hyemalis*: Passeriformes) [135]. Additionally, isolated male zebra finches housed near a single female tended to show lower

responses to PHA injection than males housed in an all-male flock [136]. Similarly, LPS-injected male zebra finches presented with a novel female showed reduced sickness behaviors [137]. In addition, subtler aspects of the social environment may also influence the magnitude of the APR. For example, house sparrows mounted higher fevers when a greater proportion of their social group was responding to LPS injections simultaneously [138]. In general, these studies suggest that at higher densities and/or in the presence of important breeding or competitive opportunities, birds may downregulate immune responses, with most of the evidence stemming from inflammatory processes (including the APR and DTH).

Although immune responses can clearly vary with social environment, the mechanisms underlying such differences remain unclear. Increased aggression or sexual displays often occur with social treatments that result in altered immune responses [131,135–137]. This pattern suggests that physiological mechanisms that underlie or respond to such behaviors may mediate links between a bird's social environment and its immune system. Although differences in hormone levels could provide such links [139,140] the few studies that have altered social environment and measured both hormonal and immune changes provide little support for this hypothesis [127,135,137]. Body mass, a proxy for the availability of resources (see Section 20.4.3), can vary with social treatment and helps explain socially-mediated differences in immune responses in some, though not all, cases [127,131,135]. Fully uncovering such mechanistic links between sociality and avian immune systems will require experiments that simultaneously manipulate both social environment and hypothesized mediators.

20.4.3 Condition, nutrition and individual quality

Physiological phenotypes represent a complex interaction between genetics and environmental influences. First principles lead to the conclusion that the immune system should be no different. Thus differences in condition and nutrition likely explain much of the variation in immune function among individuals in the wild, in part because the vertebrate immune response requires significant supplies of protein, energy, and other nutrients [65,66,141] (see also Chapter 15). However, the effects of an animal's current or prior nutritional state can be difficult to disentangle from intrinsic factors, such as overall individual quality, a broad term borrowed from evolutionary biology. In general, higher-quality individuals are those more likely to contribute their genes to subsequent generations. Individual quality is influenced not only by resource acquisition, but also by genetic background and nonheritable maternal effects. Therefore studies explicitly examining all factors yield the most information about the

relative importance of extrinsic factors in shaping immunological variation in the wild. The most robust experimental design in the wild is a partial cross-fostering manipulation, wherein nestlings are exchanged among nests, leaving each nest with a mix of both related and nonrelated individuals. This design helps to separate the effect of rearing conditions (including nutritional state) from maternal and genetic effects.

Most cross-fostering studies have reported an influence of both common origin (genetics and maternal effects) and common rearing environment on CMI responses to PHA, with the stronger effect appearing to be environmental conditions [18,88,89,142]. For example, a partial cross-fostering study in great tits found that common rearing environment explained the plurality of variation in PHA responses, mostly through its influence on nestling body mass [88]. Several other studies that did not utilize cross-fostering also found positive relationships between nestling mass and/or food availability and PHA response [92,143–147]. However, such studies cannot disentangle the effects of resource availability from genetic background or maternal effects [143]. Less is known about the role of common origin versus common rearing environment in humoral immune responses because of limited immunological development in nestlings, especially altricial nestlings. A study in serins (*Serinus serinus*: Passeriformes) found that individuals with increased food intake produced stronger humoral responses [147]. In contrast, a recent study of European starling nestlings found that experimentally increased brood sizes, which should lead to lower resources per chick, actually increased constitutive levels of IgY [148]. An important factor that might explain differences across populations, species, and experimental designs is the role of stress responses, which likely covaries with all of these factors [149–152]. The physiological influence of elevated stress hormones can interact with the direct influence of other extrinsic factors.

Studies examining variation in adults show a pattern of resource allocation tradeoffs on immune metrics, though tradeoffs likely occur only for some immune responses, but not in others. For example, adult tree swallows feeding nestlings during cold conditions and low food abundance mounted weaker CMI responses to PHA [153], suggesting that short-term differences in environmental conditions can affect cell-mediated responses. Adult house sparrows produced stronger PHA responses at night than during the day, suggesting that lower energetic demands at night (due to lower activity levels) were linked with higher PHA responses [75]. In addition, house sparrows with higher residual body mass (i.e., greater resource stores) showed stronger PHA responses [75]. In another study using house sparrows, individuals exposed to a predator downregulated the PHA responses, which

was likely due to increased levels of stress hormones or adaptive resource allocation [145]. Additionally, as revealed by a *metaanalysis*, availability of micronutrients may also play an important role, since experimentally elevated carotenoid levels in diets increased PHA responses across a number of avian species [141]. These studies indicate that CMI responses to PHA can be highly dynamic and vary not only among individuals, but within individuals due to differences in resource availability. However, other field studies have failed to find a relationship between resources, as measured by body mass, and other metrics of immunity, including the APR, *in vitro* lymphocyte proliferation, natural antibodies, complement mediated lysis, and bactericidal capacity [71,118,154]. Taken together, these results suggest that while resource availability plays an important role in immunological variation, other components of individual quality, like genetic background and/or maternal effects, must also make important contributions.

Immune responses have been found to vary with individual quality or identity, such as when immune responses are greater in larger natural broods [18,143,155,156], or as a function of individual variation. For example, early-breeding (presumably higher-quality) female tree swallows are better able to deal with trade-offs between immunological self-maintenance and offspring quality and produce stronger PHA and humoral SRBC immune responses than do later-laying females [157]. Similarly, common terns with high reproductive performance have higher levels of serum IgY than poorer performers [158]. Additionally, the ability to secure a high quality territory may translate into higher nutrient availability, and thus more robust immune responses [147], although this is not always the case [156].

Individual differences may be due to greater ability to gather resources and to differences in genetic background. Most studies find a significant influence of common origin on nestling immune responses, indicating a heritable component of immune activity [18,88,154,159,160], which is not surprising in view of genetic selection for increased immune responses and resistance to diseases in chickens [161]. However, work on inheritance of immune activity is in its infancy in free-living birds. As a consequence, little is known about variation in the strength of natural selection. Moreover, the relative effects of additive genetic variance (i.e., genetic variance associated with the average effects of substituting one allele for another) on immune function will vary depending on the strength of natural selection. Recent work on the heritability of immune responses has found significant spatial variation as well as differences between sexes and with regard to parasite exposure [162–164].

In light of emerging research on individual differences, one potential theoretical framework that could

reconcile individual variation is coping styles (aka personalities) [165–168]. Immune traits may arise through physiological integration, particularly with metabolic rate and stress responsiveness, and genetic correlations associated with behavior to lead to suite of covarying traits that include immunity [165,168]. Future work seeking to understand individual variation should consider covariation across traits.

20.4.4 Seasonality/annual cycles

A variety of immune responses differ across seasons in vertebrates [169–171]. Although many factors can influence such differences, these generally fall under two categories of hypothesized drivers: (1) life-history trade-offs (see section 20.6) and (2) environmental pressures. Under the *trade-offs hypothesis*, immune defense incurs costs in terms of energy, nutrients, or time, and animals should suppress these responses when other, costly life-history demands must take priority (e.g., during breeding or migration) [171]. Under the *environmental pressure hypothesis*, animals should bolster investment in immune defense when risk of parasitism increases (e.g., when vectors are most prevalent), or reduce investment in immune defense when abiotic conditions force investment in other physiological systems (e.g., during extreme cold) [30,169,172–174]. In birds, these hypotheses can lead to opposite predictions. For instance, in tropical ecosystems, the *environmental pressure hypothesis* might predict higher immune responses during the breeding season, when the risk of vector-borne infection is higher [175]. For those same birds, however, the *trade-off hypothesis* would predict reduced immune responses during the resource-demanding breeding season [171]. Both hypotheses have seen some support, with captive studies and recent work at tropical latitudes helping to disentangle the two, as tropical breeding seasons can overlap periods of environmental differences [175–177].

Numerous captive studies have asked how the immune system varies with photoperiod, an experimental proxy for season, which induces myriad physiological responses linked to immune defense [139,178,179]. In general, responses to PHA have been more pronounced during photoperiods mimicking winter (short days, long nights) or has shown no change across simulated seasons [170,171,180]. Data on antibody responses are somewhat mixed, although there appears to be a trend toward higher responses to novel antigens in photoperiods mimicking summer [170,171,180]. Several studies have indicated that innate immune defenses show complex patterns. In red knots (*Calidris canutus*: Charadriiformes), components of innate immunity varied inversely with one another across seasons, which could reflect prioritization of low-cost responses during energetically costly times

such as molt [181]. In contrast, no effects of season were apparent in the acute-phase responses of captive skylarks (*Alauda arvensis*: Passeriformes) treated with LPS [182]. Similarly, white-crowned sparrows (*Zonotrichia leucophrys*: Passeriformes) showed no differences in febrile response to LPS [70]. Sickness behaviors, however, were more pronounced among white-crowned sparrows in winter photoperiods, but this effect depended upon the population of origin and body condition [183]. Finally, in captive stonechats (*Saxicola torquata*: Passeriformes) a suite of innate defenses (including bacterial killing by whole blood, complement and natural antibodies, and haptoglobin) varied independently across the annual cycle, with indications of complex heritability in these patterns [184].

In addition, field studies have often, though not always, revealed seasonal variation in immune defenses. Importantly, such patterns in the field can differ from captive results (though few have tested the same species both in the wild and in the laboratory). In contrast to CMI data from captive studies [170,171,180], one study, incorporating data on 13 passerines, found that responses to PHA were more pronounced during the summer months [185], consistent with predicted increases in pathogen pressure. In contrast, free-living skylarks showed lower levels of several innate indices (e.g., complement-mediated lysis, natural antibodies, haptoglobin) during fall migration than during breeding [186], somewhat consistent with resource trade-offs during a particularly demanding life history stage. Similarly, in keeping with results from captive studies of white crowned sparrows, free-living song sparrows (*Melospiza melodia*: Passeriformes) displayed more pronounced sickness behavior in response to LPS injections in the nonbreeding season, consistent with suppression of an APR during breeding. Other studies, however, have showed no consistent pattern of variation across the annual cycle in the wild. For example, there were no differences in nitric oxide, haptoglobin, complement, or natural antibody levels detected across breeding and nonbreeding seasons in tropical African red-capped larks (*Calandrella cinerea*: Passeriformes) and rufous-naped larks (*Mirafra africana*: Passeriformes) [176].

Despite the lack of seasonal patterns in tropical larks, such tropical systems, may offer unique opportunities to disentangle the trade-off and environmental pressure hypotheses. For example, Tieleman et al. [175] used tropical house wrens (*Troglodytes aedon*: Passeriformes), which can nest across both wet and dry seasons, to test contributions of current environmental conditions versus life-history stage on immune defenses. Regardless of breeding status, microbicidal capacity of blood against *Escherichia coli* was higher in the wet than the dry season, consistent with a stronger role of environment than life-history stage in this species. In a similarly year-round,

tropical breeder, the common bulbul (*Pycnonotus barbatulus*: Passeriformes), several metrics of innate immune defense (haptoglobin, ovotransferrin, agglutination by natural antibodies, complement-mediated lysis) were best predicted by environmental conditions (and their interaction with sex) rather than life-history stage [177]. Nitric oxide levels, however, showed interactions across sex, life-history stage, and current environmental conditions [177], suggesting that environmental pressures and life-history trade-offs may likely shape particular metrics of immune defense in different ways.

Taken together, all of the above studies suggest that (1) variation in immune function across seasons is common among free-living birds and (2) both current environmental pressures and life-history trade-offs can contribute to such patterns, with the relative importance of these factors likely differing across species and types of immune defenses. Moreover, because fluctuations within a given season are also likely to play a role [177], the timing of sampling remains a critical factor in parsing variation in immune responses among wild birds. Finally, although there is evidence that some immune responses vary with predicted pathogen prevalence in the environment [175], additional work is needed to match the types of immune responses measured against the types of pathogens surveyed.

20.4.5 Parasite exposure

Parasites can have several effects on host immune systems—(1) stimulating a subset of protective immune responses, (2) reducing available resources and thus decreasing other immune responses, (3) biasing subsequent responses toward either Th1 or Th2-dominated defenses, and (4) directly manipulating host defenses. Thus parasitism can increase immune activity relative to unexposed individuals, but can also reduce certain responses. Unfortunately, we have almost no data on Th1/Th2 biases or parasite manipulation of immune responses in free-living birds. However, Vaziri et al. [187] found coinfection patterns consistent with a Th2 or T-reg bias, as song sparrows harboring more gut helminths showed reduced fevers and sickness behaviors in response to LPS challenge. Other studies have found opposite, or more complex effects of current infection on other aspects of the immune system. For example, captive red jungle fowl experimentally exposed to the intestinal nematode *Ascaridia galli* showed an increase in granulocyte levels but a decrease in CMI responses [61], consistent with multiple possible parasite effects. Similarly, a study of free-living pied flycatchers (*Ficedula hypoleuca*: Passeriformes) found that *Haemoproteus* exposure correlated positively with IgY levels but negatively with PHA responses [188]. However, correlative studies cannot

distinguish the causal relationships between current levels of parasites and current immunological metrics.

Although experimental infections of wild birds in the laboratory have shown increases in a variety of immunological defenses in response to diverse parasites [61,189–195], similar experiments are either not feasible or unethical in natural habitats. As such, medication experiments provide the best tool for examining the effects of parasites on host immune responses in the wild. For example, free-living blue tits treated with Primaquine, an antimalarial drug, exhibited lower levels of both malarial parasites and total IgY in their blood, while the total IgY levels increased in the absence of treatment with Primaquine [196]. However, in red-winged blackbirds treatment with Primaquine and Chloroquine successfully reduced loads of the malarial parasites in the *Plasmodium* genus, but did not alter haptoglobin-like activity, nitric oxide activity, or leukocyte counts [197]. Such patterns may reflect species-specific differences, or could be a result of the different defenses measured or the stage of infection (acute vs chronic) [197].

The burden of ectoparasites has, in some cases, correlated with responses to immune challenge. For example, nestling house martins (*Delichon urbica*: Passeriformes) with ectoparasite loads controlled through fumigation showed higher PHA responses than control individuals, indicating a potential role for resource depletion, Th2 bias, or parasite manipulation [198]. However, others have found no effect at all when examining ectoparasite levels [18,88,199]. In addition, variation in blood parasite levels (*Trypanosoma*, *Haemoproteus*) among pied flycatchers differed greatly between sampling years, suggesting that the effects of parasitism may depend heavily on environmental conditions [200]. Additional manipulations are clearly needed in this area, particularly in field settings, and results of studies assessing the role of parasites on immune response should also be viewed within the context of factors, like environmental conditions or life-history stage.

20.4.6 Other factors with immunomodulating effects

In addition to those factors detailed above, other environmental conditions can act as stressors that modulate avian immune defense. For example, common blackbird (*Turdus merula*: Passeriformes) nestlings anticipating increased predation risk had increased levels of ovotransferrin, total IgY immunoglobulins, and circulating lymphocytes and eosinophils [201]. This suggests a mobilization of immune defenses well-suited to rapid response following potential injury, such as during a predator attack. These effects may correspond to hormonal

alterations, which could translate into trade-offs between immunity and other developmental or self-maintenance functions. Such patterns appear to arise for some other environmental stressors with immunological effects, such as exposure to heavy metals [202,203]. For instance, in western bluebirds, heavy metal exposure was shown to decrease CMI, while a trade-off between immunity and growth manifested as increased fluctuating asymmetry of immune-challenged individuals [96]. Future research will need to thoroughly explore the fitness consequences of such environmental effects, most urgently in cases where anthropogenic changes directly influence wildlife. Recently, the effects of such novel anthropogenic stressors on immune function have been explored in birds, including the effects of noise [204] and artificial light [205].

20.5 Molecular variation and evolution in immune genes

Pathogens are among the strongest selective pressures acting on birds [206] and have driven an enormous diversity in immune defense strategies. Immune traits can be highly heritable; additive genetic effects have been shown through genealogy studies [207,208], artificial selection experiments [209], and research into avian hybrids [210]. Moreover, the genes underlying this diversity in immune strategies are among the most rapidly evolving genes in avian genomes [211,212].

Although much research in avian evolutionary ecology has focused on the Major histocompatibility complex (MHC), many immune-related genes have adapted in their expression or protein physicochemical features to respond optimally to pathogen incursion. Despite strong purifying selection driven by constraints, footprints of past and ongoing positive selection have been detected in diverse immune genes, as evidenced from the distribution of allelic frequencies [213,214], d_N/d_S substitution rates [215,216], or maintenance of long-lasting trans-species polymorphism [217]. Transcriptomic studies revealed immune genes as hotspots of shared positive selection acting in parallel across both birds and mammals [206]. Moreover, whole-genome evidence suggests more frequent positive selection namely in genes encoding immune receptors, a class of immune molecules that detects structurally variable ligands [212]. Based on host–parasite coevolutionary models we may assume that such immunological molecular adaptations take either the form of linear selective sweeps or can lead to balancing selection maintaining long-term variability within populations [218].

20.5.1 The major histocompatibility complex

The MHC forms an integral part of the vertebrate adaptive immune system and has a key role in selection for

disease resistance. Domestic chicken MHC has been well characterized and is proposed to represent a minimal essential MHC with a single MHC class I molecule dominantly expressed [219] (see Chapter 7). Compared to commercial chickens, MHC genotyping is frequently more challenging in wild avian populations, given the high numbers of MHC alleles. For example, in Eurasian coots (*Fulica atra*: Gruiformes), next-generation sequencing revealed up to 265 MHC class II alleles that are translated into 251 different amino acid sequences [220]. In most birds, MHC is under strong diversifying selection [221–223]. This holds true especially within the antigen-binding region, where positive selection is most likely to act. Therefore most of the MHC-focused research in non-model avian species has focused exclusively on the MHC class I exon 3 and MHC class II exon 2 which form the peptide-binding grooves [224,225]. Isolated pieces of evidence, such as the results from greater prairie-chickens (*Tympanuchus cupido*: Galliformes) [226], show much stronger signals for adaptation in MHC than in other immune genes, such as cytokines. Adaptive significance of variation in avian MHC is further supported by evidence for functional convergence [227] and widespread trans-species polymorphism that are consistent with balancing selection [217,224,228–234]. Paradoxically, because of selection, in bottlenecked populations, genetic variation in MHC is lost more rapidly than in neutral markers [235].

Ancient duplication events predating avian radiation diversified avian MHC (two ancient avian MHC class IIB lineages, *DAB1*, *DAB2*) [236,237], but have been followed by lineage-specific gene birth–death evolution and concerted evolution [230,233]. Most nonpasserine birds show low numbers of MHC genes (e.g., galliform birds [238,239], penguins [240], owls [241], and most birds of prey [242,243]). In these species tight linkage exists between all MHC loci that show rather fixed differences with compromised selection on combined haplotype diversity in MHC. For example, in barn owls (*Tyto alba*: Strigiformes) only 11% of all possible allelic combinations are realized in MHC class I and as little as 9% in MHC class II [244]. This is in striking contrast with the MHC evolutionary patterns in other taxa, notably passerines, where the diversity and number of MHC genes varies widely through frequent gene duplications resembling a process of Brownian motion [245]. Passerines appear to show longer introns within MHC genes [246,247], which may contribute to more frequent gene duplications [248,249]. This has led to 33 MHC class I [221] and 20 class II [249] loci in the genomes of some species. This evidence suggests that passerine birds presently exhibit the most highly duplicated MHC genes of any major vertebrate taxonomic group [231]. Such a high number of MHC loci may allow neofunctionalization, as suggested

by Biedrzycka et al. [221]. Although the expression patterns of passerine *MHC* alleles remain mostly unknown, in some species despite a high number of pseudogenes [250] many loci may be actually transcribed into functional molecules [221,249]. This raises an interesting question whether functional evolution of the passerine *MHC* class I and associated peptide-loading proteins (Tapasin and Transporter associated with antigen processing, TAP) follow the universally combinable mode, as in mammals, or the allele-specific coadaptation mode known from chickens [251]. Further research is clearly needed in the passerine *MHC* system (that highly differs from the chicken one) to show the functional relationships between *MHC* allelic variability, peptide-loading proteins, T-cell somatic variation and health and disease phenotypes.

In *MHC* class I, the strength of diversifying selection is about two-times higher in passerines than in nonpasserines [224]. Increased positive selection on *MHC* in passerines could reflect their small body size, fast metabolism, and shorter generation time, which mirror their generally rapid rate of evolution and ability to occupy a diversity of habitats. Alternatively, it is also possible that increased *MHC* diversity in passerines might have contributed to successful radiation in passerine birds [231]. In any case, this diversification happened early in passerine evolution [232] and has led to some extreme cases of *MHC* allelic variation as in the common yellowthroat (*Geothlypis trichas*: Passeriformes) with nearly a thousand *MHC* class II alleles [252], the great tit with nearly a thousand *MHC* class I alleles [253], and the sedge warbler (*Acrocephalus schoenobaenus*: Passeriformes) with over 3500 *MHC* class I alleles and up to 65 different alleles carried by a single individual [221]. Allelic variability at this scale cannot be understood using standard statistical approaches adopted in ecological immunology. For this reason, analysis of *MHC* supertypes grouping the *MHC* alleles based on their predicted peptide-binding properties [254] may be more convenient and also biologically more relevant [255] than analysis of single alleles. Nevertheless, the research in chicken has suggested a more plastic system of ligand binding in some alleles, lacking any clear *MHC* sequence signature corresponding to the pool of peptide sequences bound [256]. Further research is thus needed to clarify possible evolutionary outcomes of such promiscuous ligand binding.

It needs to be stressed that the above-mentioned patterns of *MHC* diversity differentiating passerines from nonpasserines are not universal and relatively low levels of *MHC* diversity were reported in some passerines. For example, in blue tits there are apparently only four *MHC* class I loci [228], which is similar to the white-tailed eagle (*Haliaeetus albicilla*: Accipitriformes) where there are at least three loci in the same gene [234]. Surprisingly, some taxa, such as falcons went through an

evolutionary transition from primarily highly variable towards lowly polymorphic and slow-evolving *MHC* [229]. Furthermore, in many species the number of *MHC* loci per individual varies even on intraspecific level, which further increases the complexity of the system. This pattern of increased *MHC* individual diversity [257] contrasts with theoretical models suggesting that advantages of allelic diversity (e.g., broader parasite detection) must be balanced against the costs (e.g., stronger negative selection during the maturation of the T-cells and depletion of the T-cell repertoire), leading to selection towards optimized *MHC* repertoire [258]. This may suggest variable or shifting optima for *MHC* diversity across species and even populations.

Importantly, patterns of diversifying selection appear to differ also between *MHC* class I and class II. Several studies reported stronger selection in *MHC* class II [234,244,252,259], suggesting that the evolution of *MHC* class I diversity may be constrained in birds by high rates of recombination and gene conversion. Yet, recent evidence suggests that the differences in strength of diversifying selection acting on *MHC* class I and class II may be taxon-specific, and potentially opposite between passerine (stronger positive selection on *MHC* class I) and nonpasserine birds (stronger positive selection on *MHC* class II) [225]. Current analysis of *MHC* copy number variation in more than 250 species distributed across the avian phylogenetic tree has suggested that while copy number variation in *MHC* class I shows patterns of accelerated evolution and stabilizing selection, the copy number at *MHC* class II may be shaped by fluctuating selection and drift [260].

In birds, *MHC* diversity has been linked to coevolutionary dynamics with pathogens [257,261,262]. Consistent with this idea, avian populations show more diversity in *MHC* than in selectively neutral genes [263] and geographical patterns of avian *MHC* allelic diversity are consistent with the Rapoport's rule suggesting a decrease in parasite richness with latitude [222]. It has been also proposed that migratory and colonial species show stronger signatures of diversifying selection in *MHC* class II than the nonmigratory and solitarily-breeding ones [260,264]. Among common yellowthroats, individuals from a nonmigratory population showed lower *MHC* diversity at class I, while the class II variation showed the opposite pattern, suggesting different selection pressures arising from intracellular and extracellular parasites in this species [265]. Several studies in individual bird species have also revealed relationships between disease dynamics and *MHC* alleles. For example, in house sparrows and great reed warblers (*Acrocephalus arundinaceus*: Passeriformes) associations have been found between specific *MHC* alleles and the prevalence of avian malaria [266,267]. Additionally, experimental infection of

house finches has shown that individuals expressing a higher number of class II *MHC* alleles exhibit reduced pathology in response to *Mycoplasma gallisepticum* [268] (Fig. 20.3). Similarly, individual sedge warblers with a larger number of different *MHC* supertypes had higher resistance to avian malaria [257]. Interestingly, comparison of *MHC* class I diversity and immune responses to SRBC and PHA in house sparrows revealed that one specific allele (a172) was correlated with increased primary and secondary antibody responses to SRBC and increased secondary responses to PHA [269]. Given that the CMI response to PHA is functionally independent of *MHC* specificity [270], this may suggest condition-mediated links between disease resistance, *MHC* genotypes and metrics of immunological responsiveness measurable in the field. Furthermore, in Egyptian vultures (*Neophron percnopterus*: Accipitriformes), endangered Attwater's prairie-chicken (*Tympanuchus cupido attwateri*: Galliformes) and house sparrows, specific *MHC* alleles have been associated with variation in survival [271,272] or breeding success [273]. While several, nonexclusive mechanisms have been proposed for pathogen-mediated selection and *MHC* diversity (e.g., negative frequency dependence, heterozygote advantage and pathogen diversity through space and time) [274], it remains unclear which play the most important role in free-living birds.

While not yet comprehensively investigated in birds, *MHC* genes might be highly polymorphic also for other reasons, such as random *MHC*-sheltered mutation accumulation [275] or *MHC*-based mate selection [276,277]. However, the evidence for mating based on the *MHC* genotype remains ambiguous in birds. Although research

in highly monogamous great frigatebirds (*Fregata minor*: Suliformes) and gray partridge (*Perdix perdix*: Galliformes) showed that nesting pairs tend to have more dissimilar *MHC* alleles than would be expected by chance [278,279], studies in several wild passerines species have not shown any associations between *MHC* dissimilarity and preference of social mate [280–283]. At least in some cases the selection on *MHC*-based disassortative mating may be compromised by the population-wide extreme *MHC* allelic richness [252]. However, in at least some, but not all, passerine species, the frequency of extra-pair mating may be higher among individuals whose social mates exhibit lower *MHC* heterozygosity [281,284,285]. In some birds, such as the Leach's storm-petrels it has been shown that despite random mating, *MHC* multilocus genotypes may maintain their functional diversity in offspring, thanks to high divergence between the most common alleles of each gene [286]. Finally, recent research in song sparrows *MHC* class II has shown assortative, not disassortative, *MHC*-based social mating [223]. This contrasts with the idea of pathogen-mediated balancing selection, leaving the issue of *MHC* diversity and mate choice an open question.

The *MHC*-oriented research struggles with several issues that complicate clarification of the host–pathogen molecular coevolutionary patterns. First, in many species the extreme diversity in *MHC* alleles, encoded at a variable number of loci [260], places high demands on current sequencing technologies and analytics. Second, because many pseudogenes exist at *MHC* loci, verification of allele-specific mRNA expression is required [221]. Finally, given the putative conformational plasticity

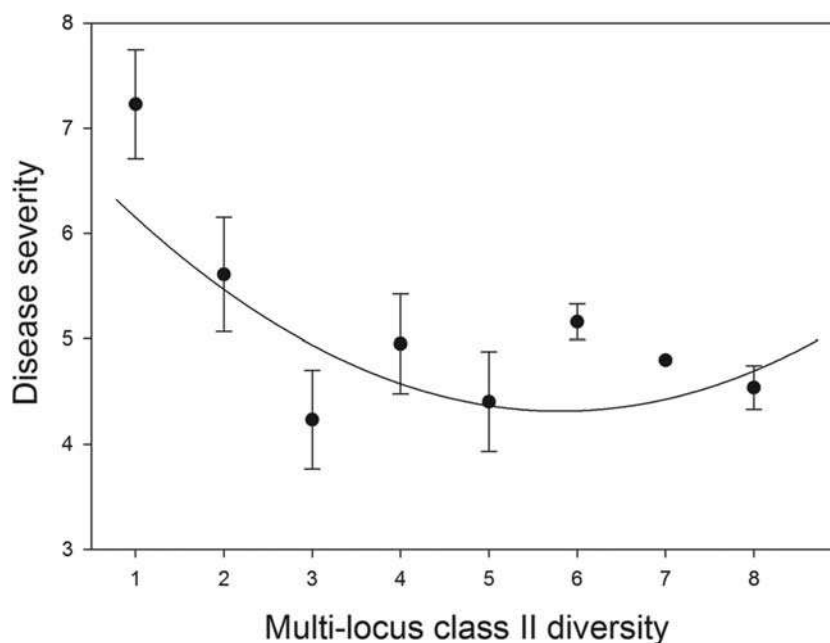


FIGURE 20.3 Increasing *MHC* class II diversity predicts decreasing eye lesion severity during *Mycoplasma gallisepticum* infection in house finches (*Haemorrhous mexicanus*). Diversity was measured by single-strand nuclear polymorphism [268]. Reprinted from Hawley D.M., Fleischer R. C. 2012. Contrasting epidemic histories reveal pathogen-mediated balancing selection on class II *MHC* diversity in a wild songbird. *PLoS One* 7: e30222, with permission.

responsible for structurally flexible ligand binding in some, but not all, MHC protein variants [256], data analysis based on supertypes may be conceptually problematic. While MHC is undoubtedly an important determinant of individual immune defense, it has been argued that focusing on this genetic region alone may not provide a comprehensive outline of all immunogenetic effects [287]. Indeed, some other genes may represent simpler models for investigation of host–pathogen molecular coadaptations.

20.5.2 Innate immune genes

Unlike the *MHC*, most avian innate immune genes appear to be single copy genes. Despite predominant purifying selection and general structural conservatism, in animals, several innate immune genes exhibit significant levels of interspecific as well as intraspecific variation and appear to be under positive selection [288]. This is especially true for the pattern recognition receptors (PRRs) which trigger inflammatory responses, broadly effective immune mechanisms that carry high costs in terms of resource allocation and host tissue damage [52]. For a range of avian pathogens it has been shown that innate immune cells from pathogen-resistant birds respond more readily to infectious challenge than those from susceptible individuals, which suggests differences in properties of their molecular repertoires [289].

Toll-like receptors (TLRs) represent the most well studied PRRs in birds. In its ancestral state the avian *TLR* family consists of ten receptors differentially adapted to distinct ligand binding [290]. Interspecific variation is observed in some avian taxa in duplication of *TLR7* (several clades within Passeriformes, Charadriiformes, Cuculiformes and Mesitornithiformes [290–293]) and pseudogenisation of *TLR5* (several clades within Passeriformes, Psittaciformes, Cariamiformes, Trogoniformes, Phaethontiformes, Eurypygiformes, and Apodiformes [290,294]). Despite gene conversion homogenizing certain regions between some *TLR* paralogues (e.g., in *TLR1* or *TLR2* [290]), strong positive selection has been repeatedly detected on interspecific level in avian TLRs. This site-specific diversifying selection appears to be stronger in the TLRs, that face the extracellular space and bind complex ligands (TLR10 [TLR1A], TLR1 [TLR1B], TLR2A, TLR2B, TLR4 and TLR5), than in TLR15 and endosomal TLRs (TLR3, TLR7, TLR21) [290].

While generally conserved across specific pathogen types, the immunogenic microbial structures (named microbe-associated molecular patterns, MAMPs) recognized by TLRs show significant structural variability [295,296]. This may create a strong selective pressure on TLRs to adapt to specific ligand variants. Indeed, despite

their much conserved general functionality [297], differences linked to disease resistance have been shown even between *TLR* alleles originating from the same species. In birds, this has been documented for chickens [298], where the existing genetic variation between breeds much exceeds human variation [299]. Similar levels of intraspecific polymorphism were also observed for *TLRs* in some free-living birds [300], including the lesser kestrel (*Falco naumanni*: Falconiformes) and the house finch, but not in all, as evidence mainly from inbred populations [292,301–303]. In Attwater's prairie-chicken it has been shown that several parameters of immune function (lysozyme activity, hemolysis) and survival are related to certain *TLR* alleles [271]. High *TLR* allelic variation can result from balancing selection [304]. Thus the pattern of adaptation through high genetic variation resembles the above-mentioned variation in *MHC* involved in the adaptive immunity. However, unlike the adaptive immune response which can take as long as 1 to 2 weeks to clear an infection [74], innate immunity is a rapid, ready-to-act defense mechanism. Thus balancing selection in these genes could provide advantage in early immunological decisions, directing subsequent responses and preventing later immunopathology.

The finite number of MAMP structural types seemingly corresponds with the frequent pattern of convergent evolution observed in avian *TLRs* [290]. Patterns of positive selection were mainly reported in extracellular domains being located either in or topologically close to ligand-binding sites reported earlier for model species [305,306]. These adaptations do not appear to affect the molecular shape of the ligand-binding region, but they alter its surface properties, namely the charge distribution [306,307]. In birds, only about 4.5% of codons experience episodic positive selection across all *TLR* loci [216], yet this variation may significantly affect the molecular phenotype. Evolutionary analysis of the *TLR4* ligand-binding region in a sample of 55 passerine species from two different geographical regions indicated phylogenetic patterns in the charge clustering, but its association to any of the tested ecological characteristics remains unsupported [307]. This is consistent with Fahrenholz's rule, in which cospeciation between hosts and microbes [308] results in their mirrored phylogenies. If this is the case, parsing signals of phylogeny from relevant environmental pressures (namely pathogen communities) can prove particularly difficult.

PRR other than TLRs, such as Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and RIG-I-like receptors (RLRs), remain poorly studied in birds other than poultry. In one study, melanoma differentiation-associated gene 5 (*MDA5*), a nonself RNA-sensing RLR, showed low variability in the helicase domain across 62 avian species [309]. Although the

pattern observed in the retinoic acid-inducible gene I (*RIG-I*) was different from *MDA5*, the sites under positive selection were located far from the RNA-binding sites. This suggests general ligand-recognition conservatism in avian RLRs, accompanied by selection on only minor adjustments in pathogen responsiveness. Interestingly, increased positive selection acting on the galliform laboratory of genetics and physiology 2 (*LGP2*) gene, a third RLR that otherwise exhibits few signs of positive selection in birds, might correspond to the loss of *RIG-I* in Galliformes [309]. Further evidence then suggests conservation of the downstream molecular machinery in birds including the *Mx* genes ([310], but see [311]). Genetic diversity of avian tetherins that are also effector molecules involved in antiviral defense remains unknown [312].

Compared to PRRs, immune signaling molecules, such as cytokines appear to evolve mostly under negative selection [212], with little evidence for directional selection [305]. This confers that these signal mediators form the structural conservatism required for their universal functionality. Despite this, some genes of pleiotropic cytokines, such as *IL1B* [313], and some cytokine receptors, such as interleukin 4 receptor alpha-chain (*IL4R alpha*) [314], have been reported to show signals of balancing selection in galliform species.

Finally, avian defense peptides and proteins (known also as antimicrobial proteins) exhibit mostly negative selection, with occasional evidence for balanced diversity, namely among β -defensins (*AvBDs*) and cathelicidins [315,316]. Despite this, site- and branch-specific episodic diversifying selection has been detected in such genes, mainly in *AvBD3*, *AvBD7*, and *AvBD10* [315]. This selection appears to affect mainly residues close to conserved cysteines forming the characteristic β sheet-rich fold. Among 44 waterfowl species, Chapman and colleagues [317] described remarkably low putative functional diversity for five *AvBD* genes. Here radical changes associated with balancing selection were observable only in the duplicated *AvBD3*. Balanced polymorphisms have also been revealed among passerine *AvBDs*, despite their otherwise conservative nature [318]. Functionally-relevant polymorphism has been confirmed in *AvBD7* in great tits, as in vitro testing revealed differential inhibitory effects of two *AvBD7* variants on gram-positive bacterium *Staphylococcus aureus* [319]. This pattern probably reflects differences in the molecular charge and hydrophobicity of the *AvBD* variants [214]. Several sites under positive selection were found also in lysozyme studies across chicken populations [320].

Despite recent progress in examining the diversity of avian innate immune genes, the list of such genes with potential ecological and evolutionary implications could certainly be expanded much further. Since virtually

nothing is known about the functional diversity of most of these genes in birds, wildlife immunogenetics clearly needs to expand its scope to arrive at a more comprehensive understanding of the evolution of avian disease susceptibility and resistance. Future investigation should also target noncoding regions involved in gene expression regulation, such as promotor regions and untranslated regions in exons.

20.6 Immune function as an evolving life history trait

20.6.1 Costs of mounting immune responses

Understanding how the immune system relates to avian life history requires knowledge of both the costs and benefits of immune defense [11,56]. For example, if reproduction and immune defense both enhance fitness, but require the same, limited resources, an organism cannot maximize expression of both traits simultaneously and they will trade-off with one another. Such perspectives have long shaped research in ecoimmunology [321] and highlight four key assumptions about the relationships among immune defenses and life history traits: (1) immune defenses incur costs in terms of resources (e.g., energy, nutrients, time) and/or damage to an organism's own tissues; (2) other life history traits require the same resources and/or respond negatively to immune-induced damage; (3) shared resources are, or have the potential to be, limited; and (4) immune systems enhance organismal fitness, presumably by increasing survival.

In the context of these assumptions, trade-offs between life history traits and immune defenses can result from two distinct, though nonexclusive processes [322]. First, physiological trade-offs should occur when immune activation monopolizes limited resources or damages an animal's own tissues, reducing the resource availability or tissue functionality essential to other life history traits. Under such conditions, the opposite case should also be true: enhancing other life history traits would compromise immune functions. Second, animals can evolve genetic trade-offs that manifest even in the absence of immediate resource limitations. These genetic trade-offs would reflect a strategy in which the genotype dictates negative covariation between the expression of immune and life history traits, such as growth rate, potentially preempting direct resource competition [323]. Because both underlying processes can function simultaneously, there is a high potential for complex genotype by environment interactions in the expression of such trade-offs [322,324]. Moreover, only the most careful of studies can reveal how each process contributes to observed correlations between immune defenses and life history traits.

Perhaps the most crucial of the above four assumptions is that immune defenses incur costs. Two approaches have been taken to quantify such costs, the first being direct measurement of resources or damage (e.g., energy expenditure, amino acid utilization, free-radical production) and the second being functional costs, such as reductions in other organismal traits related to fitness (e.g., growth, egg production, physical performance). This second approach presumes an underlying trade-off, though not its precise nature (physiological vs genetic).

Most of the work on the resource costs of immune defense in wild birds has focused on energy expenditure among captive individuals. A metaanalysis across nine species suggests that mounting CMI or antibody responses (against PHA, SRBC, or diphtheria-tetanus toxoid) increases energy expenditure by 5%–15%, with responses to diphtheria-tetanus toxoid costing significantly less than responses to the other antigens [141]. Such costs are highly variable across studies and species, potentially varying with primary versus secondary exposures [44,141]. Moreover, this metaanalysis did not include information on pronounced APRs, which can be significantly more energetically costly (a 16% increase in energy expenditure in zebra finches [325] and a 33% increase in energy expenditure in Pekin ducks (*Anas platyrhynchos*: Anseriformes [326]). The observed energetic costs of the APR likely vary with both body size and time of day; some birds exhibit fever, which is energetically costly, while others become hypothermic. For example, larger birds, including chickens and ducks, are more likely show diurnal fever [326,327] than are small songbirds. During the day, these smaller birds typically exhibit high body temperatures ($>40^{\circ}\text{C}$) and often show hypothermia during an APR [67,138,325,328–330]. This pattern may be due to a risk of overheating at already high temperatures or to metabolic challenges associated with heat loss at a high surface area to volume ratio [67,330]. When mounting an APR at night, however, several songbird species have been shown to increase body temperatures above those of healthy individuals, which typically show nocturnal hypothermia [138,328,330–332].

While the protein costs of immune activation can be substantial in poultry [66,333,334], less information exists on such costs for free-living birds. Dietary manipulations may help uncover the protein costs of immune activation, but results are currently limited and difficult to interpret. For example, supplementation with methionine increased skin swelling after PHA injection in both great tits and Eurasian magpie (*Pica pica*: Passeriformes) nestlings [335,336] although this treatment resulted in reduced growth in both cases, complicating direct calculation of protein costs. Similarly, Lochmiller et al. [337] found that CMI responses were reduced in protein-restricted, captive bobwhite quail (*Colinus virginianus*: Galliformes).

However, in another study, supplementary methionine reduced skin swelling in response to LPS among great tit nestlings [338], suggesting that protein costs cannot fully explain differences in local inflammatory responses. More recently, work in house sparrows and gray-headed sparrows (*Passer griseus*: Passeriformes) quantified incorporation of isotopically labeled amino acids into immune tissues during the APR, a promising method for uncovering protein costs [37,339]. For example, house sparrow populations that used more energy after LPS injection also showed higher incorporation of labeled leucine into liver and spleen, although comparisons with untreated birds were not available [37]. Still, such studies, especially in combination with dietary restriction, could be helpful in uncovering the role of protein costs in shaping avian immune responses.

Other resources, including micro-nutrients may also play an important role in shaping immune responses in wild birds. For example, a metaanalysis of 10 species found that increased availability of dietary carotenoids significantly increased responses to PHA [141]. While some micronutrients may be crucial components of immune defenses themselves, these carotenoid results also suggest a link to damage costs of immune responses (see below), as these molecules can act as antioxidants, buffering against self-harm from reactive oxygen species produced during an immune responses [340]. Interestingly, supplementation with another antioxidant, Vitamin E, enhanced antibody and CMI responses in domesticated species [341,342], but showed no effect on the APR or CMI in several wild species [343–345]. These results suggest that in terms of at least some micro-nutrients, free-living birds may be able to pay the costs of immune activation relatively easily.

Auto immune responses directed against self-antigens or reacting nonspecifically with host tissues represent a different type of direct cost of immune defense [6,7]. Such costs can arise through inappropriate targeting by specific defenses (e.g., self-reactive antibodies) or via the production of broadly reactive defenses, like reactive oxygen species, which can damage host as well as parasite tissues. To date, we do not know of any studies in free-living birds that address the issue of inappropriate targeting by specific defenses. Rather, most of the research has focused on the potential for oxidative damage. However, detecting such costs has proven difficult, largely because of the dynamic nature of antioxidant capacity from molecules like those mentioned above. For instance, a metaanalysis of 16 studies on 10 domestic and wild species suggested that a variety of immune challenges tended to alter oxidative damage, antioxidant capacity, or both [346]. As expected with such results, the analysis also revealed significant heterogeneity among studies [346]. Such patterns make uncovering the true damage costs of

immune responses challenging, as they highlight the two-fold nature of such costs: (1) the actual damage done and (2) the resources required to mitigate that damage. Studies that assess multiple metrics of both oxidative damage and antioxidant capacity will likely prove most insightful moving forward.

Given the direct costs of the immune system outlined above, it is not surprising that numerous studies have detected functional costs in the form of negative covariation between immune defenses and other life history traits [11,65]. One of the earliest studies to test these correlations revealed that female zebra finches rearing experimentally enlarged broods showed lower antibody responsiveness to SRBC than did controls [43]. Moreover, this downregulation appeared to be due to increased workloads because females trained to increase daily activity levels showed similar levels of immunosuppression [43]. Additionally, a metaanalysis across six different wild species showed that increased workloads reduced responses to PHA, SRBC, NDV, or diphtheria-tetanus toxoid [347]. Similarly, another metaanalysis revealed that increased effort often leads to increased parasite susceptibility, which is also suggestive of a trade-off between workload and immune defense [347].

A suite of other studies has probed such trade-offs from the other direction, asking whether immune activation reduces the expression of different life history traits. A recent metaanalysis across taxa found that immune challenge (with a diversity of antigens) reduced a range of life-history traits, including reproductive output, growth, and survival [348]. Of note, studies on birds showed the largest and least heterogeneous effect sizes, perhaps because these papers comprised the largest proportion of the dataset (21/46) [348].

In some studies, however, immune activation does not reduce expression of the life history traits under consideration. Some of such patterns may be explained by a wealth of resources—with sufficient resources to maximize expression of two otherwise competing traits, patterns of positive correlation can arise [349]. Another explanation is that birds may employ a *terminal investment* strategy [350], in which individuals maximize fitness through increased reproductive effort if the probability of survival is reduced. If infection (or immune activation) provides a physiological signal of reduced survival probability, immune challenged animals may therefore increase reproductive effort. Indeed, several authors have noted increases in metrics of reproductive effort following immune challenge, including sexual signaling [351], the probability of re-nesting [352], offspring mass and size [353], and nestling provisioning [354]. These patterns highlight an important caveat in measuring the costs of immune defense through relationships with other traits—one cannot always expect to find a negative

correlation between an immune response and another life-history trait, even if both carry physiological costs.

In the study of both resource and functional costs of immune defenses, the most common experimental design induces an immune response and measures the cost of this activation. Although activation is certainly a critical component of the cost of immunity, such assessments do not reveal the costs of developing or maintaining a well-functioning immune system [65]. In particular, there is limited knowledge of the energetic and nutrient costs of the maintenance of lymphoid tissue, removal of free radicals and the turnover of leukocytes (but see [66]). More work is needed on the basic mechanistic aspects of this question, which would greatly enhance the ability to test evolutionary hypotheses about species differences.

Effectively placing these costs of immune defense within the context of life history evolution also requires examining the additional assumptions outlined at the beginning of this section: (1) other life history traits require the same resources as immune defense and/or respond negatively to immune-induced damage; (2) these shared resources are, or have the potential to be, limited; and (3) immune systems enhance organismal fitness, presumably by increasing survival. Assumptions two and three are easily met for free-living birds. It cannot be denied that traits like growing, producing eggs, provisioning young, or migrating away from harsh environments, for example, all require energy, other nutrients, and can result in oxidative damage [355–358], although this statement admittedly omits many nuances. Similarly, availability of dietary resources fluctuates greatly across space and time, meaning that these shared physiological currencies have the potential to be limited [359]. This distinction of the potential for key resources to be limited is important for a full understanding of immune-life-history trade-offs. Specifically, evolved trade-offs can lead to negative correlations between immune activation and other life-history traits even in the absence of immediate resource limitation [360]. For example, variation in immune defenses across the annual cycle can persist under captive conditions, even with *ad libitum* food access and when thermal stress (which alters energy allocation) is applied [181]. In such cases, patterns of annual variation presumably reflect long-term selection for optimal resource allocation, rather than immediate resource limitation.

While the assumptions of shared and potentially limited resources are easily accepted, the fourth assumption, that immune defenses enhance survival, remains somewhat challenging to accurately quantify [56]. In nestlings, some immune responses have correlated with increased survival probability. For example, high total immunoglobulin levels in nestling house martins predicted survival, reflected in recapture rates, although CMI responses did

not [198]. Higher CMI responses did, however, predict return rates in the year following fledging among pied flycatchers and blue tits [361,362]. In contrast, Eurasian collared doves (*Streptopelia decaocto*: Columbiformes) challenged with LPS as nestlings were less likely to survive the postfledging period [363]. Such contrasting patterns hint that intermediate levels of immune investment could provide optimal survival benefits.

Among adult free-living birds, the magnitude of responses to immune challenge have shown variable patterns with survival. For example, female tree swallows producing strong secondary immune responses to SRBC had higher return rates [364]. Additionally, female collared flycatchers with reduced NDV responses were more susceptible to *Haemoproteus* and had increased mortality rates [365], while barn swallows with strong humoral responses to SRBC had increased survival rates [366]. However, such correlations may be specific to the type of response and intermediate responses to immune challenge can also predict higher survival. For example, among blue tits, higher survival probabilities correlated with higher secondary responses to tetanus-toxoid, but intermediate primary responses to diphtheria-toxoid [46] (refer to Fig. 20.1).

Taken together, these results show that there are likely survival benefits to immune responses that are intermediate or high. However, studies that activate the immune system with novel, nonreplicating antigens miss a critical component of the core question—what are the survival benefits of low, intermediate, or high levels of various

mechanisms of immune defense in the face of true infection [2,56,321,367]? Certainly, mounting an immune response in the absence of a true danger would carry all resources and damage costs with none of the benefits, meaning it would be most likely to diminish survival. The opposite should also prove true—during a true infection, mounting no response at all should reduce survival. What remains most challenging is understanding the balance between these two extremes, something that studies of inert antigens alone cannot tease out. Only by pairing such studies with experimental infections can we begin to quantify how the costs of different immune phenotypes balance against the functional protection they offer, and thus how these phenotypes truly predict survival probabilities.

20.6.2 Parasite-mediated natural selection and immune function

Although it may be methodologically more feasible to investigate immunological variation within a single species, natural selection also shapes variation in immunological traits across species. Similarly, it is important to distinguish between intra and interspecies interactions of parasites with the immunological phenotypes. On the intraspecific level, theory predicts selection for intermediate levels of well-balanced immunological investments [7,52] (see Fig. 20.4). Yet, empirical data show that in certain environmental contexts, and within certain

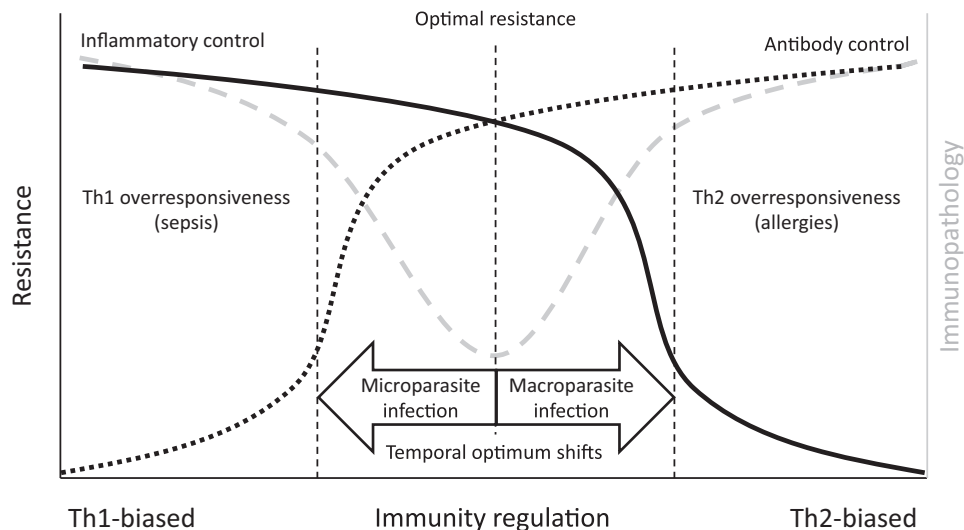


FIGURE 20.4 Optimal host resistance is evolutionarily shaped by pressures from different types of pathogens, against which a balance of different types of immune responses is most efficient, here indicated as Th1-regulated inflammation (solid line) and Th2-regulated antibody responses (dotted line). Since deficient and excessive responses decrease host fitness through either susceptibility or immunopathology (gray dashed line), intermediate, well-balanced immunological phenotypes may be selected instead of maximal immune responsiveness. Despite this, temporal shifts to this optimum alter the immunological balance during infections (arrows schematically linking microparasite infections to Th1 inflammatory control and macroparasite infections to Th2 antibody control).

immunological pathways, the strongest immune responsiveness may result in lowest mortality rates [55,198]. Similar trends may be elusive on interspecific level where, over the evolutionary time, greater levels of parasite-induced mortality select for improved immune functions that eventually reduce mortality levels. Thus, an individual's level of immune function reflects both the (1) species-level differences in strength of natural selection through parasite exposure (causing greater investment into the immune defense in highly parasitized species relative to species that experienced low parasite levels) and (2) individual-level variation.

Parasite pressures may be linked to spatial distribution of the host populations and may shape life history variation among species [3]. In general, animals in tropical regions are believed to have higher yearly survival rates, but also experience higher parasite pressures [368]. Therefore tropical birds show a suite of life history adaptations that indicate a lower investment *per breeding attempt* and greater investment in self-maintenance, consistent with their predicted longer lifespan. Researchers frequently assess the immunological performance both as an indicator of immunological self-maintenance as well as a selective force driving larger life history patterns. These ideas have coalesced on the paradigm of "*pace of life*" suggesting that species are distributed along a gradient of relative investments into reproduction and lifespan. Species that invest heavily in each breeding attempt (fast-living strategy) have fewer resources available for self-maintenance or face greater physiological trade-offs such as stress-induced immunosuppression [180]. Consequently, they show comparatively lower investment in immune function [63]. Yet, this perspective is somewhat oversimplified, because differential investment across arms of the immune system as a function of the life history has been found [63]. We should therefore consider individual defenses, rather than entire immune systems, when predicting differential investment along a pace of life continuum. For example, despite the phylogenetic signal, the *pace of life hypothesis* appears applicable to the avian H/L ratio data [15]—species with longer residual lifespans that are likely to encounter more diverse parasitic challenges throughout their lives had lower H/L ratios, possibly indicating higher investments into adaptive immunity. Other evidence suggests correlations between inflammatory immune responsiveness, growth rate, and lifespan in birds [369]. A brood size manipulation experiment across the geographic distribution range of tree swallows found population-level differences in investments into CMI and antibody responses consistent with the life history predictions: a northern population, with higher mortality and more intense reproductive bouts, increased feeding effort and reduced immune responses, while a southern population did not [370].

However, it remains to be shown if such differences can result from phenotypic plasticity reflecting the response to distinct local environmental conditions. In an elegant common garden experiment comparing tropical and temperate populations of house sparrows (as a surrogate for species-level differences), tropical house sparrows raised under the same conditions as temperate individuals showed different patterns of PHA responses (see Fig. 20.5) [371]. While tropical individuals maintained similar immune responsiveness throughout the year, temperate individuals showed a peak during the late breeding season.

Endocrine-immune interactions may underlie differences across populations and species. In many situations, high levels of stress hormones, such as corticosterone, suppress immune responses [150]. In locations where the probability of exposure to parasites is both high and seasonally invariant, individuals may have evolved a lower sensitivity to the immunosuppressive effects of such stress hormones. For example, temperate house sparrows experimentally implanted with corticosterone showed reduced immune responses, while tropical house sparrows did not [149]. This suggests that tropical individuals have evolved different physiological pathways for addressing the selection pressures associated with greater immune threats. The neuroendocrinological regulation of immunity may be also selected with respect to animal personality—proactive individuals can be predicted to meet a larger pool

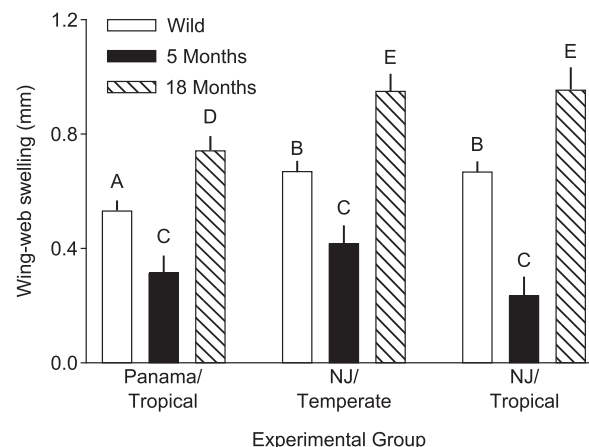


FIGURE 20.5 Phytohemagglutinin (PHA)-induced wing-web swelling during the nonbreeding season in wild and common garden-housed house sparrows from New Jersey and Colon, Panama. Open bars represent free-living values, solid bars represent 5-month captive values; hatched bars represent 18-month captive values. Labels for experimental groups indicate latitude of origin and climate of captivity. The letters above the bars indicate significant differences between groups ($P < .05$) based on a variety of statistical tests. Numbers inside the bars indicate sample sizes. Histogram bars depict means ± 1 SE [371]. Reprinted from Martin L.B., Pless M., Svoboda J., Wikelski M. 2004. Immune activity in temperate and tropical house sparrows: A common-garden experiment. *Ecology* 85:2323–2331, with permission.

of parasites and more likely become exposed to infections. While the *pace of life hypothesis* predicts that proactive behavioral style is associated with shorter lifespans and therefore reduced investment in immune function (possibly as a trade-off), the alternative *risk of parasitism hypothesis* suggests that the exploratory individuals invest more in immune defense to minimize these risks [372]. Although rarely tested in behavioral studies, the results on natural antibody titers in superb fairy-wrens (*Malurus cyaneus*: Passeriformes) [372] and inflammatory responsiveness in Carib grackles (*Quiscalus lugubris*: Passeriformes) [373] are rather consistent with the *pace of life hypothesis*.

One major reason that introduced species thrive in novel environments is the lack of natural parasites (*Enemy release hypothesis*) [374]. Relaxed parasite pressure has been suggested especially for islands [375,376]. Here, consistently with the *Evolution of increased competitive ability hypothesis* [58], selection against excessive costs may drive evolution of certain traits involved in immune defense towards weakened immune-responsiveness. When compared to ancestral populations, invaders may then show reduced immunological investments, which can be predicted namely for costly innate immunity. In support to this hypothesis, captive spectacled Thrushes (*Turdus nudigenis*: Passeriformes) from neotropical island populations showed lower levels of inflammation elicited by LPS injection compared to their mainland conspecifics [58]. Interestingly, a comparison of 21 avian species from two tropical islands and a neighboring African mainland indicated lower acquired (immunoglobulins), but not innate (haptoglobin) humoral immunity on the islands [377].

Preselected differences among species in immune performance may help to explain why some species are more successful as invaders than others, as well as why there are differences in natural ranges among closely related species. Successful invaders were predicted to less likely suffer from the cost of inflammatory responses when encountering novel pathogens, relying more on less costly humoral responses [378]. Experimental work comparing the successful invader, the house sparrow, with its congener, the tree sparrow (*Passer montanus*: Passeriformes), found that house sparrows showed less costly inflammatory responses to LPS than did tree sparrows [379]. This difference may be relevant in view of potential challenges with Gram-negative bacteria, many of which have a high LPS content in their membranes.

These patterns, however, need not necessarily be universal and differences in the costs and benefits of different immunological pathways may be predicted. Although individual studies may observe positive relationships between different immunological components [380], because of the balanced antagonistic regulation of type 1

and type 2 immunity, in certain contexts, it may be difficult to produce both strong cell-mediated and humoral immune responses simultaneously [7,375]. To support this notion, a study examining a wide diversity of avian orders found no relationship when comparing species pairs in their ability to produce responses in both these arms simultaneously [381]. Captive Red Jungle fowl with high IgY levels had low levels of DTH, suggesting a condition-dependent trade-off between cell-mediated and humoral immunity [61]. Results from the field suggest that trade-offs directing the immunological balance may differ across populations, as individual tree swallows at low latitude produced both strong humoral responses to SRBC and strong CMI to PHA, whereas those at higher latitudes showed negative correlation between the two [162]. Interspecific and population differences in immunological regulation may also depend on the local microbial environment. The general phenomenon of decreasing H/L ratio in lower latitudes suggests that interspecific variation in avian H/L ratios may be associated with exposure to diverse parasites [15]. In larks (Alaudidae) differing in life histories and habitat selection it has been shown that pace of life explains only little of the variation in immune investment, while proxies for *environmental antigen exposure* predict the defense strategy [382]. Haptoglobin and ovotransferrin levels as well as complement-mediated hemolysis in larks are stronger in mesic environments than in arid ones, which are traditionally thought of as parasite-poor. Recently, it has been even suggested that unlike energy metabolism, the immune system does not vary with life history among species closely enough to mediate the classical trade-offs [174]. This idea shifts the long-established ecoimmunological paradigm outlying immune function as a costly trait to stress the benefits of specific immune mechanisms in a specific environmental and ecological context, which further accents the need for qualitative and quantitative data to the immunobiotic pressures present in diverse environments. However, this task is not a simple one. It needs to be stressed that most microorganisms inhabiting host bodies likely have only minimal direct contact with the host cells and those that do trigger mainly the innate immunity that further codirects the adaptive responses [74]. Moreover, measuring pathogen abundance in an environment is easily confounded. First, measuring pathogens within hosts is confounded by the hosts' functional immune systems, which likely kill off many potential pathogens before detection. Second, measuring microbes in the environment assumes that those microbes capable of surviving outside of hosts also infect hosts. As such, we still know relatively little about how immunological regulation is linked to the actual pathogen threats in different environments. This clearly highlights the need for future research to identify the costs and benefits of

different specific regulatory pathways in specific contextual ecological situations.

Evolution of immunological regulation appears optimized to actual balance between benefits arising from pathogen resistance and costs that may arise through immunopathology [7]. Some species that face similar pathogen challenges may evolve common adaptations regulating this balance. For example, recent genomic research has indicated convergent genomic immunological adaptations between Old World and New World vultures that split about 60 million years ago [383]. On the other hand, distinct parasite pressures may select in different species and populations for different adaptations. Although revealing the link between parasite infections and evolving immunological traits is a difficult task on the interspecific level, a basic understanding of this complex issue can be obtained from episodes of temporal host–pathogen coevolution tracked across populations on ecological time scales. Research on rapid host adaptation to newly emerged pathogens may prove particularly valuable [384]. Few model systems of this kind are known in birds. One of those is the Hawai'i honeycreeper 'amakihi (*Chlorodrepanis virens*: Passeriformes) adapting to avian malaria caused by a haemosporidian parasite *Plasmodium relictum*. Followed by severe population declines, 'amakihi have adapted to the parasite at low altitudinal elevations [385]. This adaptation is observable as signatures of positive selection in several immune related genes (e.g., *MHC*, *FMR1* interacting protein 2 involved in T-cell adhesion, *TLR5*, *TLR7*, attractin, *CD59*, several cytokines and their receptors, beta-defensin, or immunoglobulin receptors), but also gene classes with no immunological function (e.g., genes related to metabolism). This evidence suggests possible evolution of *infection tolerance* rather than resistance to the pathogen, wherein milder immune response may minimize the risk of immunopathology, enabling enhanced fitness [386]. Another valuable model system for such questions has been that of the house finch coevolving with its bacterial parasite *Mycoplasma gallisepticum*. *M. gallisepticum* triggers local inflammation with systemic effects that are tractable based on mRNA expression of key regulatory cytokines (*IL1B*, *IL6*, *IL10*, *IL18*, *TGFB2*, *TNFSF15*, and *CXCL2*, syn. *IL8L*) [80,81] in periocular lymphatic tissues or in blood. In the spleen, the infection alters expression of other genes, such as heat shock proteins, *CD74* or T-cell immunoglobulin mucin 1, as well as large number of genes that are still lacking their proper annotations [77]. In the past 25 years, the pathogen has evolved, increased virulence, which potentially facilitates its transmission, and increases the strength of the host inflammatory response (especially in the periocular tissues) [387–390]. Since the house finch populations with distinct evolutionary histories with the pathogen seemingly

differ in their responsiveness to the mycoplasmal infection [78,80,391], it is possible, that the host responds to this selective pressure by decreasing its pro-inflammatory signaling, which may lead to optimizing the costs of the response. Together with evidence on immune gene regulatory sequence evolution [392] and shifts in costs of the immune response [393], the evidence available in this model system suggests evolution of balance of host resistance and tolerance [394]. As such phenotypes are likely polygenic, challenges arise in identifying genetic drivers of such observed patterns, which are central to our understanding of disease dynamics, evolution of virulence, coevolutionary dynamics, and the prediction of future outbreaks [385].

20.6.3 Links with male secondary characters

Parasites are known as rapidly evolving agents, especially when compared to their hosts. If immunological balance is altered by parasite pressure, costs arising from inappropriate levels of investments into the immune functions may arise due to this time lag in evolutionary change between hosts and parasites. Since Darwin [395], researchers have hypothesized that sexual dimorphism in bright color patterns is due to sexual selection (i.e., differential mating success). Although the purported underlying mechanism for female benefits from mating with bright males has varied over the years, an idea emerged that sexual selection leading to shifts in offspring allele frequencies may allow rapid adaptation of the host population to a changeable immunobiotic environment. According to the *Parasite-mediated sexual selection hypothesis* mating to partners better capable to strive against the present parasite pressure brings direct and indirect benefits [396], which can drive mate preference. Avian models with female mate choice have played a central role in this investigation [397]. They helped to postulate the *Viability indicator model* of sexual selection which suggests that mating partners can use ornaments to reliably assess the health status of their bearers [398]. Mating signals are considered honest because the costs of ornament expression are condition-dependent, predictably reflecting variation in immune performance among individuals. Thus it is hypothesized that through secondary sexual characters animals can choose mates based on disease resistance and immune responsiveness.

The link between secondary sexual characters and immune function was proposed for the first time by Hamilton and Zuk who hypothesized that individuals with elaborately developed ornaments (bright males) signal their greater health state, which results from some genetic advantage decreasing their susceptibility to parasites ("good genes") [397]. According to their hypothesis, bright colors can be expected to evolve mainly in species

with high levels of parasitism. On an intraspecific level, the Red-queen-type of coevolution between the host and parasite leading to oscillations in parasite genotypes [218] likely induces fluctuations in allele frequencies in host resistance genes (such as *MHC* genes or *TLRs*). This balancing selection on health-affecting genes linked with condition-dependent ornaments can be then predicted to ensure maintenance of variation between males in a population [399].

In several avian species specific immunological genotypes linked to ornament expression have been reported [400,401]. Supporting this, relationships between ornamentation and parasite loads, health, or physiological stress have been found in various species [402–406]. Despite this, the prediction that bright colors signal a genetic link with parasite resistance has proved difficult to test. For this reason, most studies now focus on the phenotypic associations between condition-dependent immune responses and sexually selected traits, making the inference that traits are heritable. Rich evidence supports the idea that male ornaments evolved as immune-correlated traits allowing females to assess male's appropriate immune performance during courtship. For example, male barn swallows with naturally long tails (a preferred ornamental character in European barn swallows) were reported to show higher survival-linked immune response to SRBC, while short-tailed males with experimentally elongated tails showed reduced immune responses [366]. Supporting this, belly coloration was linked to various immunological parameters such as antibody titers or bactericidal capacity in barn swallows [407] and also tree swallows [408].

The complex physiological role of carotenoids had led much of ecoimmunological research in birds to focus on carotenoid-based ornaments [409,410]. Experimental evidence shows that carotenoid-supplemented male zebra finches increase their actual blood carotenoid levels, resulting in improvement of both the bill color and mating success, as well as the responses to novel antigens (SRBC) and mitogens (PHA) [411]. Decreased ornamentation has been found, for instance, in European blackbirds [412] and mallard ducks (*Anas platyrhynchos*: Passeriformes) [413] after stimulation with SRBC or in zebra finches after LPS [414] or KLH [415] treatments. Interestingly, zebra finches that mounted a stronger antibody response lost less red coloration after the challenge than those mounting a weaker antibody response [415]. In some cases the adverse effects of immunity activation may have even long-term impacts [415].

Similar links to immune activity has been also reported for some nonmorphological traits. Although still fragmentary, much evidence suggest that acoustic signals included in avian song may be subject to parasite-mediated sexual selection [416]. In white-browed sparrow

weaver (*Plocepasser mahali*: Passeriformes) song performance positively correlated with pro-inflammatory responsiveness and decreases in the time of the immune challenge [417]. Additional evidence in canaries (*Serinus canaria*: Passeriformes) links song learning to parasitic infections. For example, malaria (*Plasmodium relictum*) infection reduces development of the high vocal center song nucleus in avian brains [418]. In addition, on the interspecific level, song complexity is linked to high levels of pro-inflammatory responsiveness [419].

Although much of the reported evidence shows a positive relationship between ornament expression and immune responsiveness, which is intuitively consistent with the “good genes” concept [420–422], other results show occasional negative associations between the plumage color and various immune components [423–425]. Studies with mixed results [426,427] together with the results showing no correlation between immunological traits and male color traits or reproductive success [47] indicate interspecific variation exists in the generality of parasite-mediated sexual selection. Furthermore, differences also exist in links between individual immunological mechanisms and sexual selection. For example, responses to PHA, but not antibody response to SRBC, correlated with train length (a sexually selected trait) in male blue peacocks (*Pavo cristatus*: Galliformes) [428]. In European blackbirds, SRBC response had no correlation with bright bill color in males (a sexually selected trait) but PHA response did [429]. Importantly, depending on the specific context, both high and low responses may be interpreted as positive traits, depending on the parasite-linked association to the mechanism of their regulation. For example, cutaneous hypersensitivity (induced e.g., by PHA) is linked to large skin infiltration with basophiles [49] (a disease-related and not parasite-resistance trait) in some species [423], but not in others [430].

The precise physiological mechanisms linking ornamentation to immunity appear to be complex and context-dependent, suggesting that honesty of the signaling may be ensured by several mutually nonexclusive mechanisms related to foraging capacities, immunomodulation, or redox balance [431]. In general, all individuals seek to produce the maximum ornamental signals, but cheating is prevented by ornamentation-linked costs that only a small number of individuals can bear, creating individual variation in signal expression. This is consistent with resource allocation trade-off and/or the *handicap principle* [432] (*Immunocompetence handicap hypothesis* [360,399], *Oxidation handicap hypothesis* [433], or *Carotenoid maintenance handicap hypothesis* [431]; but for current conflicting evidence see e.g., [434]). Frequently, as part of the *Immunocompetence handicap hypothesis*, the immunosuppressive effects of testosterone hormonal regulation are considered. While the evidence for this

immunosuppression appears generally ambiguous [435], recent metaanalysis revealed that unlike in correlational studies, manipulative experiments show a medium-sized immunosuppressive effect of testosterone on immune activity [436]. However, it remains unclear whether this is a direct effect of the hormone or if indirect links to stress hormones may be responsible for this physiological pattern.

Alternatively, to the “good gene” model, genetic diversity may provide immunological benefits. For example, in the endangered crested ibis (*Nipponia nippon*: Pelecaniformes), the plumage-coloration-based mate choice supports selection on *MHC* heterozygosity which shows fitness benefits [437]. In savannah sparrow (*Passerculus sandwichensis*: Passeriformes) mating based on *MHC* dissimilarity affects female fidelity [284] and in the tree swallows the mating patterns have been suggested to relate to the β -defensin heterozygosity at *AvBD2* [438]. Although some of these diversity patterns have been linked to ornamentation [401], this *complementary gene model*, does not necessarily assume sexual signaling through ornaments, but allows for other, less well-understood means of immunological genotype assessment between the partners. For example, in song sparrow larger singing repertoire size was related to intermediate levels of *MHC* diversity [439].

20.7 Priorities for future research

Understanding the immune defenses of free-living birds in an evolutionary and ecological context is essential to build a solid conceptual framework for avian immunobiology and will facilitate tests of meaningful hypotheses in the future. As the field matures, we continue to refine the original ecoimmunological approaches and supplement these with evidence from a broad variety of biological disciplines, opening new dimensions and scales of research. However, because of the fragmentary nature of studies, greater integration of results and interpretations is needed. Here, we suggest areas of future work that are most critical to moving the field forward.

- *Continue to develop and validate methods used to measure immune activity.* Avian wildlife immunology mostly lacks specific molecular tools. Many of the standard techniques to assess immune responses are nonspecific, older methods that originated in poultry. However, they are relatively crude compared to modern poultry techniques. Work is badly needed to create new assays specific for free-living species and to determine, in a controlled setting, how well field-based measures assess immune performance. Moreover, as many ecological and evolutionary
- questions require comparative work, we should continue to strive for techniques that function across a broad array of species.
- *Test the central assumption that immune response to an inert antigen predicts immune performance against actual disease threats.* Many studies in wildlife immunology have assumed that higher levels of immune activity indicate an increased ability to clear infections. Since not all types of immune responses affect all types of pathogen, this generalization appears too broad. To test this assumption, more studies must directly compare immune performance with the ability to resist (or tolerate) ecologically relevant pathogens.
- *Link variation in immune phenotypes to polymorphisms in immune-related genes and regions regulating their expression.* Although much work has described inter and intraspecific differences in immune traits and other efforts have mapped sequence variability in immune genes, the connections between genotypes and phenotypes remain elusive. Disentangling these relationships would facilitate a better understanding of the processes that shape the evolution of avian immunity.
- *Describe microbiota and other symbionts across avian species and populations.* While the avian immunobiome is formed by a complex network of interacting organisms that shape immune function, these relationships remain mostly unknown in the free-living birds. The experimental use of antibiotics and probiotics may be adopted to characterize links among various commensal and pathogenic symbionts with immune function. Furthermore, the impact of variation in avian immune genes on evolutionary interactions with such microbes remains unknown.
- *Compare immunological ontogeny.* There may be marked differences in the extent and rate of immunological development among bird species. Specifically, different natural selection pressures can underlie such patterns. Systematic studies of immune system development across a range of bird species are still needed to determine how the immune system interacts with other life history traits.
- *Understand the costs and benefits of maternal antibody transmission.* Given the critical role of antibody transfer in influencing trade-offs in offspring development, more research is needed on the costs and benefits of maternal transmission and on how species-level differences reflect adaptive responses to selection pressures. Specifically, research on the complex interactions among maternal antibodies, immune system ontogeny and early disease exposure will help elucidate variation among and within species in patterns of immune system activity, effectiveness and polarization.

Acknowledgment

We dedicate this chapter to the memory of Victor Apanius, an early pioneer of ecoimmunology.

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Advances in genetic engineering of the avian genome

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21.1 Methods to manipulate the avian genome

21.1.1 Viral vectors

The first genetically modified birds were produced using viral vectors derived from avian retroviruses, which were excellent candidates for gene transfer vectors because they naturally integrate into their hosts' chromosomes as a normal process of their life cycle. These retroviruses were replication-competent vectors derived from avian leucosis virus (ALV) [1] and replication-defective vectors derived from reticuloendotheliosis virus [2] and, more recently, an ALV replication-defective vector [3] and an avian spleen necrosis virus vector [4]. The efficiency of production of transgenic chickens using these vectors was very low (~0.5% of G1 progeny); however, they were capable of generating germline modified birds, including transgenic hens that express low levels of a therapeutic protein in egg white [3].

Attempts to make transgenic quail using ALV were unsuccessful [5]. However, in 2001 Mizuarai et al. successfully produced germline transgenic quails using a vesicular stomatitis virus (VSV)-pseudotyped retroviral vector. In this study, a replication-defective retroviral vector based on Moloney murine leukemia virus-pseudotyped vesicular stomatitis virus G protein was injected into the subgerminal cavity at the blastodermal stage of embryogenesis. Using this method, an average survival rate to hatch of 28% was observed across three studies. Viral vector sequence was detected in all the quails that hatched and germline transmission of greater than 80% was reported in the birds which were mated [6]. One drawback of this work was transgene silencing, while expression of

the neomycin resistance gene was observed, no green fluorescence protein (GFP) expression was detected.

McGrew et al. [7] reported a major advance in chicken transgenic technology by investigating the efficiency with which lentiviral vectors could be used to introduce a transgene into the chicken germline [7]. Naldini et al. (1996) [8] reported the use of a novel group of vectors derived from the lentivirus class of retroviruses. These have some advantages over the previous vectors that were used to develop transgenic chickens, including the ability to infect non dividing cells, and reduce the levels of transgene silencing postgenome integration. More significantly with respect to generating transgenic animals, transgenic mammals, including mice, pigs, and cattle, had been efficiently generated using lentiviral vectors, and reliable transgene expression was successfully achieved after germline transmission ([9–11]; reviewed in [12]). McGrew et al. [7] developed lentivectors carrying reporter transgenes (GFP and LacZ) which would facilitate analysis in transgenic birds. The vectors were packaged using an envelope coat protein from vesicular stomatitis virus, concentrated to give high titers, and injected into the subgerminal cavity below the developing embryonic disk of newly laid eggs [stage X Hamburger & Hamilton (HH)]. These embryos consist of an estimated 60,000 cells, approximately 50 of which are thought to give rise to primordial germ cells (PGC). Injected embryos were subsequently incubated through to hatch. Twelve males were generated and all were chimeric for the transgene. Ten of these birds were mated with wild-type hens and transgenic chicks were detected in the G1 offspring from all the tested roosters at frequencies from 4% to 45%. Stable transmission to the G2 generation was demonstrated and analysis of expression of the reporter

transgene showed a conserved expression profile between individuals from several transgenic lines and GFP fluorescence was readily detected in live G1 chicks and G2 progeny. The majority of G1 birds contained a single lentiviral insertion, but several contained multiple insertions, with a maximum of four, observed in one bird. These data demonstrate that the lentiviral vectors can be used to generate transgenic lines with an efficiency in the order of 100-fold higher than any previously reported method, with no detectable silencing of transgene expression between generations.

Following this advance in the use of viral vectors to develop transgenic chickens, the same team then proceeded to apply the improved efficiency of the lentiviral system to test different transgene constructs. The first transgenes were designed to express therapeutic proteins for incorporation in the egg white of transgenic layer hens. Lillico et al. [13] delivered transgene constructs comprising regulatory sequences from the ovalbumin gene to specifically drive the expression and synthesis of associated therapeutic proteins to the oviduct [13]. Ovalbumin makes up greater than half of the total protein in the white of a laid egg and expression of the ovalbumin gene is restricted to the tubular gland cells of the oviduct. The transgenes investigated consisted of codon-optimized coding sequences for either a humanized ScFv-Fc mini-antibody (miR24) or human IFN- β -1a. Transgenic hens were successfully generated that expressed recombinant versions of both therapeutic proteins specifically in the oviduct of the laying hens as a component of egg white. Expression analysis for both transgenes from tissues from G1 and G2 progeny showed that, as expected, expression was limited to the oviduct. The amount of recombinant protein secreted into the egg white varied between 15 and 50 $\mu\text{g/mL}$ for miR24 and 3.5 to 426 $\mu\text{g/mL}$ for hIFN- β -1a. hIFN- β -1a was also shown to be functional via demonstrable antiviral activity in an antiviral/cytopathic effect inhibition assay.

The next type of lentiviral transgene was designed to express a decoy RNA that can inhibit and block influenza virus replication and thereby lead to the development of transgenic chickens with increased resilience to avian influenza [14]. Disease resilience is a breeding trait of major interest to the poultry industry and one potential strategy to control viral diseases such as avian influenza is to use transgenic technology to introduce novel genes that confer increased resistance to infection [15]. Of equal importance is the protection of humans from potentially devastating zoonotic disease caused by avian influenza and controlling the virus in poultry will help reduce the risk of a future bird flu pandemic. Lyall et al. [14] developed a transgene designed to express 3'- and 5'- terminal sequences of influenza A virus genome segments that contain the binding site for the viral encoded polymerase

and that can act as a decoy to interfere with the virus infection process. This RNA decoy, designated D5, corresponds to an absolutely conserved sequence that is essential for the regulation of viral transcription, replication, and packaging of all strains of influenza A viruses, offering pan-subtype protection. A D5 lentivector was used to generate transgenic chickens with a single transgene integration on chromosome 2. One transgenic rooster was crossed with wild-type hens and the resulting transgenic (test) and nontransgenic (control) progeny were evaluated in virus challenge studies. Although the D5 transgenic birds succumbed to the initial experimental challenge, they did exhibit a marked prevention of transmission of infection to unprotected nontransgenic chickens housed in direct contact with them. This introduced trait could have a major impact on the susceptibility and transmission of infection at the flock level and supports the concept of transgenic technology for controlling avian influenza infection in poultry.

21.1.2 Transposons

Further advances in chicken transgenesis utilized “cut-and-paste” transposons in cultured PGCs. These are a particular class of mobile genetic elements that transpose genetic information by a “cut-and-paste” process from one genomic location to another within the host genome [16]. Transposons were investigated as a potential improvement to retrovirus- or lentivirus-mediated transgenesis in chickens on the basis that they may improve on the relatively low and variable rate of germline transmission, reduce transgene silencing, and remove perceived safety issues related to the use of viral vectors for some transgenic applications. Macdonald et al. [17] demonstrated that chicken PGCs can be efficiently modified in vitro using both the piggyBac and Tol2 transposable elements [17]. The piggyBac transposon was originally isolated from the genome of the cabbage looper moth [18] and the Tol2 transposon from the genome of medaka fish [19]. Both of these transposons are functional in a wide range of host species, including chickens [20], and have been used to modify the germline of several animal species, such as the mouse, zebrafish, rat, and pig. Macdonald et al. [17] reported that both transposon vectors could efficiently transpose in chicken PGCs. The frequency of stable transfection of PGCs obtained was 10.5% and 42.5% using piggyBac and Tol2 transposon vectors. Tol2 integration sites were spread throughout both the macro- and minichromosomes of the chicken genome and were prevalent in gene transcriptional units and intronic regions, consistent with characterized Tol2 integrations in other species. They also reported that flanking insulator elements (HS4) were not required for reporter gene expression from an integrated transposon.

They then proceeded to demonstrate that modified PGCs containing integrated transposons colonized the gonad of host embryos and formed functional gametes via the successful generation of transgenic offspring that correctly expressed a reporter gene carried in the transposon.

The successful application of transposable elements as efficient vectors for the genetic manipulation of PGCs and the chicken genome was further demonstrated by Park and Han [21]. They reported that piggyBac transposition into cultured gonadal PGCs improved the efficiency of transgenic chicken production and led to a high level of transgene expression [21]. A PGC line was created that contained one stable piggyBac integration on chromosome 20. Based on insertion site analysis of sequence flanking the integrated transposon, it was predicted that no functional gene or transcript was disrupted by the transgene. The transposon encoded the GFP gene and GFP-positive PGCs were injected into recipient embryos to produce donor-derived progeny and to assess the transmission efficiency of the transposon-modified PGCs. The efficiency of germline transmission of the donor PGCs ranged from 90.4% to 98.9% from six male chimeric G0 founders that received the donor PGCs. In total, 437 out of 459 hatched G1 progeny were GFP positive (95.2%) and therefore derived from the modified donor PGCs. GFP expression was stable and strong confirming that piggyBac transposition into chicken PGC lines is a very efficient and effective approach to generating transgenic chickens.

21.1.3 Direct in vivo transfection

Tyack et al. [22] reported another method for producing transgenic birds via direct in vivo transfection of PGCs [22]. The results presented in this paper demonstrate a simple procedure for the in vivo transfection of PGCs with miniTol2 transposon plasmids to generate stable germline transgenic male chickens capable of passing the transgene onto the next generation. The method is based on a significant improvement to a previously published method which demonstrated that transfection of PGCs can be achieved by injecting plasmid DNA–liposome complexes into the bloodstream of stage 14 HH embryos [23]. This previous study showed that although it was possible to introduce exogenous DNA into gonadal germ cells by transfecting circulating PGCs in vivo, it was a very inefficient and unstable process. Furthermore, they were unable to demonstrate that this approach was able to generate transgenic birds. Tyack et al. [22] adopted the latest developments in lipofection technology and significantly advanced this approach to stably transform PGCs in vivo and successfully and efficiently generate transgenic offspring expressing the enhanced green fluorescence protein (EGFP) gene carried in a transposon. They used the miniTol transposon system made up of two plasmids; the

first plasmid contained the EGFP transgene under the control of the CAGGS promoter and flanked by the Tol2 ITRs (pMiniTol-EGFP) and the second plasmid (pTrans) encoded the Tol2 transposase under the control of the cytomegalovirus (CMV) immediate-early promoter for in trans expression of the transposase and subsequent transposition of miniTol-EGFP from plasmid to chromosome in transfected PGCs. pMiniTol-EGFP and pTrans were combined and formulated with lipofectamine 2000 and intravenously injected into stage 14 HH embryos. Successful transfection was confirmed by the visualization of EGFP expression in the gonads of ED 14 embryos. Forty percent of the remaining embryos survived to hatch and the male chicks were grown to sexual maturity. Semen was then collected from all roosters and tested using polymerase chain reaction (PCR) for the presence of the miniTol-EGFP transgene and 45% of the males were found to have transgenic semen. Three males with the highest levels of miniTol DNA in their semen were selected as founder roosters to breed for G1 germline transgenic offspring. The selected roosters were each mated with hens of the same line and a total of 419 G1 chicks were hatched and screened for visual whole-body EGFP expression. A total of 5 out of the 419 chicks were positive for EGFP expression confirming stable integration of miniTol-EGFP into transfected PGCs of the founder roosters and germline transmission of the transgene to the G1 offspring. Two of the three roosters had germline transmission of approximately 1.5%. Southern blot analysis of genomic DNA from the five positive G1 chicks revealed that a single transposition event had occurred in four of the five chicks and a double transposition event had occurred in one chick.

This technology has now been applied to several functional genomics studies in chickens such as examining the effects of overexpressing known antimicrobials on the colonization of known poultry pathogens [24] and effects of overexpression of aromatase and anti-Müllerian hormone on gonadal development and sex development [25,26].

This simple method provides a new opportunity for researchers without extensive PGC culturing facilities or skills with retroviruses to undertake functional genomics studies within the chicken and will also help to drive the development of new applications for avian transgenesis in biotechnology and agriculture such as ducks, turkeys, and other avian species of agricultural importance where there is the potential for improvement in production traits and disease resilience.

21.1.4 Sperm transfection-assisted gene editing

Another germ-cell culture-free approach that could be used for avian genome engineering is sperm transfection-assisted gene editing (STAGE) [27]. This method

involves transfecting sperm with Cas9 mRNA and guide RNA and then using the transfected sperm for artificial insemination in hens. STAGE was designed to harness the ability of sperm to deliver nucleic acids and combine that with recently developed gene-editing systems such as CRISPR/Cas9. In the past, researchers have attempted to use sperm as a delivery mechanism for transgene constructs [28]. While the sperm proved very effective at delivering DNA constructs, transgene integration into the genome remained a major hurdle [29]. This research did lay the foundation to show that sperm cell transfection is possible and that transfected sperm are both viable and capable of fertilization.

STAGE is particularly relevant to avian species as the current methods that could be used to produce gene-edited birds take two generations. Editing PGCs in culture then transferring them to developing embryos [30,31] and directing *in vivo* transfection of circulating PGCs in embryos [22] both result in gonadal mosaic birds. STAGE is designed to cause mutations in the early zygote, preferably occurring in the single-cell zygote to generate full knockout animals in a single generation; however, it can also cause gene mutations in the multicell zygote, leading to mosaicism [27]. STAGE enables editing in the early zygote because it delivers Cas9 mRNA and synthesized sgRNAs as opposed to plasmids containing these components. The STAGE method utilizes RNA-based components because avian oocytes and early embryos, like oocytes and early embryos from most species, are transcriptionally quiescent [32]. Early zygotic development is directed by maternal RNA deposited in the cytoplasm before ovulation, with the embryo eventually becoming transcriptionally active. This process is known as the maternal to zygotic transition, and in chickens, it occurs when the embryo reaches stage X and contains greater than 20,000 cells [33]. Recent research has shown that in the chicken the male also contributes RNA to the zygote that helps direct early embryonic development [34]. How RNA is packaged and stored in chicken sperm is unknown; however, understanding and being able to mimic this process may improve the efficiency of STAGE.

The majority of the gene mutations generated when using STAGE are different from those typically observed when delivering CRISPR/Cas9 components to cells in culture or to mammalian oocytes or fertilized zygotes. While most CRISPR/Cas9-induced mutations are clustered within 10 to 15 base pairs of the protospacer adjacent motif (PAM) site, the mutations induced by STAGE often occur in 50 to 200 base pairs away from the PAM site [27]. It is not clear why this occurs, which is further compounded by the lack of knowledge about the DNA repair mechanisms present in the early chicken zygote. These mutations may occur as a result of errors taking place during DNA break repair, but additional studies

investigating DNA repair in avian zygotes are needed to further elucidate the mechanisms underpinning the mutations resulting from STAGE.

Overall STAGE is a promising new method that has the potential to be applied to many different avian species. However, STAGE generates a high proportion of single-base mutations between 50 and 200 base pairs distal to the PAM site, making it still effective for gene knockouts, but less effective for precise editing. To better understand the mechanism underlying these mutations, further research is needed to elucidate the DNA repair mechanisms present in the early chicken zygote. In addition, as a recent protocol, there is significant scope for optimizing the STAGE method to improve its use in gene editing.

21.1.5 Primordial germ cells

Even though a lot of attempts have been made to generate genetically modified chickens, obtaining precise modifications has been hampered by the lack of technologies in comparison to embryonic stem cell cultures in mice. Though it is possible to isolate and cultivate chicken embryonic stem cells, they only contribute to somatic tissue and cannot be used for the production of genetically modified chickens [35,36]. Early experiments using PGCs isolated by density gradient centrifugation and subsequent reinjection into chicken embryos enabled the generation of germline chimeras by using chicken PGCs [37].

PGCs are the precursor of spermatogonia or oogonia. Like in mammals, PGCs are also described for a long time in birds [38] but the origin of avian germ cells was not clear [39]. Eyal-Giladi et al. [40] were able to show by using quail–chicken chimeras that avian PGCs derive from the epiblast [40] and translocate during primitive streak formation. From stage XIII epiblast PGCs migrate into the germinal crescent of stage X chicken blastoderm [41]. In contrast to mammals, chicken PGCs migrate from an anterior location toward the genital ridge compartment. They travel from the extraembryonic located germinal crescent via the anterior part of the sinus terminalis and the anterior vitelline vein to the intraembryonic vascular system [42]. The overexpression of the chemokine SDF-1, as well as knockout of its receptor CXCR4, demonstrated the crucial role of SDF-1/CXCR4 interaction in the migration of PGCs into the gonads [43,44]. However, the knockout of CXCR4 in PGCs was not able to completely eliminate colonization of the avian gonads by PGCs, suggesting that other factors are involved in PGC migration in chickens [43].

However, genetic modification of these cells was not possible for a long time. In 2006 van de Lavoie et al. finally succeeded in developing a cell culture system to propagate undifferentiated chicken PGCs [45]. Using

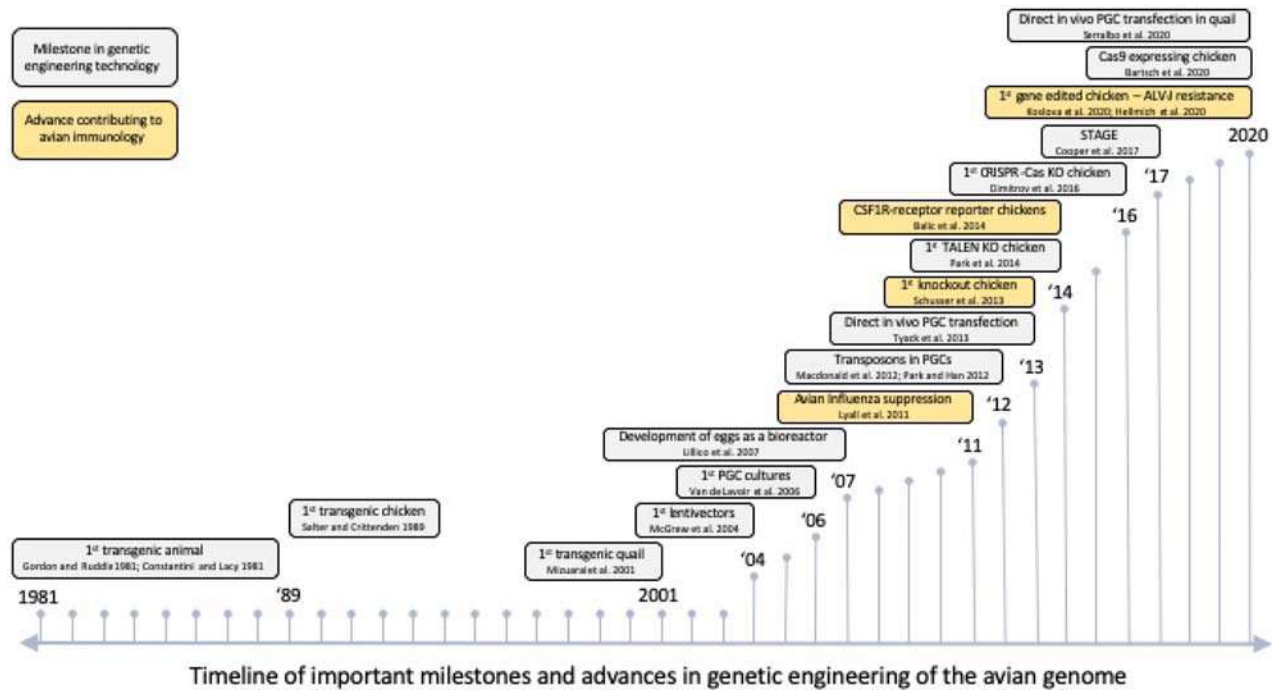


FIGURE 21.1 Timeline of important published milestones and advances in genetic engineering of the avian genome. Significant advances that have contributed to avian immunology are highlighted in yellow boxes.

conditioned KO-DMEM inactivated feeder cells, and a combination of different growth factors, it became feasible to derive, expand, transfect, and individually select edited PGC clones. By stable integration of the eGFP gene into chicken PGCs, green fluorescent PGCs were generated and have been used to generate eGFP-expressing chicken embryos. The availability of this technology was a game changer. Shortly after the availability of PGC cell cultures, Leighton et al. used the system to generate a PGC line with a precise knockout of the immunoglobulin light-chain genes [46]. Nevertheless, it took five more years till the same technology led to the generation of immunoglobulin heavy-chain J segment knockout chickens via homologous recombination [47]. Due to the laborious cell culture system, the required training in embryology and biotechnology of PGC culture was at that point successful in a handful of laboratories worldwide. A milestone making chicken PGC cultures accessible to more research groups was the development of a defined culture medium specific for avian PGCs [48]. The conducted research was based on adapting the osmolarity of commercial KO-DMEM to the osmolarity measured in blood samples from chicken embryos; this included reducing the amount of Ca^{2+} and the addition of FGF2, insulin, activin, B-27 (serum-free supplement normally used for neural cell cultures), and ovotransferrin, to replace chicken serum. It became possible to derive, expand, and manipulate chicken PGCs without the need for feeder cells and a preconditioned medium.

Also, the growth of female PGCs is indistinguishable from male PGCs using this new cell culture protocol. Not having to rely on a specific lot of serum as well as using less variable growth elements that can negatively influence the culture conditions makes PGC technology available to more laboratories now. It also allows the cultivation of PGCs under specific-pathogen-free (SPF) conditions for the production of genetically modified chickens in SPF housing.

First attempts are also made to use the different PGC derivation and cultivation protocols for interspecies germ-line transfer for species conservation as well as for cryopreservation of rare chicken lines [49,50].

An overview of the different technologies described in this chapter is given in Fig. 21.1.

21.2 Genetically modified chickens

Since the generation of the first transgenic chicken by Salter and Crittenden [1], several different genetically modified bird lines have been generated mainly for basic research in the context of infectious diseases, immunology, developmental biology, and agriculture (Fig. 21.2). The development of genetically engineered chicken lines is constantly increasing particularly since the availability of PGC cultures as well as CRISPR/Cas9-mediated genome editing [51]. Currently available genetically modified chicken lines are listed in Table 21.1.

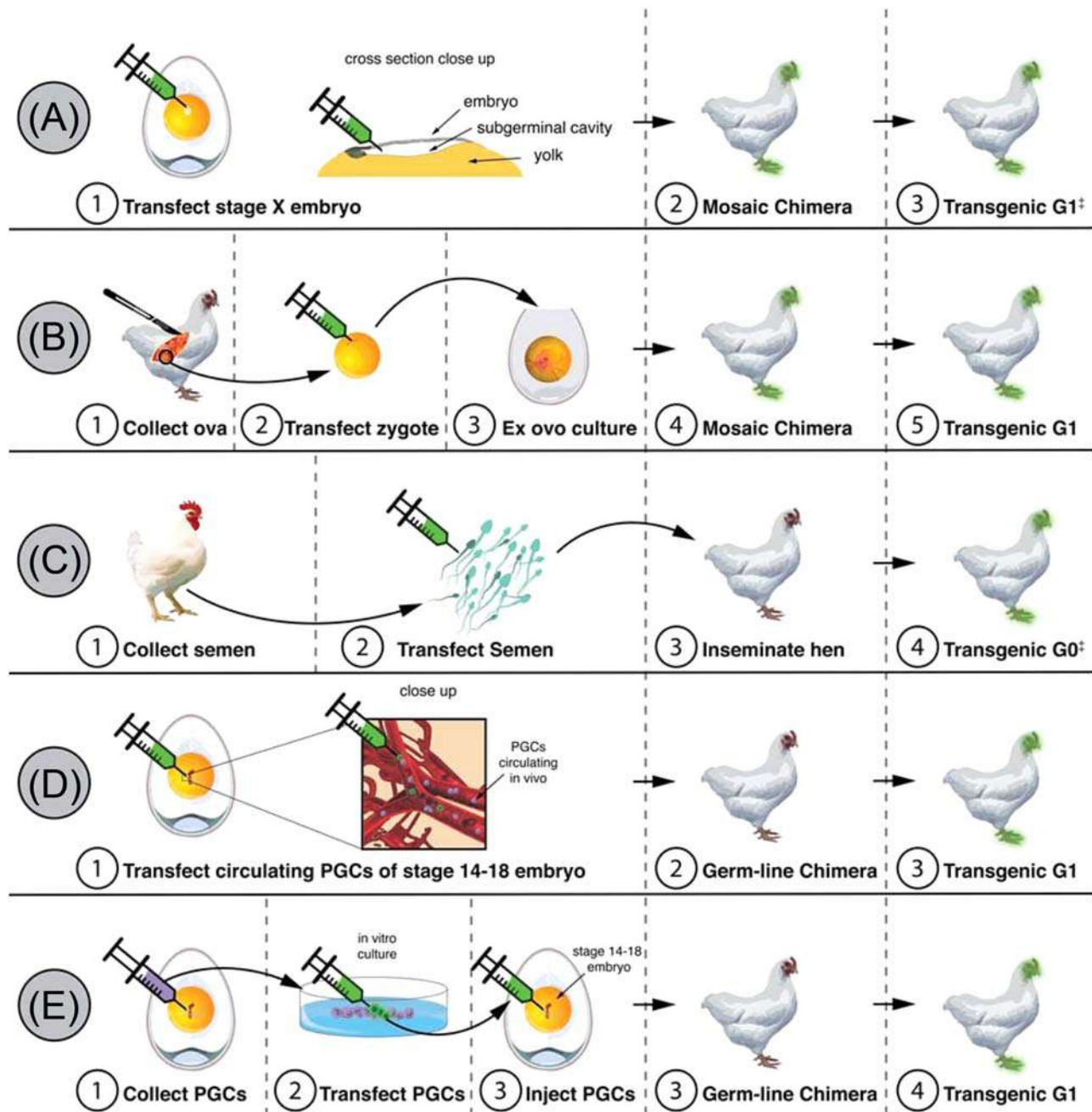


FIGURE 21.2 Reported methods for genetic engineering of the avian genome. Illustrated examples are based on the generation of transgenic GFP chickens but are also applicable methods for other types of transgenes and also genome editing applications: (A) manipulation of stage X (HH) embryos, most often with lentivirus vectors; (B) zygote transfection and *ex ovo* embryo culture; (C) sperm transfection followed by artificial insemination. This is a similar approach to sperm transfection-assisted genome editing (STAGE); (D) direct injection of stages 14–18 (HH) embryos via the dorsal aorta and transfection of circulating PGCs; (E) in vitro culture and manipulation of PGCs followed by injection into recipient embryos. HH, Hamburger & Hamilton; PGCs, primordial germ cells.

21.2.1 Chicken models for immunological research

The first knockout in a nonmammalian vertebrate species by homologous recombination was performed in chicken PGCs leading to the first knockout chicken line [47].

Chickens without productive rearrangement of the immunoglobulin heavy-chain locus have been generated by targeting the J segment of the immunoglobulin heavy chain. Birds with a homozygous modification showed complete depletion of peripheral B lymphocytes as well as antibodies. No side effects on different lymphocyte populations

TABLE 21.1 Worldwide availability of genetically modified chickens.

Transgenic chicken	Affiliation of the research group	Country	References
Transgenic chickens carrying a benign defective subgroup A leukosis virus	Avian Disease and Oncology Laboratory, USDA Agriculture Research Service	United States	[1,52]
Transgenic chickens expressing active β -lactamase in the egg white	AviGenics, Inc., Georgia BioBusiness Center, Athens	United States	[53]
eGFP-expressing chickens	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	[7]
	Department of Physiology, Catholic University of Daegu School of Medicine	South Korea	[54]
	University of Utah School of Medicine, Department of Neurobiology and Anatomy	United States	[55]
	Crystal Bioscience/ Ligand Pharmaceuticals Inc	United States	[45]
	Technical University Munich, School of Life Sciences Weihenstephan, Department of Molecular Life Sciences, Reproductive Biotechnology	Germany	[56]
Hens specifically expressing therapeutic proteins in the oviduct	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	[13]
Production of transgenic chickens expressing a tetracycline-inducible eGFP gene	Department of Physiology, Catholic University of Daegu School of Medicine, Daegu	South Korea	[57]
Short-hairpin RNA against Influenza expressing chickens	Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge	United Kingdom	[14]
Transgenic chickens expressing human extracellular superoxide dismutase	Laboratory of Dermatology-immunology, The Catholic University of Korea	South Korea	[58]
Immunoglobulin heavy-chain (JH) KO chickens	Crystal Bioscience/Ligand Pharmaceuticals Inc	United States	[47]
	Technical University Munich, School of Life Sciences Weihenstephan, Department of Molecular Life Sciences, Reproductive Biotechnology	Germany	
Transgenic chickens expressing the human urokinase type-plasminogen activator	Department of Animal Biotechnology, Bio-Organ Research Center, Konkuk University	South Korea	[59]
CSF1R-receptor reporter chickens	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	[60]
Membrane-localized eGFP-expressing chickens	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	[61]
Immunoglobulin light-chain (IgL) KO chickens	Crystal Bioscience/Ligand Pharmaceuticals Inc	United States	[62]
	Technical University Munich, School of Life Sciences Weihenstephan, Department of Molecular Life Sciences, Reproductive Biotechnology	Germany	
Cre-recombinase-expressing chickens	Crystal Bioscience/Ligand Pharmaceuticals Inc	United States	[63]
Ovalbumin and ovomucoid KO chickens	Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology and Animal Breeding and Reproduction Research Division	Japan	[31]
Aromatase-overexpressing chickens	Department of Anatomy and Developmental Biology, Monash University, Clayton	Australia	[26]
mCherry-expressing chickens	Technical University Munich, School of Life Sciences Weihenstephan, Department of Molecular Life Sciences, Reproductive Biotechnology	Germany	[56]
	BIOPHARM, Research Institute of Biopharmacy and Veterinary Drugs and Institute of Molecular Genetics of the Czech Academy of Sciences	Czech Republic	
Lox-stop-lox chicken. Inducible expression of eGFP by Cre-mediated recombination	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	[64]
Chameleon chicken. Ubiquitous expression of nuclear-blueFP, replaced after Cre-mediated recombination by expression of eYFP or tdTomato or mCerulean	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	[64,65]

(Continued)

TABLE 21.1 (Continued)

Transgenic chicken	Affiliation of the research group	Country	References
Flamingo chicken. Ubiquitous cytoplasmic expression of tdTomato	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	[64,65]
Runt-related transcription factor 1 (<i>RUNX1</i>)-eGFP transgenic reporter chicken	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	[64]
<i>DDX4</i> KO chickens	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	[66]
3D8 single-chain variable fragment (scFv)-expressing chickens	Animal Biotechnology Division, National Institute of Animal Science and Department of Avian Disease Laboratory, College of Veterinary Medicine	South Korea	[67]
Notch reporter chicken	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	[68]
Chickens with humanized immunoglobulin genes	Crystal Bioscience/ Ligand Pharmaceuticals Inc	United States	[69]
Human interferon β -producing chickens	Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology, Osaka	Japan	[70]
Switch gene 2 knockout chickens	Seoul National University, Pyeongchang-gun, Gangwon-do	South Korea	[71]
Myostatin knockout chickens	Seoul National University, Pyeongchang-gun	South Korea	[72]
ALV-J-resistant CB line chickens	Institute of Molecular Genetics of the Czech Academy of Sciences	Czech Republic	[73]
ALV-J-resistant commercial chicken line	Technical University Munich, School of Life Sciences Weihenstephan, Department of Molecular Life Sciences, Reproductive Biotechnology	Germany	[74]
Cas9-expressing chickens	Technical University Munich, School of Life Sciences Weihenstephan, Department of Molecular Life Sciences, Reproductive Biotechnology	Germany	[75,76]
iCaspase9-inducible sterile surrogate chickens	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	[77]
Chickens with a knockout constant region T-cell receptor γ	Technical University Munich, School of Life Sciences Weihenstephan, Department of Molecular Life Sciences, Reproductive Biotechnology	Germany	Unpublished
Chickens with a knockout constant region T-cell receptor β	Technical University Munich, School of Life Sciences Weihenstephan, Department of Molecular Life Sciences, Reproductive Biotechnology	Germany	Unpublished
Chicken with a knockout of the androgen receptor	Technical University Munich, School of Life Sciences Weihenstephan, Department of Molecular Life Sciences, Reproductive Biotechnology	Germany	Unpublished
Split GFP chickens: ubiquitous expression of large subunit of eGFP (GFP1–10) that fluoresces when the small subunit (GP11) is supplied, for example, delivered by an infecting virus	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	Unpublished
Runt-related transcription factor 1 (<i>RUNX1</i>)-Cre-recombinase chicken	The Roslin Institute and Royal Dick School of Veterinary Studies, University of Edinburgh	United Kingdom	Unpublished

except B cells have been observed. Homozygous edited animals develop normally and reach sexual maturity under clean housing conditions. Surprisingly, B cells without surface immunoglobulin have been able to colonize the bursa of Fabricius and proliferated within B-cell follicles. However, after hatching, the surface Ig-negative B cells are neither able to emigrate from the bursa of Fabricius nor to cross the basal membrane of bursal B-cell follicles. Four weeks after hatch, nearly all B cells

have undergone apoptosis in the bursa of homozygote JH animals. JH-KO^{-/-} chickens have been so far used for in-depth analysis of the chicken immune system as well as to have a closer look at the role of B cells and antibodies in the context of various infectious diseases in poultry. In a first infection experiment, JH-KO^{-/-} chickens have been challenged with a deadly Marek's disease virus (MDV). To our surprise, no obvious differences were observed between wild-type chickens and those without

B cells, demonstrating that peripheral B cells are dispensable for MDV pathogenesis [78].

Besides the deletion of the J segment of the immunoglobulin heavy chain, Schusser et al. (2016) [62] also depleted the V, J, and C segment of the immunoglobulin light-chain and generated chickens without productive immunoglobulin light-chain rearrangement [62]. In contrast to JH-KO^{-/-} chickens, the IgL^{-/-} chickens showed a small population of peripheral B cells, separation of B-cell follicles in the bursa of Fabricius as well as antibodies. Immunization with KLH even resulted in antigen-specific IgM and IgY antibodies. More detailed analysis showed a deletion of the CH1 segment in the constant region of the immunoglobulin heavy chain allowing the B cells to produce heavy-chain-only antibodies. Even though these results have been unexpected, they clearly demonstrated that the immunoglobulin heavy chain alone can support B-cell development in chickens.

Besides the deletion of B cells, more recently $\gamma\delta$ T-cell knockout chickens have been generated by deletion of the constant region of the T-cell receptor γ chain. Targeting was performed by a combination of CRISPR/Cas9 and homologous directed repair. Resulting TCR $\gamma^{-/-}$ chickens show a complete lack of $\gamma\delta$ T cells (unpublished data). Although chickens belong to the so-called $\gamma\delta$ T-cell high species, the lack of this T-cell population did not lead to any negative phenotype. TCR $\gamma^{-/-}$ birds were completely healthy under clean housing conditions and did not show differences in lymphocyte populations, antibody titers, and general development and reproduction compared to wild-type birds.

Balic et al. [60] generated the so-called MacReporter chicken lines by expression of green (MacGreen) or red fluorescent (MacRed) reporter genes under the control of the chicken colony-stimulating factor receptor 1 promoter and the FIRE enhancer [60]. Expression of the reporter genes was proven to be restricted to the macrophage lineage and the usefulness of these chicken lines for developmental biology as well as studies regarding host–pathogen interaction was shown by multiple publications [79–81].

21.2.2 Disease-resistant chickens

Poultry is threatened by a plethora of dangerous pathogens, including microorganisms with zoonotic potential such as influenza A viruses. Infections are causing huge economic losses every year and also a serious problem concerning animal health and welfare. Currently, biosecurity measures as well as vaccination are the only ways to protect poultry flocks worldwide. With the availability of technologies to modify the avian genome, first attempts are made to engineer chickens to become less susceptible and/or resistant to certain pathogens.

By overexpression of a short-hairpin RNA functioning as a decoy inhibiting influenza A virus polymerase, Lyall et al. [14] produced for the first time birds that did not transmit the virus to both transgenic and nontransgenic birds [14]. This was the first step toward the generation of disease-resistant birds. Another promising approach is the manipulation of ANP32A, a protein that was shown to be essential for the activity of the influenza A virus polymerase. Encouraging in vitro data showed that a knockout of ANP32A in PGCs differentiated to fibroblasts abolished influenza A virus polymerase activity of human and avian origin. Whether chickens with genetically engineered ANP32A are resistant or less susceptible to influenza A virus infection has still to be determined [82–84].

While resistance toward some ALV subgroups was accomplished by breeding programs, there is still no efficient vaccination as well as genetic selection against ALV subgroup J (ALV-J). Nevertheless, a genetic difference between naturally resistant versus susceptible bird species was observed. A nonconserved tryptophan at amino acid position 38 of the chicken sodium/hydrogen exchanger 1 (NHE1) protein seems to be the origin of a resistant phenotype against ALV-J [85]. In vitro deletion of W38 led to complete resistance of chicken cells against ALV-J [86,87]. This was the first report of a single receptor and its modification being responsible for disease resistance in chickens. Two chicken lines have been generated with a deletion of W38 of the chNHE1 via precise genome editing to obtain ALV-J-resistant chickens. Subsequent challenge experiments of fibroblasts derived from NHE1 Δ 38^{-/-} embryos as well as in vivo challenge experiments showed a complete resistance against ALV-J in edited chicken lines [73,74]. Extensive whole-genome sequencing of edited PGCs, founder animals, and edited animals did not show any off-target effects caused by the CRISPR/Cas9 treatment (unpublished data). Genetically, engineered chicken lines resistant to ALV-J develop normally compared to wild-type siblings and show no side effect of the genetic manipulation as shown by two independent studies [73,74].

These are the first described examples of the use of genome manipulation to increase animal health and animal welfare in poultry production. Challenges for the future are the identification and characterization of additional possible targets for gene editing in poultry to generate disease-resistant lines.

21.2.3 Genetically engineered chickens for basic research and agriculture

Besides the development of chicken lines for immunological research and the generation of disease-resistant chicken lines, several bird lines useful for basic research,

agriculture, as well as bioreactors for recombinant protein production have been generated in the past couple of years.

The ability to quickly and easily purify large amounts of protein out of the egg makes the genetically engineered chicken a perfect bioreactor for the pharmaceutical industry. Lillico et al. [13] successfully generated chickens with an oviduct-specific expression of a humanized ScFV-FC miniantibody (miRNA24) for melanoma treatment and human IFN- β -1a at the same time [13]. Expression was shown to be stable over a laying period of more than 150 eggs. Similar results were obtained by precise targeting of the chicken ovalbumin locus and subsequent knockin of the human interferon-beta gene [31,70].

To increase feed efficiency and muscle growth, Kim et al. used for the first time D10-Cas9 nickase to knockout chicken myostatin, resulting in faster and increased muscle growth [72].

More recently, Bartsch et al. generated chickens with ubiquitous expression of Cas9 nuclease [75]. The resulting birds are healthy and show functional Cas9 nuclease in all examined tissues. The Cas9 chicken line is a useful tool for basic research. It was shown that, with plasmid DNA, hard to transfect primary cells derived from these chicken lines can be easily modified by transfection with small synthetic gRNAs. Also, gRNAs can be delivered to Cas9-expressing embryos by in vivo electroporation or infection with viral vectors allowing tissue-specific knockouts without the need for generating a new chicken line. The availability of this chicken line will help to further promote the chicken as a model for various research areas.

An important application of all described technologies and especially PGC culture techniques is the cryopreservation of rare chicken lines and the conservation of endangered bird species. However, it is challenging to obtain exclusive colonization of the gonads with the transplanted PGCs. Genetic manipulation of genes needed for germ-cell development and/or maintenance offers the chance to generate “universal” recipients for germ-cell transplantation of different species. A gene essential for mammalian germ-cell development and also expressed by chicken PGCs is the chicken vasa homolog DDX-4 [88]. The knockout of DDX-4, which is essential for mammalian germ cells, resulted in a reduced number of male and female germ cells but failed to produce sterile male chickens only resulting in sterile female birds [66,89]. Post hatch germ cells were completely absent in DDX-4 knockout female chickens leading to failure in laying eggs. The transplantation of cryopreserved PGCs from rare chicken breeds allowed the restoration of egg production in homozygous DDX-4 female chickens. Egg production started at 22 weeks of age and continued till 80 weeks of age. Insemination with semen from the same chicken line the PGCs were derived from allowed the complete restoration of rare chicken lines using DDX-4 knockout chickens as surrogate hens [90].

Overall, an increasing number of genetically engineered chicken lines have become available as models for basic research as well as for applied science and industrial usage.

21.3 Genetically modified quails

The Japanese quail (*Coturnix coturnix japonica*) is becoming an increasingly attractive research alternative to the chicken. Quail and chickens are close relatives (Order: Galliformes; Family: Phasianidae), sharing approximately 95% homology at the gene sequence level, and of critical importance to avian immunology, quails are susceptible to most chicken diseases [91]. However, a clear advantage/attraction of quail over chicken when it comes to genetic engineering applications is that they reach sexual maturity in 6 weeks which is significantly shorter than 26 weeks for chickens. Their smaller size also makes them more compatible with the frequently limited space available for animal facilities in research institutions.

Currently, there is no reliable method described to culture quail PGCs for genetic engineering applications. Transgenesis in quail has relied on the infection of blastoderm stage embryos (or circulating PGCs; [92]) with viral vectors carrying fluorescent marker genes under ubiquitous or tissue-specific promoters, leading in recent years to the generation of several quail lines [92–98]. In 2001 Mizuarai et al. were the first to successfully produce germline transgenic quails using a VSV-pseudotyped retroviral vector. In this study, a replication-defective retroviral vector based on Moloney murine leukemia virus-pseudotyped vesicular stomatitis virus G protein was injected into the subgerminal cavity at the blastodermal stage of embryogenesis. Using this method, an average survival rate to hatch of 28% was observed across three studies. Viral vector sequence was detected in all the quails which hatched and germline transmission of greater than 80% was reported in the birds which were mated [6]. One drawback of this work was transgene silencing, while expression of the neomycin resistance gene was detected, no GFP expression was observed.

The use of lentivirus was also a breakthrough in the production of transgenic quail [99]. Shin et al. [99] examined the use of lentivirus to produce transgenic quail by the injection of ex vivo lentivirus transduced gonadal PGCs (gPGCs) into a recipient embryo as well as the direct injection of lentivirus into the subgerminal cavity of blastodermal stage embryos. In this study, they reported similar transgenesis rates using both methods (1.6%–1.9%) but suggested advantages with the transplantation of ex vivo transduced gPGCs such as color selection and a greater potential to produce an efficient system compared to the mosaic integration observed in the early undifferentiated cells of blastodermal embryos. Although Shi et al. described the advantages of this method, its uptake in the literature is absent most likely

due to difficulties associated with its high technical requirements. The requirement to isolate gPGCs from stage 28 embryos, culture the gPGCs to allow for the lentivirus transduction, and inject the gPGCs into the dorsal aorta of a stage 13 recipient embryos is not trivial.

In 2012 a method that involved the microinjection of lentivirus into the bloodstream at stages HH 13–15 of embryogenesis was described [92]. In this study, they compared injection into the bloodstream at stages 13–15 to injection into the subgerminal cavity of blastoderm embryos and found a much higher hatch rate of transgenesis in the G1 progeny, 13% compared to 1.7%. Although the results using direct injection into the bloodstream look very promising, recent studies using transgenic quail have used subgerminal cavity injections into blastoderm stage embryos. This is most likely due to the higher skill requirement to inject into the dorsal aorta of an early-stage embryo compared to the subgerminal cavity.

Even though large inserts (> 10 kb) can theoretically be packaged using lentivirus, a significant reduction in viral titer is observed with inserts >4–5 kb, likely representing the upper limit of construct size that can be used to generate transgenic birds [100]. Viruses are sufficient for a number of genetic engineering applications in quail, but the size limitation of inserted constructs may be a significant restriction for their use to generate more complex transgenes to study avian immunology. To overcome this limitation, Serralbo et al. [101] used the Tol2 transposon system and direct in vivo transfection of PGCs to develop three novel quail transgenic lines carrying fluorescent proteins under ubiquitous and tissue-specific promoters [101]. This technique of liposome-mediated PGC transfection, with its simplicity and flexibility, more easily allows the design of complex constructs and the use of large tissue-specific promoters that would be very challenging to accommodate in lentiviruses.

An important advance in quail was the recent use of adenoviral vectors to perform the first CRISPR/Cas9-mediated targeted gene knockout by direct infection of blastoderm cells [102].

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Genetic stocks for immunological research

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A.1 Introduction

Avian genetic resources in the form of living bird stocks—predominately chickens, which are valuable for use in immunology and avian health research—are listed in [Tables A1.1–A1.6](#). Brief explanations for the categories of lines listed in the tables are provided in the following sections. The main subdivisions are based on breeding format or history (inbred, congenic, randombred, and selected) and the key genetic characteristics of the stocks, such as the major histocompatibility complex (MHC) haplotype. Expanded explanations for the categories and further details can be found in Delany and Pisenti [1] and Pisenti et al. [2] and at <http://grcp.ucdavis.edu/publications/doc20/full.pdf>. These sources also provide additional references, including historical and informative reviews. Researcher/curator names and institutions (i.e., principal investigators/researchers and academic or government institutions) are provided in the tables. The curators suggested the entries for the lines given. They should be contacted for exact details about specific lines and their availability.

A.2 Major histocompatibility complex lines

A large number of poultry stocks have been selected and studied regarding specific MHC haplotypes. They are useful for immunological research on the molecular and cellular basis for genetic resistance and susceptibility to pathogens ([Table A1.1](#)). The lines were originally developed based on their haplotype. Although their initial development and selection were based on the use of allo-antisera reactions, many MHC haplotypes can be ascertained using one microsatellite locus, LEI 0258, [51]

providing an important verification opportunity. These stocks come in the form of inbred or congenic inbred lines as well as selected lines, which are described next.

A.2.1 Inbred lines

An inbred line is a genetic stock of highly related individuals that share their genetic sequence. A common breeding scheme to create highly inbred lines is full-sib mating for a period of 10 or more generations. The calculated inbreeding coefficient (F) expresses the relatedness of the individuals. The true level of inbreeding can be lower than that calculated if one is selecting the “best” individuals for breeding, because heterozygous loci may persist. Two generations of full-sib (brother $\times$ sister) mating results in an F value of 0.375, which is considered the lower boundary for calling a stock inbred. When the F value is at this level, the stock can be referred to as “partially inbred,” whereas stocks with F values 0.85–0.99 are referred to as “highly inbred” ([Table A1.2](#)).

A.2.2 Congenic lines

Congenic lines differ from one another at a small region of the genome, the “selected” gene or complex plus closely linked regions which are not disrupted by recombination because of their tight physical linkage. Numerous chicken congenic lines exist that differ for their MHC haplotype while sharing a genetic background. Pairs of MHC-congenic lines are created by crossing an inbred line with an MHC of interest (selected MHC) into a highly inbred “parent” line having a different MHC. The resulting MHC heterozygous progeny are backcrossed to the inbred parent line with continued selection for the heterozygous MHC combination, typically for a

TABLE A1.1 MHC-defined lines.

Location (curator)	Stock name	Origin/history	Description/notes	References
INRA-France (P. Quéré)	LD1	BL: originated from Edinburgh	evBL1 (no production of p27)	[3–5]
	B13 Histo	White Leghorn (WL) GB1 (Athens): highly inbred	MHC: B13; highly sensitive to MD	[6,7]
	B21 Histo	WL Cornell N strain: highly inbred	MHC: B21; resistant to MD	[6,7]
	B19 Histo	WL: originated from Birmingham (to France, 1975); highly inbred	MHC: B19 sublines selected for resistance/sensitivity to Rous sarcoma subline env0	[8–10]

TABLE A1.2 Inbred lines.

Location (curator)	Stock name	Origin/history	Description/Notes	References
Aarhus University, Department of Animal Science (ANIS) (T.S. Dalgaard)	ANIS B12-2	White Leghorn (WL): intercrossed for line maintenance	MHC: B12	[11]
	ANIS B131-131	Scandinavian WC	MHC: B21-like	[12]
	ANIS B13-133	WL: GB1	MHC: B13	[12]
	ANIS B14-34	WL: Hy-Line origin	MHC: H.B14A	[13]
	ANIS B15-22	WL: Cornell K Line origin	MHC: B15	[14]
	ANIS B19-111 Scandinavian	Scandinavian WL	MHC: B19	[15]
	ANIS B19-131 Scandinavian	Scandinavian WC	MHC: B19	[15]
	ANIS B-19-21	American WL	MHC: H-B19	[11]
	ANIS B21-21	WL: Hy-Line origin	MHC: H-B21	[11]
	ANIS B2-32	Scandinavian WL	MHC: B2	[16]
	ANIS BW1-111	Red Jungle Fowl (RJF): Copenhagen Zoo	MHC: B21-like	[12]
	ANIS BW3-13	RJF: Copenhagen Zoo	MHC: BW3	[17]
	ANIS BW4-14	RJF: Copenhagen Zoo	MHC: BW4	[17]
	ANIS BR4	WL: Hy-Line origin	MHC, formerly named R4-1	[16,18,19]
	ANIS BR5	WL: Hy-Line origin	MHC, formerly named R5-1	[18,19]
National Avian Research Facility (NARF) (Lindsay Henderson)	IAH 6 ₁	WL: ADOL 1972	MHC: B2; susceptible to ALV -A, -B, -C, -D; resistant to ALV tumor development and Marek's disease (MD); expresses chB1 on B cells. Histocompatible with IAH 7 ₂ , inbreeding coeff. (<i>F</i>) = 0.99	[20,21]
	IAH 7 ₂	WL: ADOL 1972	MHC: B2; susceptible to ALV -A, -B, -D, -E; resistant to ALV tumor development; highly susceptible to MD; expresses chB6b on B cells; histocompatible with IAH 6 ₁ ; <i>F</i> = 0.99	[20,21]
	IAH N	WL: ADOL; originated Cornell 1982	MHC: B21; highly resistant to MD	[21]
	IAH P2a	WL Lelystad; originated Cornell 1996	MHC: B19; highly susceptible to MD	[21]
	IAH 0	WL: imported from ADOL 1985	MHC: B21; free of endogenous ALV genes (ev loci) by DNA hybridization; susceptible to infection by ALV subgroups A, B, C, and D	
	IAH C	WL: imported from Cambridge; originated Reaseheath Poultry Breeding Station (RPBS), 1969	MHC: B4 and B12; susceptible to ALV subgroups B, C, and D; resistant to MDV; <i>F</i> = 0.99	

(Continued)

TABLE A1.2 (Continued).

Location (curator)	Stock name	Origin/history	Description/Notes	References
	IAH 151	WL: imported from ADOL 1962	MHC: B15; susceptible to ALV -A and -C; segregating for B, D, and E; moderately susceptible to MD	[21]
	IAH W	WL: imported from Wellcome Research Laboratories 1962	MHC: originally 4 congenic lines—B14A, B14B, B14C, B14D; represents each combination of two alleles at both IgM and IgY constant regions	
Iowa State University (ISU) (S.J. Lamont)	ISU 8–15.1	Single Comb White Leghorn (SCL): derived from crosses of 1920s ISU inbreds before 1935	$F > 0.99$; MHC: B15.1	[22–24]
	ISU M15.2	Fayoumi: originated in Egypt; imported to USA in 1954; congenic with line ISU M5.1	$F > 0.99$; MHC: B15.1; original stock thought to be resistant to lymphoid leukosis (LL)	[22–24]
	ISU M5.1	Fayoumi: originated in Egypt; imported to USA in 1954; congenic with line ISU M15.2	$F > 0.98$; MHC: B5.1; original stock thought to be resistant to LL	[22–24]
	ISU Sp21.2	Spanish chicken: originated in Spain; imported to USA 1954	$F > 0.99$; MHC: B21.2	[22–24]
University of California, Davis (UCD) (R.A. Gallardo)	UCD 001	RJF: original stock Malaysia to Hawaii to Cornell to UC Berkeley to UCD	MHC: BQ (B21-like); $F > 0.80$; wild-type jungle fowl (phenotype: seasonal breeder, small brown eggs, appropriate body size and plumage, red ear lobes); parent line used for East Lansing reference-mapping population	[1,25,26]
	UCD 003	SCWL: full-sib crosses since 1956	MHC: B17; $F > 0.99$; A4/E7, C2, P3 blood cell alloantigens; resistant to Rous sarcoma (RS) and susceptible to MD. Serves as background line for UCD congenic lines (MHC and mutant); parent line used for East Lansing reference-mapping population	[1,25,26]
	ISU M15.2	Fayoumi: originated in Egypt; imported to USA in 1954; congenic with line ISU M5.1. Acquired from Iowa State University	$F > 0.99$; MHC: B15.1; original stock thought to be resistant to lymphoid leukosis (LL)	[22–24]
USDA Avian Disease and Oncology Laboratory (ADOL) (H.H. Cheng)	ADOL 6 ₃	SCWL: initiated 1939	$F > 0.99$; MHC: B2; endogenous ALV ev1 and ev3; resistant to ALV and MD; specific-pathogen-free (SPF)	[27–29]
	ADOL 7 ₁	SCWL: initiated 1939	$F > 0.99$; MHC: B2; endogenous ALV not defined; susceptible to MD; SPF	[27–29]
	ADOL 7 ₂	SCWL: initiated 1939	$F > 0.99$; MHC: B2; endogenous viruses ev1 and ev2; resistant to ALV-A, -B, and -E; susceptible to MD; SPF	[27–29]
	ADOL 15B ₁	SCWL: initiated 1939	$F > 0.95$; MHC: B5, B15; no endogenous ALV; susceptible to all ALV; SPF	[28]
	ADOL 15I ₅	SCWL: initiated 1939	$F > 0.99$; MHC: B15; endogenous ALV ev1, ev6 and ev10 or ev11; susceptible to ALV-A, -B, and -E, and MD; SPF	[27–30]

minimum of 6–10 generations. Thus, each successive generation gains more of the background parent genotype, while the two MHC types and tightly linked genetic regions are retained through selection.

Originally many of the selected congenic inbred lines were selected for the MHC haplotype using the classic hemagglutination assay (serological) for detecting B-G (MHC class IV) alloantigens, although now a polymerase chain reaction assay (LEI 0258) can be used for verification. [51] Following the 10th

generation backcross, the resulting heterozygous birds are crossed inter se and the resulting alternate homozygous birds for each MHC type are used as the founding population for a pair of MHC-congenic lines which share the same parent background (of the original inbred parent line) but differ in MHC type, with one line having the donor or selected MHC; the other, the original parent MHC. Each line is perpetuated by within-line breeding and should be verified for MHC haplotype on some regular basis (Table A1.3).

TABLE A1.3 Congenic lines.

Location (curator)	Stock name	Origin/history	Description/notes	References
USDA Avian Disease and Oncology Laboratory (ADOL) (H.H. Cheng)	ADOL 100B	SCWL: initiated 1962; congenic with ADOL 7 ₂	MHC: B2; endogenous viruses ev1 and ev2; resistant to ALV-A, -B, and -E; specific-pathogen-free (SPF)	[29]
	ADOL 15.6-2	SCWL: congenic with ADOL 15I ₅ ; initiated 1979	MHC: B2 from ADOL 6-1; SPF	[18,27]
	ADOL 15.7-2	SCWL: congenic with ADOL 15I ₅ ; initiated 1979	MHC: B2 from ADOL 7-2; SPF	[18,27]
	ADOL 15.15I-5	SCWL: congenic with ADOL 15I ₅ ; initiated 1979	MHC: B5 from ADOL 15I ₄ ; SPF	[18,27]
	ADOL 15.C-12	SCWL: congenic with ADOL 15I ₅ ; initiated 1979	MHC: B12 from Reaseheath line C; SPF	[18,27]
	ADOL 15.P-13	SCWL: congenic with ADOL 15I ₅ ; initiated 1979	MHC: B13 from Cornell JM-P; SPF	[18,27]
	ADOL 15.N-21	SCWL: congenic with ADOL 15I ₅ ; initiated 1979	MHC: B21 from Cornell JM-N; SPF	[18,27]
	ADOL 15.P-19	SCWL: congenic with ADOL 15I ₅ ; initiated 1979	MHC: B19 from Cornell JM-P; SPF	[18,27]
Iowa State University (ISU) (S.J. Lamont)	ISU Ghs-13 (also known as G-B1)	SCWL: congenic with ISU Ghs-6	$F > 0.99$; MHC: B13	[22–24]
	ISU Ghs-6 (also known as G-B2)	SCWL: congenic with ISU Ghs-13	$F > 0.99$; MHC: B6	[22–24]
University of California, Davis (UCD) (R.A. Gallardo)	UCD 253	SCWL: congenic with UCD 003, Kimber-derived B-system	MHC: B18; resistant to RS and MD	[25,31]
	UCD 254	SCWL: congenic with UCD 003; Hy-Line B-system origin	MHC: B15; susceptible to RS and MD	[25,31]
	UCD 312	SCWL: congenic with UCD 003; MHC from New Hampshire breed	MHC: B24; susceptible to RS and MD	[25,31]
	UCD 330	SCWL: congenic with UCD 003; MHC from Australorp inbred line, Hy-Line-B-system origin	MHC: B21; resistant to RS and MD	[25,31]
	UCD 331	SCWL: congenic with UCD 003; MHC from dwarf SCWL	MHC: B2; resistant to RS and MD	[25,31]
	UCD 335	SCWL: congenic with UCD 003; MHC from commercial (Hy-Line origin) Richardson Mt. Hope SCWL	MHC: B19; susceptible to RS and MD	[25,31]
	UCD 336	SCWL: congenic with UCD 003; MHC from RJF	MHC: BQ (similar to B21); resistant to RS and MD	[25,31]
	UCD 342	SCWL: congenic with UCD 003; MHC from cross of Ceylon Jungle Fowl and RJF	MHC: BC, susceptible to RS; sex ratio problem, excess males at hatch (2M:1F)	[25,31]
	ISU Ghs-6 (also known as G-B2)	SCWL: congenic with ISU Ghs-13. Acquired from Iowa State University	$F > 0.99$; MHC: B6	[22–24]
INRA-France (P. Quéré)	Congenic B4	WL: originated from Prague (34)	MHC: B4	[32]
	Congenic B13	WL: congenic B12 (Prague) × B13 Histo; inbred	MHC: partly B13; sensitivity to MD: intermediate	
	Congenic B21	WL: congenic B12 (Prague) × B21 Histo; inbred	MHC: partly B21. Sensitivity to MD: intermediate	

A.2.3 Randombred lines

Randombred stocks are unselected, closed populations established and maintained to provide control stock for comparison to related “selected” lines. Being an unselected line means that the dams and sires mated to create the next generation are not chosen for any specific criteria; that is, intentional pairing of breeders is avoided. Line maintenance typically requires relatively large numbers (150–200 birds) to minimize inbreeding (Table A1.4).

A.2.4 Selected lines

Selected lines can be defined by the breeding schema wherein the selection of breeders (mating groups) for use in reproduction is based on specific criteria, often for quantitative traits governed by multiple genes.

Breeders are selected for specific traits (phenotypes)—for example, resistance or susceptibility to disease, body size, egg traits, and antibody response. Many of them have a “line-bred” breeding scheme (vs “outbred” or crossing with a different line) in that mating is between animals within a population exhibiting extreme high or low values for a desired trait (e.g., small versus large body size; see Table A1.5).

A.3 General lines

Lines in this category do not fit one specific genetic definition but are included because they have been found useful for immunological research. Often, they are closed breeding stocks—for example, line-bred with no intentional crossing between other unrelated lines (Table A1.6).

TABLE A1.4 Randombred lines.

Location (curator)	Stock name	Origin/history	Description/notes	References
University of Arkansas Autoimmune disease lines/models (G. Erf and S. Orłowski)	Arkansas Smyth Line B101	SL: acquired from Smyth, University of Massachusetts, (UMass) 1996; vitiligo-susceptible; derived from Massachusetts Brown line	MHC-B101; vitiligo-expressing line of Smyth autoimmune vitiligo model; 70%–95% incidence of autoimmune vitiligo	[33]
	Arkansas Brown Line B101	BL: acquired from Smyth, UMass, 1996; subline of ancestral parent stock for SL	MHC-matched (B101), control/parental line for Arkansas Smyth line; autoimmune vitiligo-susceptible; 1%–2% incidence of vitiligo	[33]
	Arkansas Light-Brown Leghorn Line B101	LBL: acquired from Smyth, UMass, 1996	MHC-matched (B101) pigmentation control for Arkansas SL and BL; not vitiligo-susceptible; 0% incidence	[33]
	Obese strain	OS: acquired from Kerje, Uppsala University, Sweden; 2015	OS MHC B13; develops Hashimoto's Thyroiditis	[33]
	UCD 200	SCWL; acquired from Kerje, Uppsala University, Sweden, 2015	UCD 200; MHC B2; develops Scleroderma/Systemic Sclerosis	[33]
	UCD 206	SCWL; acquired from Sgonc, Innsbruck, Austria, 2015	UCD 206; MHC B15; develops scleroderma/systemic sclerosis	[33]
University of Arkansas (S. Orłowski)	AR Progressor	WL; AR-PG 13 line; derived from 1965 cross of Giant Jungle fowl and WL	MHC B13; Rous sarcoma progressorMHC subline maintained since 1994	[34]
	AR Regressor 221	WL; AR-RG 221 line; derived from 1965 cross of Giant Jungle fowl and WL	MHC 221; Rous sarcoma regressorMHC subline maintained since 1994	[34]
	AR Regressor 13	WL; AR-RG 13 line; derived from 1965 cross of Giant Jungle fowl and WL	MHC 13; Rous sarcoma regressorMHC subline maintained since 1994	[34]

TABLE A1.5 Selected lines.

Location (curator)	Stock name	Origin/history	Description/notes	References
Agriculture and Agri-Food Canada (F. Silversides)	UBC-RES	Japanese Quail: from NCSU 1988	Resistant cholesterol-induced atherosclerosis	[35–37]
	UBC-SUS	Japanese Quail: from NCSU 1988	Selected for susceptibility to cholesterol-induced atherosclerosis	[35–37]
Aarhus University, Department of Animal Science (ANIS) (T.S. Dalgaard)	ANIS MBLHigh-10	Danish WC Stock	20 + generations of selection for high MBL plasma levels	[38]
	ANIS MBLLow-10	Danish WC Stock	20 + generations of selection for low MBL plasma levels	[38]
Institute of Farm Animal Genetics, Friedrich-Loeffler-Institute (S. Weigend)	RSV RES Line Gr	WL: derived from crossbred parents from long-established American commercial breeding program, 1965	Resistant to RSV-A and -B; segregating for B2, B13, B14, B19, and B21	
	RSV RES Line Mr	WL: derived from commercial pure line WL (Cashmean) 1967	Resistant to RSV-A and -B; homozygous B2	
	RSV RES Line Rr	WL: derived from Cornell K Line 1965	Resistant to RSV-A and -B; homozygous B15	
University of California, Davis (UCD) (R.A. Gallardo)	UCD-Cornell Trisomic	SCWL: Acquired from Cornell 1995	Chromosomal variant; line-bred; trisomy or tetrasomy of the MHC/NOR chromosomes, GGA16; such aneuploids can hatch and reach maturity, but tetrasomics often small and with poor production characteristics	[39–41]
University of Georgia (S.E. Aggrey)	Athens AR	SCWL: derived from Athens AR2.5–AR3.0 cross 1997	Selected for resistance to aflatoxin	[42]
	Athens AR3.0	Japanese Quail	Selected for resistance to aflatoxin	[43]
USDA Avian Disease and Oncology Laboratory (ADOL) (H.H. Cheng)	ADOL Line 0	SCWL: initiated 1979	MHC: B21; closed line selected for absence of endogenous ALV-E proviral genes and resistance to endogenous but susceptibility to exogenous ALV; specific-pathogen-free (SPF)	[28]
	ADOL 0.44-VB*S1	SCWL: initiated 2000	MHC: B21; closed line selected for the absence of endogenous ALV-E proviral genes and for susceptibility to endogenous and all exogenous ALV; SPF	
	ADOL 0. ALV6	SCWL: initiated 1989; transgenic	Transgene is ALV-A envelope gene; transgenic progeny resistant to ALV-A and ALV-E	[44]
Virginia Tech (P. Siegel)	Virginia Antibody Line-High	Derived from Cornell randombred SCWL, starting 1977	Selected over 47 generations for high antibody response to sheep red blood cells	[45]
	Virginia Antibody Line-Low	Derived from Cornell randombred SCWL, starting 1977	Selected over 47 generations for low antibody response to sheep red blood cells	[46]

TABLE A1.6 General lines.

Location (curator)	Stock name	Origin/history	Description/notes	References
University of Georgia (S.E. Aggrey)	ACRB	Ranombred control population since 1956	Immune parameters	[47]
INRA-France (P. Quéré)	PA12	WL (ranombred)		[48]
Iowa State University (ISU) (S. Lamont)	ISU Broiler	Closed population. line-bred since early 1990s, origin: commercial broiler male line	Male parent line of advanced intercross	[49,50]
	ISU Advanced Intercross, BrXM15.2	Advanced intercross formed from mating one broiler male with multiple inbred dams of Fayoumi M15.2 line; in F32 generation in 2021	Useful for fine-mapping of quantitative trait loci	[49,50]

A.4 Genetically modified lines

Recently, genetically modified chicken lines became available for avian immunology research. Currently available lines are summarized and described in detail in Chapter 21.

Notes on the tables:

Please refer to the following notes, which apply to Tables A1.1–A1.6:

- Curators and institutions listed in the first column are current as of March 2021. See <http://animalscience.ucdavis.edu/AvianResources> for contact information and for a larger listing of avian research resources, curators, and contact information.
- The stock name, as listed in the second column is as designated by the curators of the lines.
- In the third column, additional information on the stocks is provided by the curators.
- The fourth column lists review and research articles provided by curators for background on the stocks indicated; this is not an all-inclusive listing. These publications are listed in the References section.

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Abbreviations

2D-PAGE	two-dimensional polyacrylamide gel electrophoresis	BCMA	B-cell maturation antigen
3D8 scFv	3D8 single-chain variable fragment	BCR	B-cell receptor
4-TBP	4-tertiary-butyl-phenol	Bd	bursal duct
A549	adenocarcinomic human alveolar basal epithelial cells	BDCA-2	blood dendritic cell antigen 2
Ab	antibody	BF	bursa of Fabricius
AcLDL	acetylated low-density lipoprotein	Bl	bursal lumen
ACTH	adrenocorticotrophic hormone	BL	Massachusetts Brown line of chickens
AECA	anti-endothelial cell antibodies	BL101	BL chickens with major histocompatibility complex genotype 101
AEV	avian encephalomyelitis virus	BLUP	best linear unbiased prediction
AFB1	aflatoxin B1	BM	bone marrow
Ag	antigen	BMDM	bone marrow-derived macrophage
AGP	α -1-acid glycoprotein	bp	base pair
AI	avian influenza	BPI	bactericidal permeability-increasing
AID	activation-induced cytidine deaminase	BrdU	bromodeoxyuridine
AIDS	acquired immunodeficiency syndrome	BSA	bovine serum albumin
AIV	avian influenza virus	BSDC	bursal secretory dendritic cell
AIF	allantoic fluid	BW	body weight
ALV	avian leukosis virus	C	constant
AMA-1	apical membrane protein-1	C4bC2b	C3 convertase
AmF	amniotic fluid	Ca	calcium
AMP	antimicrobial peptides	CAGE	cap analysis of gene expression
AMPV	avian metapneumovirus	CALT	conjunctiva-associated lymphoid tissue
AMV	avian myelocytomatosis virus	CAM	chorioallantoic membrane
ANA	antinuclear antibodies	CARD	caspase activation and recruitment domain
ANP32A	acidic leucine-rich nuclear phosphoprotein 32 family member A	Cas9	CRISPR associated protein 9
APC	antigen-presenting cells	CATH-2	cathelicidin 2
APEC	avian pathogenic E. coli	CBH	cutaneous basophil hypersensitivity
APP	acute-phase proteins	CCL	CC Chemokine (suffixed by a number)
APR	acute-phase response	cCL-K1	chicken Collectin Kidney 1
APRIL	A proliferation inducing ligand	cCL-L1	chicken Collectin Liver 1
Arg	arginine	CCR	CC chemokine receptor (suffixed by a number)
ARP	avian respiratory macrophages or phagocytes	CD	cluster of differentiation (suffixed by a number and sometimes Ligand: L)
ARV	avian reovirus	cDCs	conventional dendritic cells
ASC	antibody-secreting cell	cDNA	complementary DNA
Asn	asparagine	CDR	complementarity-determining region
ATP	adenosine triphosphate	CEF	chicken embryonic fibroblast
AvBD	avian beta-defensins	CFA	complete Freund's adjuvant
AVP	arginine vasopressin	CFSE	carboxyfluorescein succinimidyl ester
B101	major histocompatibility complex genotype 101	CFU	colony forming units
B19-2D8	chicken cell line	ch	chicken (suffixed by another abbreviation, e.g., chIFN)
BAC	bacterial artificial chromosome	CG	conglutinin
BAFF	B-cell activating factor of the TNF family	cGAS	cGMP-AMP synthase
BAFF-R	BAFF receptor	cGAS-STING	cGMP-AMP synthase-stimulator of interferon genes
BALT	bronchus-associated lymphoid tissue		

chBMDC	chicken bone marrow-derived DC	DA	dopamine
chIL	chicken interleukin	DAdV	duck aviadenovirus
ChIP	chromatin immunoprecipitation	DAM	delayed amelanosis
CHIR	chicken immunoglobulin-like receptor	DAMP	danger-associated molecular pattern
chMBL	chicken MBL	DC	dendritic cells
chSTAU1	chicken staufer protein 1	DCAR	dendritic cell activating receptor
chTLR	chicken TLR	DCIR	dendritic cell inhibiting receptor
CIIV	chicken infectious anemia virus	DcR3	decoy receptor 3
CL-43	collectin of 43 kDa	Dct	dopachrome tautomerase
CL-46	collectin of 46 kDa	DDX-4	DEAD-box helicase 4
CLDN1	claudin 1	DEF	duck embryonic fibroblast
CLEC	c-type lectin receptor	DEG	differentially expressed gene
CLIP	class II invariant chain associated peptide	DES	diethylstilboestrol
CL-K1	collectin Kidney 1	DEV	duck enteritis virus
cLL	chicken lung lectin	DF-1	douglas Foster-1 chicken embryonic fibroblast cell line
CL-L1	collectin liver 1	DFM	direct fed microbials
CL-P1	collectin placenta 1	DH	delayed hypersensitivity
CLR	C-type lectin receptors	DHBV	duck hepatitis B virus
CM	continuous membrane	DHV	duck hepatitis virus
cMGF	chicken myelomonocytic growth factor	Dil	fluorescent lipophilic marker
CMI	cell-mediated immunity	DISC	death-inducing signaling complex
cMIF	chicken macrophage migration inhibitory factor	DMEM	Dulbeccos's modified Eagle's medium
CMV	cytomegalovirus	dN/dS	non-synonymous substitution rate to synonymous substitution rate ratio
CNPF	canarypox virus	DNA	deoxyribonucleic acid
CNS	central nervous system	DNP	dinitrophenol
CNV	copy number polymorphism	DON	deoxynivalenol
Con A	concanavalin A	dpi	days post infection
CORT	corticosterone	DPV	duck plaque virus
COX-2	cyclooxygenase-2	DRV	duck reovirus
Cp	ceruloplasmin	ds	double-stranded
CpG-ODN	unmethylated deoxycytosine-deoxyguanosine-oligodeoxynucleotide	DTH	delayed-type hypersensitivity
CR	complement receptors	du	duck (suffixed by another abbreviation, e.g., duIFN)
CR1	chicken retroviral-like insertion element	E	embryonic day
CRD	carbohydrate recognition domain	E. coli	<i>Escherichia coli</i>
CREMP	cysteine-rich eggshell membrane protein	EAC	ellipsoid-associated cells
CRF	corticotrophin-releasing factor	EBV	epstein Barr virus
CRH	corticotropin-releasing hormone	ECM	extracellular matrix
CRISPR	clustered regularly interspaced short palindromic repeats	ECs	endothelial cells
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/ Caspase 9	ED (or EDS)	egg drop syndrome
CRISPRa	clustered regularly interspaced short palindromic repeats activation	ED	embryonic day
CRISPRi	clustered regularly interspaced short palindromic repeats interference	eGFP	enhanced green fluorescent protein
CRP	C-reactive protein	EHT	endothelial-to-haematopoietic transition
CSF	colony-stimulating factor	EID	embryonic incubation day
CSF1R	colony-stimulating factor 1 receptor	ELISA	enzyme-linked immunosorbent assay
CSS	capsule of the Schweigger-Seidel sheath	ELISPOT	enzyme-linked immunospot technique
CT	cecal tonsil	ENS	enteric nervous system
CTD	C-terminal regulatory or repressor domain	EP	epinephrine
CTL	cytotoxic T lymphocytes	ER	endoplasmic reticulum
CTLA-4	cytotoxic T lymphocyte-associated protein 4	ERAP	endoplasmic reticulum amino peptidase
Cu	copper	ERC	epithelial reticular cells
CXCL	CXC chemokine (suffixed by a number)	ES	eggshell
CXCR	CXC chemokine receptor (suffixed by a number)	ESM	eggshell membrane
D	diversity	EST	expressed sequence tag
		EV	extracellular vesicle(s)
		EW	egg white

EY	egg yolk	Hb	hemoglobin
F	filial	HBV	hepatitis B virus
F gene	fusion gene	H-chain	heavy-chain
FAANG	functional annotation of animal genomes	HCs	hematopoietic cells
FAdV	fowl aviadenovirus	HD11	chicken macrophage-like cells
FAE	follicle associated epithelium	HDP	host defense peptides
FARM	free avian respiratory macrophages	HEK293T	human embryonic kidney 293 cells with SV40 T-antigen
FB	fibrinogen	HEPA	high-efficiency particulate air (filter)
FB₁	fumonisin B ₁	HET	heterophil extracellular trap
FCN	ficolin	HEV	high endothelial venule
FcR	fc receptor	HEV	hemorrhagic enteritis virus
FcRL	fc receptor-like protein (Fc receptor lacking IgY reactivity)	HG	harderian gland
FcRY	fc receptor for IgY	HH	hamburger and hamilton stage (of embryonic development)
FDC	follicular dendritic cell	HI	hemagglutination inhibition
FFE	feather follicle epithelium	HIV	human immunodeficiency virus
FGF	fibroblast growth factor	Hp	haptoglobin
FGFR	fibroblast growth factor receptor	HPA	hypothalamic-pituitary- adrenal
FIRE	fms-intronic regulatory element	HPAIV	highly pathogenic influenza A viruses
FITC	fluorescein isothiocyanate	HPV	human papilloma virus
FMT	fecal microbial transplant	HSC	hematopoietic stem cells
FN	fibronectin	HSP	heat-shock protein
FoxP3	forkhead box P3 transcription factor	HVT	herpesvirus of turkeys
FPV (or FWPV)	fowlpox virus	Hx	hemopexin
ft3	free triiodothyronine	IAHC	intra-aortic hematopoietic cluster
GalNAc	N-acetylgalactosamine	IBD	infectious bursal disease
GALT	gut-associated lymphoid tissue	IBDV	infectious bursal disease virus
GBLUP	genomic best linear unbiased prediction	IBH	inclusion body hepatitis
GC	germinal center	IBV	infectious bronchitis virus
G-CSF	granulocyte colony-stimulating factor	ICAM	intercellular adhesion molecule
GEBV	genomic estimated breeding value	IDC	interdigitating dendritic cell
GF	growing feather	iE-DAP	γ -glutamyl diaminopimelic acid
GFP	green fluorescent protein	IEL	intraepithelial lymphocytes
gg-pIgR	chicken polymeric Ig receptor	IFE	interfollicular epithelium
GI	gastrointestinal	IFIT	interferon-induced proteins with tetratricopeptide repeat
GILZ	glucocorticoid-induced leucine zipper	IFN	interferon (suffixes by e.g., - α , - β , - γ)
GITR	glucocorticoid-induced TNF receptor	IFNAR	IFN-alpha/beta receptor
GITRL	glucocorticoid-induced TNFR ligand	Ig	immunoglobulin (may be suffixed by A, D, E, G, M, Y or light chain: L and prefixed by surface: s)
Gln	glutamine	IGF	insulin like growth factor
GM-CSF	granulocyte/macrophage colony-stimulating factor	IgSF	immunoglobulin superfamily
gp	glycoprotein	IH	immediate hypersensitivity
gPGC	gonadal primordial germ cells	Ii	invariant chain
GPI	glycophosphatidylinositol	IL	interleukin (suffixed by a number)
GS	genomic selection	ILT	infectious laryngotracheitis
GST	glutathione S-transferase	ILTV (or ILV)	infectious laryngotracheitis virus
GVH	graft-versus-host	IML	infiltrating mononuclear lymphocytes
GvHR	graft versus host response	IMP-1	immune mapped protein-1
GWAS	genome-wide association study	iNOS	inducible nitric oxide synthase
H	heavy	INRA	institut national de la recherche agronomique
H/L	heterophils to lymphocytes ratio	IRF	interferon regulatory factor (suffixed by a number)
H₂O₂	hydrogen peroxide	IRG	interferon regulated gene
H3K27ac	histone H3 lysine 27 acetylation	ISG	interferon-stimulated gene
H3K27me3	histone H3 lysine 27 trimethylation	ITAM	immunoreceptor tyrosine-based activation motif
H3K4me1	histone H3 lysine 4 monomethylation	ITIM	immunoreceptor tyrosine-based inhibition motif
H3K4me3	histone H3 lysine 4 trimethylation		
H5N1	avian influenza virus strain H5N1		
HA	hemagglutinin		

J	junctional or joining	miR24	humanized single-chain variable fragment-constant fragment mini-antibody
JNK	jun NH ₂ -terminal kinase	miRNA	microRNA
kb	kilo base pairs	MIS	multiple-head injector system
kDA	kilodalton	ML	myeloid leukemia
KIR	killer immunoglobulin-like receptor	MLN	mural lymph nodes
KLH	keyhole limpet hemocyanin	MLR	mixed lymphocyte reaction
KO	knockout	Mn	manganese
L	light	MoDCs	monocyte-derived DCs
LAG-3	lymphocyte-activation gene 3	MPPs	multipotent progenitors
LAMP1	lysosomal-associated membrane glycoprotein 1	MPS	mononuclear phagocyte system
LBL	light brown leghorn	mRNA	messenger RNA
LBL101	LBL chickens with major histocompatibility complex genotype 101	MUC	mucin
LBP	LPS (lipopolysaccharide)-binding proteins	MVB	multivesicular body
L-chain	immunoglobulin light-chain	MW	molecular weight
LC-MS/MS	liquid chromatography–tandem mass spectrometry	Mx	interferon-induced GTP-binding protein
LD	linkage disequilibrium	N	neuraminidase
LE	linkage equilibrium	Nabs	natural antibodies
LILR	leukocyte immunoglobulin-like receptor	NAD	nicotinamide adenine dinucleotide
LITAF	lipopolysaccharide-induced TNF- α factor	NaI	sodium iodine
LL	lymphoid leukemia	NALP3	NLRP3 inflammasome
LOX-1	lectin-type oxidized LDL receptor 1	NALT	Nasal-associated lymphoid tissue
LP	lipoproteins	NCBI	national Center for Biotechnology Information
LPAI (or LPAIV)	low pathogenic avian influenza	nd	non-defective
LPS	lipopolysaccharide	NDV	newcastle disease virus
LRC	leukocyte receptor complex	NE	norepinephrine
LRR	leucine-rich repeat	NF	nuclear factor
LT	lymphotoxin	NF-κB	nuclear factor kappa light chain enhancer of activated B cells
LTA	lipoteichoic acid	NGS	next generation sequencing
LTi	lymphoid tissue inducer cells	NGVEV	new type gosling viral enteritis virus
LTR	long terminal repeat	NHE1	sodium/hydrogen exchanger 1
LYZ	lysozyme	NK	natural killer
M cells	microfold cells	NKC	natural killer complex
mAb	monoclonal antibody	NLR	NOD-like receptors
MAC	membrane attack complex	NLRC4	IPAF inflammasome
MALT	mucosa associated lymphoid tissues	NLRP1	NALP1 inflammasome
MAMP	microbe-associated molecular pattern	nNOS	neuronal NOS
MAP	MBL-associated protein	NO	nitric oxide
MAPK	mitogen-activated protein kinase	NOD	nucleotide-binding oligomerisation domain
MARCO	macrophage receptor with collagenous structure	NOS	nitric oxide synthase
MAS	Marker-assisted selection	O₂	oxygen
MASP	MBL-associated serine protease	OAS	oligoadenylate synthetase
MATSA	Marek's disease tumor-associated antigens	OASL	oligoadenylate synthetase-like
MAVS	mitochondrial antiviral-signaling protein	OC	ovocleidin
Mb	megabase	OCX	ovocalyxin
MBL	mannose-binding lectin	ODN	oligodeoxynucleotide
M-CSF	macrophage colony-stimulating factor	OPG	osteoprotegerin
MD	Marek's disease	OPL	outer perivitelline membrane
MDA	maternally derived antibodies	ORF	open reading frame
MDA5	melanoma differentiation-associated factor 5	OS	obese strain
MDV	Marek's disease virus	OVT	ovotransferrin
MeOPN	O-methyl phosphoramidate	P	palindromic
MEQ	MDV <i>EcoRI</i> -Q	PALS	periarterial lymphatic sheath
MHC	major histocompatibility complex	PALT	pineal-associated lymphoid tissue
MIF	migration inhibition factor	PAM	protospacer adjacent motif
MIP	macrophage inflammatory protein	PAMP	pathogen-associated molecular pattern
		PBL	peripheral blood lymphocytes

PBMC	peripheral blood mononuclear (or monocytic) cells	SCARF1/	scavenger receptor class F, member 1/2
PCD	programmed cell death	SCARF2	
PCR	polymerase chain reaction	SCF	stem cell factor
PD-1	programmed cell death 1	SCFA	short chain fatty acids
PD-L1	programmed cell death ligand 1	SCN	suprachiasmatic nuclei
PEMS	poult enteritis and mortality syndrome	scRNA-seq	single-cell RNA-sequencing
PGC	primordial germ cell	SDF	Stromal cell derived factor
PGN	peptidoglycan	SFB	segmented filamentous bacteria
PHA	phytohemagglutinin	sgRNA	single guide RNA
PHL	Poultry Health Laboratory	slg	surface immunoglobulin (may be suffixed by A, G, M or Y)
PI	post infection	sIgA	secretory immunoglobulin A
PI3K	phosphatidylinositol 3-kinase	SL	Smyth line
pIgR	polymeric immunoglobulin receptor	SL101	SL chickens with major histocompatibility complex genotype 101
PL	perivitelline layer	SLAM	signaling lymphocyte activation molecule
PLC	peptide-loading complex	sLe^x	Sialyl Lewis ^x
PLpro	papain-like protein	SLV	Smyth line vitiligo
PLS	periellipsoidal lymphoid sheath	SNP	single nucleotide polymorphism
PLUNC	palate, lung and nasal epithelium clone (domaine)	SOCS	suppressor of cytokine signaling
PNAd	peripheral lymph node addressin	SP-A, SP-D	surfactant protein A or D
polyI:C	polyinosinic:polycytidylic acid	SPF	specified pathogen free
PP	Peyer's patches	SPI	<i>Salmonella</i> pathogenicity island
Pr	proctodeum	SR	scavenger receptors
pre-TCR	pre-T cell receptor	SRBC	sheep red blood cells
PRM	poultry red mite	SRCL	scavenger receptor with C-type lectin
PRR	pattern recognition receptor	SSc	systemic sclerosis
PSM	pre-somitic mesoderm	STAGE	sperm transfection assisted gene editing
PTCRA	pre-T- cell receptor α chain (pT α) gene	STING	stimulator of interferon genes
PTX3	pentraxin 3	STm	<i>S. enterica</i> serovar typhimurium
PWP	periellipsoidal white pulp	STR	short tandem repeat
q	quantitative	SV40	Simian virus 40
qPCR	quantitative polymerase chain reaction	T3	triiodothyronine
QTL	quantitative trait loci	T4	thyroxine
RACE	rapid amplification cDNA end	TACI	transmembrane activator, calcium modulator, cyclophilin ligand interactor
RAG	recombination-activating genes	TadV	Turkey aviadenovirus
RANTES	regulated on activation, normal T cell expressed and secreted	TALEN	transcription activator-like effector nuclease
RCAS	avian sarcoma-leukosis virus	TAP	transporter associated with antigen processing
REV	reticuloendotheliosis virus	TAPBP	TAP binding protein
RFLP	restriction fragment length polymorphism	TCR	T-cell receptor ($\alpha\beta$ -TCR or $\gamma\delta$ -TCR)
RIG-I	retinoic acid-inducible gene I	TdT	terminal deoxynucleotidyl transferase
RISC	RNA interference silencing complex	TERT	telomerase reverse transcriptase
RIR	RIG-I-like receptor	Tfh	follicular T helper cell subset
RNA	ribonucleic acid (may be prefixed by ds, m or ss)	Tg	thyroglobulin
RNA-seq	ribonucleic acid sequencing	TGF	transforming growth factor
ROS	reactive oxygen species	TGFβRII	TGF- β type II receptor
RSCA	reference strand mediated conformational analysis	Th	T helper cell (1, 2, 9 or 17)
RSS	recombination signal sequence	THP	Turkey heterophil peptide
RSV	Rous sarcoma virus	Thr	threonine
RT	reverse transcriptase	TIM1	T-cell immunoglobulin and mucin domain 1
RT-PCR	reverse transcriptase polymerase chain reaction	TIR	toll interleukin-1-like receptor
RT-qPCR	reverse transcription-quantitative polymerase chain reaction	TLR	toll-like receptor
SAA	serum amyloid A	TM	transmembrane
SAT	spontaneous autoimmune thyroiditis	TNF	tumor necrosis factor
SC	secretory component	TNFR	TNF receptor
SCARA5	scavenger receptor class A member 5	TNFRSF	TNF receptor super-family
SCARB1/	scavenger receptor class B type 1/type 3	TNFSF	TNF super-family
SCARB3			

TNP	trinitrophenol	V	variable
Tol2	oryzias latipes tyrosinase gene active transposon	VLR	variable lymphocyte receptor
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate	VMO	vitelline membrane outer layer protein
TRAF	TNFR-associated factor	VN	virus neutralizing
Treg	T regulatory cell subset	VP	viral protein
TREM	triggering receptor expressed on myeloid cells	VSV	vesicular stomatitis virus
TRIM	tripartite motif	vTR	virus-encoded RNA telomerase
TSH	thyroid stimulating hormone	vv	very virulent
TSL	trilaminar substance	WNV	West Nile Virus
TSLP	thymic stromal lymphopoietin	WT	wild-type
TSS	transcription start site	YS	yolk sac
Tyrp1	tyrosinase-related protein-1	ZEA	zearelenone
UA	University of Arkansas	Zn	zinc
UCD	University of California Davis	ZP	zona pellucida
UCD 200/206	UCD chicken line 200 and 206	ψV	pseudo-V
United States	United States (of America)		
UTR	untranslated region		

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Avian Immunology

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Avian Immunology, third edition, contains a detailed description of the avian lymphoid tissues, innate and adaptive immune systems, encompassing the enteric, respiratory, and reproductive systems. The diseases and disorders it covers include immunosuppressive diseases and immune evasion, autoimmune diseases, and tumors of the immune system. Practical aspects of vaccination are examined as well. Resources of inbred chicken lines are appended, and extensive tables in the respective chapters provide an overview of transgenic chicken lines and avian cytokines.

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