

Chicken immune response genes, post-genome

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Summary

Most mechanisms of adaptive immunity evolved during the divergence of vertebrates. The immune systems of extant vertebrates therefore represent different successful variations on the themes initiated in their earliest common ancestors. The genes involved in elaborating these mechanisms have been and continue to be subject to exceptional selective pressures in an 'arms-race' with highly adaptable pathogens, resulting in highly divergent sequences of orthologous genes, and the gain and loss of members of gene families, as different species find different solutions to the challenge of infection. As a result it has been difficult to transfer to the chicken detailed knowledge of the molecular mechanisms of the mammalian immune system, and thus to enhance the already significant contribution of chickens towards understanding the evolution of immunity.

The availability of the chicken genome sequence means we can begin to resolve outstanding questions concerning which molecular components of the immune system are shared between mammals and birds, and which represent their unique evolutionary solutions.

We have now integrated genome data with existing knowledge to make a new comparative census of members of cytokine and chemokine gene families, the Toll-like receptors and the defensins, amongst others, distinguishing the core set of molecules, likely to be common to all higher vertebrates, from those particular to these 300 million-year-old lineages. Some differences can be explained by the different architectures of the mammalian and avian immune systems. For example, chickens lack functional eosinophils, and also lack IgE, the eotaxins and the eotaxin receptors, molecules crucial for the function of eosinophils in mammals. Chickens also lack lymph nodes, and knockout mouse models have shown that the development of lymphoid organs, such as lymph nodes, is dependent on expression of the lymphotoxin and lymphotoxin receptor genes, absent from the chicken genome. Other differences (for example in the chemokine repertoires) suggest that the lineages have developed different strategies to cope with similar pathogen challenges.

Introduction

The adaptive immune system is one of the more recent products of evolution peculiar to vertebrates. Genes involved in regulation of immune responses directed towards the control of invading pathogens are subject to intense selective pressure by the countervailing evolution of escape mechanisms by pathogens. This pressure results in exceptionally high sequence divergence in this class of genes in different species (Murphy, 1993), and in accelerated expansion and contraction of membership of their gene families, by which newly duplicated genes provide the substrate for the evolution of new functions.

There are substantial structural and functional differences between the immune systems of birds and mammals, such as the architecture of the MHC (Kaufman et al., 1999), the absence of lymph nodes in birds (Higgins 1996) and different modes of somatic recombination in the generation of antibody diversity (Reynaud et al., 1985; 1987; 1989). Even mouse and man may employ different genes for equivalent functions in immunity (Long, 1999). Thus the extent to which birds and mammals have the same complement of gene products involved in the regulation of immune responses is a question of primary importance, both for understanding of the evolution of immune systems, and for the practical development of chicken immunology.

Demonstration of the crucial importance of the innate immune response is one of the major

advances in immunology in the past few years (Imler & Hoffman 2001). Rather than just being an ancestral, immune response, it is now obvious that it both provides an important initial response to pathogens that can limit or even prevent infection, and crucially determines the course of an adaptive immune response, and hence the ability to respond to future infection with the same pathogen. There are polymorphisms in genes controlling innate responses in mammals, which affect function (e.g. in the TLR4 gene between He/J and He/N mice). Further, different lines of chickens also differ in innate immune responses to pathogen challenge, and this correlates with resistance to infection (Ferro et al., 2004; Swaggerty et al., 2003; 2004; 2005). It therefore seems reasonable to suppose that this differential response is under genetic control and that similar mechanisms may in part explain the different resistance profiles of the inbred lines of chickens available at the Institute for Animal Health to all the pathogens so far tested (viral, bacterial and protozoan parasites) (Bumstead et al., 1991; Bumstead, 1998).

The identification of innate immune resistance mechanisms conserved across avian species also has strategic industrial relevance. The EU poultry industry faces numerous challenges in order to remain sustainable. These include the imminent move to more extensive rearing systems, the withdrawal of prophylactic and many therapeutic antibiotics and other drugs such as anti-coccidials, and resistance and residue problems with anti-helminthics. These challenges will all have an impact on poultry health. It is important that poultry breeders are able to select for genetic improvement in performance when birds are reared in such environments and one obvious phenotype would be a general resistance to infectious disease. Improved innate resistance would contribute to this phenotype and would be a general phenomenon. By its very nature the innate immune response does not differentiate between different pathogens, but rather recognises and responds to pathogen-associated molecular patterns (PAMPs) that are conserved across pathogen classes.

Many avian viral infections are currently controlled by vaccination, but there is evidence that the vaccination regimes may be driving the viruses to higher virulence, causing vaccine breaks due to the emergence of "very virulent" strains (Witter, 1998; Muller et al., 2003). For many other viral infections, such as avian influenza, vaccines are not in common usage. The ability to select for improved innate immune responses to viral infection should reduce the incidence of those infections, as the innate immune response recognises viral components, such as their genomic nucleic acid, in which it would be virtually impossible to select escape mutants. In recent years poultry and other birds have been a source of new infectious diseases (SARS, Avian Influenza, etc), in particular in Asia. Thus selection for improved innate resistance in poultry may also provide a new tool in our defence against infectious disease and so benefit human health.

The recent availability of the chicken genome sequence (Hillier et al., 2004) marks a major shift in our ability to understand the general biology of this important model and commercial organism, and in particular, its immune response to infection. Pre-genome, genes involved in immune function have not always been easy to identify, as they are under heavy selective pressure due to the ongoing host-pathogen "arms-race" and thus have limited amino acid identity to their mammalian orthologues. But now, we are able to use these genome resources to clone and identify genes related to innate immunity. For example, we have cloned/identified those genes encoding defensins, chemokines and their receptors, pro-inflammatory cytokines, the type I IFN's, NK cell receptors and Toll-like receptors (Smith et al., 2004; Hillier et al., 2004; Kaiser et al., 2005; Kogut et al., 2005). Some of these will now be discussed in more detail.

Cytokines and chemokines

Coordination of the immune response is mediated by secreted intercellular messenger molecules, among which are the (glyco)peptide molecules known as cytokines. Cytokines are produced by many different types of cells and exert regulatory effects on both haematopoietic cells and immune system cells involved in host defence and homeostasis. The panoply of cytokines includes the IFNs, interleukins, CSFs, TGFs, TNFs and the small peptide chemokines, which are

the products of different gene families each with many members in mammalian species.

Simply put, interleukins have functional activity involving lymphocytes and IFNs have anti-viral effects. The role of CSFs, TGFs and TNFs appear obvious, yet TGF- β has a crucial role in the regulation of inflammatory responses, and the TNFSF members have a wide spectrum of activities other than anti-tumor.

New members of the cytokine gene families continue to be discovered even after the advent of complete genome sequences of human and rodents, such as new members of the IL-1, IL-10 and IL-17 families, and nomenclature changes as the biological and structural properties of cytokines are elucidated. Thus, for example, IL-28A, IL-28B and IL-29, recently shown to constitute a new family of type I IFNs, have consequently been renamed as IFN- λ 1-3 (Kempuraj et al., 2004).

Chemokines are peptides of 67-127 amino acids of which most are secreted, except for CXCL16 and CX3CL1, which can be both membrane-bound and secreted. Their primary function is to regulate leukocyte traffic (Moser et al., 2004). They are classified according to biological role and molecular structure. Some have a role in inflammation, while others regulate homeostasis, and yet others have dual functions. Their two main structural families are distinguished by the arrangement of the first two N-terminal cysteine residues, either adjacent in the CCL chemokines, or separated by a single amino acid residue in the CXCL chemokines. The latter are further distinguished according to the presence or absence of an ELR (Glu-Leu-Arg) tripeptide motif, which determines receptor specificity. There are two minor structural types, XCL chemokines with an isolated cysteine, and CX3CL chemokines in which two cysteines are separated by three amino acid residues.

The TNFSF includes molecules with a variety of functions both in the regulation of immune responses and in the development of the architecture of the immune system and other organs. They are mostly type II transmembrane proteins. Many have an extracellular site for specific proteolytic cleavage which releases the active soluble cytokine, while others require cell-cell contact for signalling through the complementary receptors of the TNFRSF. The active form of TNFSF molecules is a non-covalent trimer of the TNFSF domain encoded in the last exon of the TNFSF genes. There are five chicken orthologues of human TNFSF genes described as cDNAs in public databases, including TNFSF5 (CD40L), TNFSF8 (CD30L), TNFSF10 (TRAIL), TNFSF11 (RANKL) and TNFSF15 (VEGI), and one that does not appear to have a human orthologue (TRAIL-Like). A number of publications have described TNF-like activities in the chicken (Arnold et al., 1996; Byrnes et al., 1993; Qureshi et al., 1990; Rautenschlein et al., 1999; Zhang et al., 1995), but no definitive sequence of the responsible factor(s) has been reported.

Progress in cloning and characterising the avian equivalents of these molecules has been slow because of the evolutionary distance between birds and mammals, and the rapid divergence of orthologous sequences under selective pressure from pathogens. Amino acid sequence identities are often as low as 25%. Mammalian reagents and bioassays generally do not cross-react. Some more conserved avian cytokines were cloned first (e.g. TGF- β (Burt & Paton, 1991; Burt & Jakowlew, 1992; Burt et al., 1995; Jakowlew et al., 1988; 1990)) simply on the basis of sequence similarity. Others required expression cloning techniques (Digby & Lowenthal, 1995; Sundick & Gill-Dixon, 1997; Weining et al., 1998). EST libraries enhanced progress in the cloning of cytokines whose sequences could be recognised (Lillehoj et al., 2001; Min & Lillehoj, 2002; 2004; Schneider et al., 2000; 2001), and a genomic approach based on the conservation of synteny was used to clone chicken Th2 cytokines, GM-CSF and IL-12 β (Avery et al., 2004; Balu & Kaiser, 2003). Without complete genome sequence, however, it was never possible to know how many cytokine genes remained to be identified.

In some respects the chicken immune system may be considered simplified relative to that of mammals. Lacking functional eosinophils (Vegad & Katiyar, 1995) and IgE, chickens lack components of the anti-helminthic worm and allergic responses that are controlled by Th2 cytokines in mammals, and thus may have reduced requirement for this class of cytokines. Chickens have a

reduced repertoire of polymorphonuclear cells, neutrophils, 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. Chickens lack lymph nodes, the primary site of antigen presentation in mammals, whose development is controlled by members of the TNFSF, TNF- α and lymphotoxin- α and - β (Fu & Chaplin, 1999; Ruddle, 1999). While these differences may lead to the expectation of a reduced cytokine and chemokine repertoire, chickens also have specialised lymphoid tissues and patterns of development absent in mammals, notably the bursa of Fabricius, where the immunoglobulin repertoire is developed by a process of gene conversion, and the much larger complement of circulating $\gamma\delta$ T-cells (Arstila & Lassila, 1993). The regulation of these cells and processes might require a specialised set of cytokines whose function is not part of the mammalian immune system.

To address these questions concerning the evolution of the cytokine repertoire in concert with the evolution of different developmental and responsive modes of the immune system, we used the recently released chicken genome sequence to approach, as far as is possible, a complete catalogue of chicken cytokines and chemokines (Table 1). Many of the cytokine genes are identified as such in the initial ENSEMBL annotation, but detailed analysis revealed genes that were not recognised by the automatic annotation process and others where errors in predicted gene structures compromise their utility. These results should encourage others to participate in the careful annotation effort that will be necessary to maximise the usefulness of the genome sequence and form the basis for functional investigations that will test the expected activities of the proteins encoded by the identified genes.

Cytokine family	No. of genes		Ensembl gene set ^a	
	Human	Chicken	Predicted incorrectly ^b	Missed
Interferons	18	9		3
Interleukins	39	26		8
Transforming growth factors	3	3		3
Tumour necrosis factors	19	12	9	
Colony stimulating factors	3	3		2
Chemokines	C	2		
	CC	28		5
	CXC	16		
	CX3C	1		
Total	129	77		19

Table 1. Cytokines and chemokines present in the chicken genome sequence.

^aThe Ensembl gene set quoted is based on ensembl release 22.1.1 (March 2004) which is assembly WASHUC1 at <http://www.genome.gov/11510730>

^bThis analysis was only carried out for the tumour necrosis factors.

The membership of gene families evolves by gene duplication, providing the substrate for specialisation of function, and the loss of genes whose functions have become redundant or been

taken over by other genes. We might expect to see a conserved set of direct orthologues of genes responsible for the oldest indispensable functions necessary for all vertebrate immune systems, and conversely, different surviving members of gene families representing differences in the physical architecture and functional repertoire of the mammalian and chicken immune systems. Generally, the families we have investigated have smaller numbers of genes in chickens than in mammals. This is consistent with the trend towards minimalism in avian immune system genes most clearly evident in the 'minimal essential MHC'. Although some genes may have been missed because of incomplete coverage, this is unlikely to explain all our observations. Perhaps the most surprising absence would be that of TNF- α itself (TNF2). The lack of lymphotoxin genes (TNF1 and 3), whose primary functions in mammals may be in the generation of lymph nodes, would be less surprising, as the chicken has no lymph nodes. However, TNF2 is present in many species of fish, chickens do appear to have its receptor, and there are several reports of TNF- α -like activity in chickens. It is likely that this chicken gene remains to be discovered.

Except in the case of the chemokines, nearly all the genes that are present in families with reduced membership are readily identified as direct orthologues by sequence similarity and, often, conserved syntenic location. This does imply that the chicken immune system has dispensed with, or never developed, the functions of the missing genes. While the chemokines are also reduced in number, especially the CCL chemokines, they also reveal a pattern of replacement of one set of paralogous genes by another set which are not direct orthologues of the mammalian genes. This points to a more dynamic evolution of the chemokine genes than that of the other gene families. This may be a reflection of substantial differences in architecture, such as the lack of lymph nodes, greater numbers of circulating $\gamma\delta$ T-cells, and different secondary lymphoid organs, using different sets of signals to control cell migration.

In the majority of cases, the absence of a homeostatic chemokine gene is matched by the absence of the corresponding receptor. For example, the chicken lacks CXCL9-11, which in mammals are IFN- γ -induced chemokines that attract Th1 and NK cells and are recognised by CXCR3; the chicken genome lacks CXCR3. Similarly, the chicken lacks obvious orthologues of CCL11, CCL24 and CCL26 (eotaxins 1-3), which in mammals primarily attract eosinophils and basophils and are recognised by CCR3; the chicken genome lacks CCR3. This latter observation fits with the biology of the avian immune response, in that chickens lack functional eosinophils and the chicken orthologue of IL-5 appears to be a pseudogene. The only exceptions are the apparent absence of CCL25, but the presence of CCR9, the relevant receptor.

There is one exception to the generally smaller numbers of members of gene families in chickens than mammals. The chicken has three apparent orthologues of the B-cell chemotactic chemokine CXCL13. The course of B-cell development is very different in chickens and mammals. The B-cell compartment in chickens depends absolutely on colonisation of the gut-associated lymphoid organ, the bursa of Fabricius, where the repertoire is elaborated. As the bursa involutes at sexual maturity, the adult B-cell compartment depends on replenishment from an as yet uncharacterised 'post-bursal stem cell'. Investigation of the involvement of the new CXCL13 orthologues in the biology of bursal and post bursal B-cells is likely to be fruitful. Determination of the functions of the new paralogous chemokines will also shed light on the ways in which evolution of these gene families correlates with the evolution of new functional scenarios.

To summarise (Kaiser et al., 2005; Table 1), in the chicken genome we can identify the genes for 24 interleukins, 8 type I IFNs, IFN- γ , two colony stimulating factors (GM-CSF and MGF), two of the three known transforming growth factors, 24 chemokines (1 XCL, 14 CCL, 8 CXCL and 1 CX3CL) and 10 TNFSF members. Receptor genes present in the genome suggest the likely presence of 2 other interleukins, 1 other CSF, and 2 other TNFSF members.

The availability of the draft chicken genome sequence has taken us a large step towards a complete comparison of the repertoire of molecules involved in regulating immune processes in this species, towards knowledge of common and separate pathways taken by their evolution, and

towards more informed immune manipulation. With careful enhancement of the results of automatic annotation, this resource can be used to focus on specific questions about the evolution and function of the chicken immune system.

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