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Developmental and Comparative Immunology 41 (2013) 377–388

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Defense genes missing from the flight division

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ARTICLE INFO

Article history:
Available online 24 April 2013

Keywords:
RIG-I
pathway
TLR8
Lymph node
Duck
Chicken
Major Histocompatibility Complex

ABSTRACT

Birds have a smaller repertoire of immune genes than mammals. In our efforts to study antiviral responses to influenza in avian hosts, we have noted key genes that appear to be missing. As a result, we speculate that birds have impaired detection of viruses and intracellular pathogens. Birds are missing TLR8, a detector for single-stranded RNA. Chickens also lack RIG-I, the intracellular detector for single-stranded viral RNA. Riplet, an activator for RIG-I, is also missing in chickens. IRF3, the nuclear activator of interferon-beta in the RIG-I pathway is missing in birds. Downstream of interferon (IFN) signaling, some of the antiviral effectors are missing, including ISG15, and ISG54 and ISG56 (IFITs). Birds have only three antibody isotypes and IgD is missing. Ducks, but not chickens, make an unusual truncated IgY antibody that is missing the Fc fragment. Chickens have an expanded family of LILR leukocyte receptor genes, called *CHIR* genes, with hundreds of members, including several that encode IgY Fc receptors. Intriguingly, LILR homologues appear to be missing in ducks, including these IgY Fc receptors. The truncated IgY in ducks, and the duplicated IgY receptor genes in chickens may both have resulted from selective pressure by a pathogen on IgY FcR interactions. Birds have a minimal MHC, and the TAP transport and presentation of peptides on MHC class I is constrained, limiting function. Perhaps removing some constraint, ducks appear to lack tapasin, a chaperone involved in loading peptides on MHC class I. Finally, the absence of lymphotoxin-alpha and beta may account for the observed lack of lymph nodes in birds. As illustrated by these examples, the picture that emerges is some impairment of immune response to viruses in birds, either a cause or consequence of the host-pathogen arms race and long evolutionary relationship of birds and RNA viruses.

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1. Birds have a reduced complement of immune genes

A survey of genomic resources demonstrates that the avian immune gene complement is reduced compared to mammals. An initial investigation of the immune genes in the chicken genome, a Red Jungle Fowl, suggested that birds have a reduced immune gene repertoire (Consortium, 2004). As this sequence assembly and annotation has been improved, some of these missing genes have been identified, however others are clearly not present. As genomes are sequenced for other birds, including turkey (Dalloul et al., 2010), zebrafinch (Warren et al., 2010) and duck (http://pre.ensembl.org/Anas_platyrhynchos/Info/Index), synteny along the chromosome allowed identification of genes. Thus, immune genes could be identified even if significantly diverged. A comparison of immune genes between three species of birds, confirmed that immune genes show greater divergence between species than other genes, with higher dN/dS ratio than other parts of the genome and evidence of positive selection on specific codons within genes (Ekblom et al., 2010). The sequencing of cDNA libraries as expressed sequence tags (ESTs) (Carre et al., 2006), and BLAST homology searches helped to identify the genes. Nonetheless, some genes are still unaccounted for. This appears true for all birds, although species differences exist. For some of these genes missing from the avian defense arsenal, the evidence is overwhelming, while others are less certain. In all cases, the completion and quality of the genome sequence and annotation determines whether a gene can be identified or not. Gaps exist in the genome sequences, and immune genes are often present in gene families, which are particularly prone to problems with assembly. EST libraries are incomplete, and immune gene expression may be restricted to certain tissues or cell types, and most importantly, only following immune activation. Thus, until genomes are complete and error-free it may be premature to say that a gene is not there. Nonetheless, claiming that a gene is missing certainly inspires research aimed at confirming or disproving this, or demonstrating that another gene plays an analogous or compensatory role. Thus, it is worth highlighting the genes that appear to be missing.

The contracted immune gene repertoire of birds was discussed in recent review of the progress in avian immunology since the

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0145-305X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.dci.2013.04.010

availability of the chicken genome (Kaiser, 2010, 2012). In comparison with mammals, birds have partial repertoires of pattern recognition receptors including TLR receptors (Boyd et al., 2007; Brownlie and Allan, 2011; Cormican et al., 2009) and RIG-like receptors (Barber et al., 2010; Karpala et al., 2012). Others have extensively examined the repertoire of avian cytokines (Kaiser et al., 2005) and chemokines (Hughes et al., 2007; Kaiser et al., 2005) interferons (Schultz et al., 2004) (Schultz and Magor, 2008) and defensins (Lynn et al., 2007) noting the genes missing from these repertoires. The immunoglobulin locus been characterized in ducks (Lundqvist et al., 2001), and encodes just three antibody isotypes (Magor, 2011). Finally, the chicken Major Histocompatibility Complex (Kaufman, 2013) is a minimal MHC, where only the most essential genes have been retained. These reviews of each system, although excellent, do not dwell on the genes not found.

Over the course of our analysis of immune systems of ducks, we have often invested significant effort to identify homologues of the chicken or mammalian immune system. Despite our best efforts, some genes have eluded our search. Here we will focus on components of three parts of the immune system that we are investigating in ducks (pattern recognition, antibodies and MHC) and identify the genes that are not there in the duck or the chicken or both. We will assess the strength of the data suggesting the absence of the gene, and consider the effect of the gene loss on the immune system of the animal. Finally, we will speculate on the selective forces that may have led to the loss of the gene.

2. Toll-like receptors

Innate immunity provides the first line of defense against pathogens. Recognition of the pathogen through the molecular patterns of conserved pathogen components, or pattern recognition activates a signaling cascade to turn on genes for the effectors of the immune response. Toll like receptors (TLRs) detect foreign invaders by sensing pathogen-associated molecular patterns. Binding of agonists to TLRs on the cell surface, or within the endosomal compartment, activate signal transduction pathways to turn on antimicrobial peptides, cytokines, interferons and cellular killing mechanisms. Birds possess genes for ten TLRs. These include two *TLR1* genes, two *TLR2* genes, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR15* and *TLR21*. Several excellent reviews have been written recently on avian *TLR* genes (Brownlie and Allan, 2011; Cormican et al., 2009). Two genes are missing in comparison to fish and mammals, *TLR8* and *TLR9*. *TLR9*, which detects CpG, has been functionally compensated by *TLR21* (Brownlie et al., 2009; Keestra et al., 2010).

2.1. TLR8 is missing in birds

TLR7 and *TLR8* are phylogenetically related as the product of an ancient gene duplication and both can recognize single-stranded RNA, oligoribonucleotides, and nucleic acid analogues in the endosomal compartment (reviewed by Cervantes et al., 2012). *TLR8*, which is present in fish and mammals, is absent in birds. Sequencing downstream of chicken *TLR7* showed only fragments of *TLR8* (Philbin et al., 2005). Further, PCR evidence suggested that *TLR8* was disrupted in all the galliform birds, but not anseriform birds, and there was speculation that this could account for the increased susceptibility of chickens to influenza relative to ducks (Philbin et al., 2005). To follow up on this observation, we cloned duck *TLR7* cDNA, and isolated a genomic clone for duck *TLR7*, sequenced it, and examined the region downstream. As seen for chickens, we could identify only small fragments of *TLR8*, and a CR1 element disrupted the gene in both ducks and chickens (MacDonald et al., 2007). *TLR8* is also absent from the zebra finch (Cormican et al., 2009) and turkey genome (Ramasamy et al., 2012). Given the evolutionary distance of galliform birds and zebra finch, *TLR8* is likely missing from the entire avian lineage.

For several years, mouse TLR8 had been presumed non-functional, based on the lack of response to TLR7/8 agonists in the *TLR7−/−* mouse (Hemmi et al., 2002). However, when peripheral blood monocytes from mice are treated with selective TLR8 agonists, imidazoquinoline 3M002 and poly T oligonucleotides, mouse TLR8 activation is demonstrated while TLR7 is suppressed (Gorden et al., 2006). TLR8 is expressed in monocytes/macrophages and myeloid DCs, while TLR7 is expressed in pDCs and B cells (Hornung et al., 2002). TLR8 also plays a role in detecting bacterial RNA, including RNA from *Borrelia burgdorferi*, the agent of Lyme disease, inducing production of IFN-beta through IRF7 (Cervantes et al., 2011). *TLR8* is upregulated by the phagocytosis of *Mycobacterium*, including the attenuated BCG vaccine strain, *Mycobacterium bovis*, and *Mycobacterium tuberculosis* (Davila et al., 2008). Human *TLR8* allelic variants are associated with increased susceptibility to pulmonary tuberculosis (Davila et al., 2008). The protective allele is associated with decreased translation of *TLR8*, presumably resulting in a decrease in sensing and activation, and less inflammation (Davila et al., 2008). Effectively, loss of TLR8 expression is protective against tuberculosis.

It is not clear why the loss of TLR8 was selected for in birds. The simplest explanation is the similarity of function between TLR7 and TLR8 rendered the second gene non-functional. In this scenario, however, there is no selection for the deletion of the gene. Alternatively, TLR8 became detrimental, perhaps by recognizing self-antigens and initiating autoimmunity. Negative selection would then likely lead to the loss of this receptor. TLR7 has been implicated in induction of autoimmunity (Mills, 2011). Early experiments used chickens to demonstrate thyroid autoimmunity (Sundick et al., 1992; Wick et al., 1974) but it is not known to what extent avian species suffer autoimmunity in nature. Ironically, knockout of *TLR8* in mice leads to autoimmunity through overexpression and disregulation of TLR7 (Demaria et al., 2010). Intriguingly, nucleic acid sensing TLRs are implicated in preventing reactivation of host retroviral elements and consequent tumor production (Yu et al., 2012). TLR7 has been directly implicated in this immunosurveillance, as lack of antibodies against endogenous retroviral elements correlates with absence of TLR7 in knockout mice strains. This crucial role of TLR7 in immunosurveillance of endogenous retroviruses would provide the selective pressure to retain TLR7 in the genome, regardless of how TLR8 was lost. Since TLR8 appears to have been inactivated by a CR1 repetitive element, it is tempting to speculate that TLR8 was lost in a hypothetical reactivation of endogenous retroviral elements that disrupted the genome in a distant avian ancestor. Jim Kaufman has alluded to such a catastrophic 'avian big bang' in describing the loss of several avian MHC genes (Kaufman and Wallny, 1996). Another theoretical possibility is that TLR8 became the target of a pathogen that subverted it for its own benefit (discussed in Barber, 2011). Viral subversion of TLR3 is such that its absence increases host survival from many pathogens. TLR3-induced host proinflammatory cytokines allow West Nile Virus to cross the blood brain barrier (Wang et al., 2004). TLR3 activity has also been implicated in influenza-induced pneumonia (Le Goffic et al., 2006) and morbidity from vaccinia infection (Hutchens et al., 2008). Along these lines, we can envision a pathogen that subverted avian TLR8 for increased susceptibility. This could include viral targeting of TLR8 receptor for increased inflammation and pathology, or subversion of an endosomal TLR8 for entry of a mycobacterial pathogen into the cell. Mycobacteria are initially engulfed by macrophages, but survive and multiply intracellularly. Thus, bacterial or viral subversion of a PRR may drive selection to disable the gene.

Whether cause or effect, the lack of TLR8 in avian monocytes/macrophages likely does contribute to the susceptibility of birds

to RNA viruses (West Nile virus, Newcastle disease virus, influenza virus and others) and intracellular bacterial infections, including mycobacteria. *Mycobacterium avium* is a significant pathogen of birds, particularly those raised in small flocks, while modern flock hygiene has reduced the incidence in commercial poultry. Susceptibility to mycobacteriosis in birds varies, with chickens, pheasants, partridges being most susceptible, ducks and geese moderately resistant, and pigeons being very resistant (reviewed in Tell et al., 2001).

3. RIG-I signaling pathway

RIG-I is a cytoplasmic pattern recognition receptor for single-stranded 5′-triphosphate RNA with short double-stranded conformation, such as panhandle structures of viral genomes (Hornung et al., 2006; Pichlmair et al., 2006; Schlee et al., 2009; Yoneyama et al., 2004). Both RIG-I, and the related pattern recognition receptor for intracellular RNA, MDA5, share the same pathway signaling through MAVS on the mitochondrion (Fig. 1). After detection of viral RNA by RIG-I, a conformational change releases the CARD domains (Kolakofsky et al., 2012; Kowalinski et al., 2011; Luo et al., 2011; Takahasi et al., 2008). TRIM25, an E3 ubiquitin ligase, interacts with the CARD domains of RIG-I to activate it through attached (Gack et al., 2007) or unanchored K63-polyubiquitin chains (Jiang et al., 2012; Zeng et al., 2010). The relative importance of these two mechanisms in the activation of RIG-I is still controversial, but activation leads to oligomerization and RIG-I translocation to the mitochondria. Translocation of RIG-I and TRIM25 to the mitochondrial membrane involves the mitochondrial chaperone 14-3-3ε (Liu et al., 2012), allowing interaction with MAVS at the mitochondrion. This interaction induces prion-like aggregates of MAVS (Hou et al., 2011) that initiate signaling leading to IRF3/7 translocation and the production of type I interferons and proinflammatory cytokines.

3.1. RIG-I is missing in chickens

The gene encoding RIG-I, *DDX58*, is not annotated in the chicken genome sequence, and is missing in some fish species, but MDA5 homologues are present in all vertebrate families (Zou et al.,

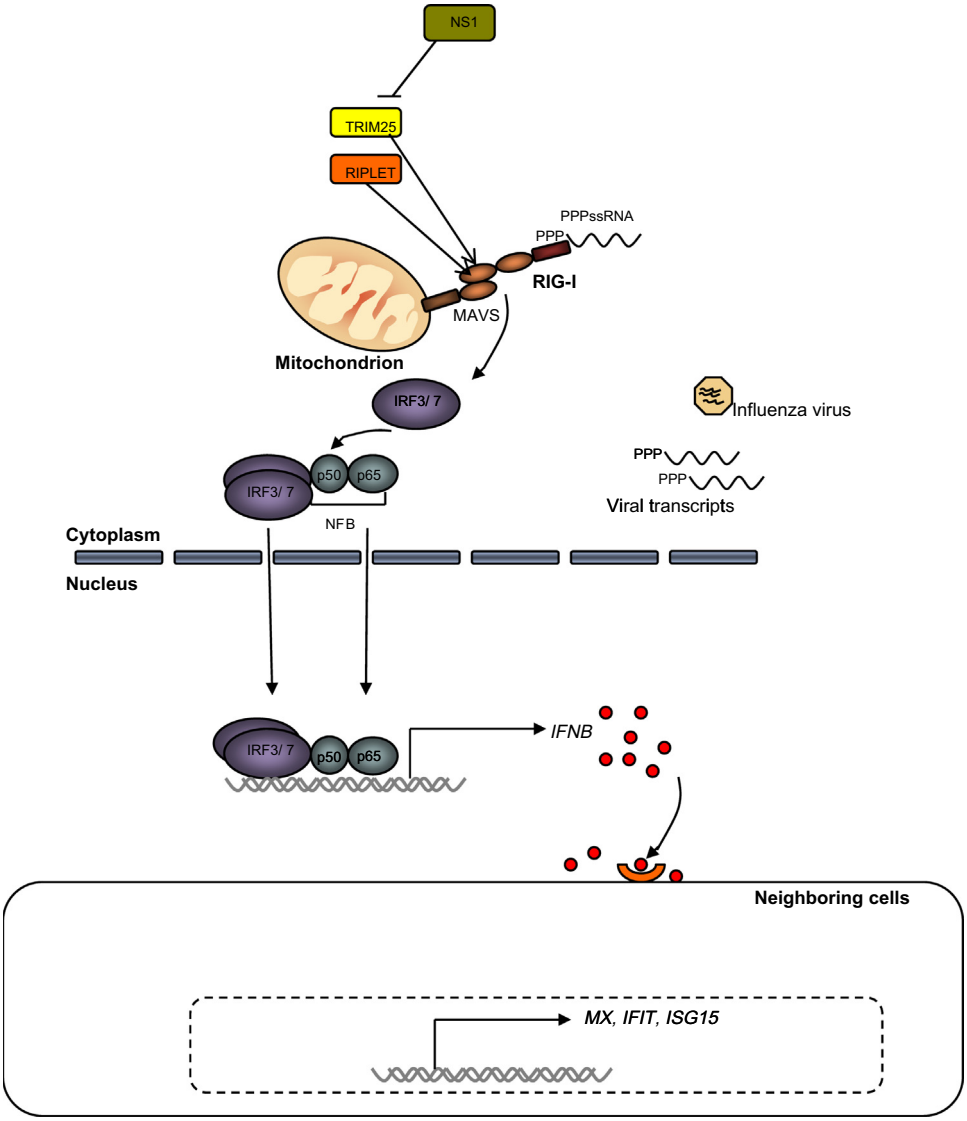

Fig. 1. A simplified schematic of the RIG-I signaling pathway. RIG-I detects 5′ triphosphate RNA and undergoes conformational changes that allow it to engage MAVS on the mitochondrion. TRIM25 and Riplet/RNF135 are E3 ubiquitin ligases involved in activation of RIG-I. MAVS multimerization initiates a signaling cascade that ultimately results in dimerization and translocation of IRF3 or IRF7, along with NFkB into the nucleus to activate IFNβ. Type I IFNs acting on neighboring cells turn on downstream interferon stimulated genes including MX, IFIT, ISG15 and many others. This figure is adapted from Bowie and Unterholtzner (2008).

2009). We demonstrated that ducks have a functional RIG-I (Barber et al., 2010). In contrast, the *DDX58* gene appears absent in chickens by analysis of the syntenic region of the Z chromosome, although we can identify the flanking gene. We also cannot find *RIG-I* in a search of the expressed sequence tag database for chickens. Thus *RIG-I* is missing in the genome of the ancestral chicken represented by the Red Jungle Fowl, and the sequences from modern commercial chicken breeds. Our Southern blots show a duck RIG-I probe cross-hybridizes with pigeon DNA, but not with chicken DNA (Barber et al., 2010). We also cannot detect the gene in DNA of turkey or partridge, suggesting that the gene is missing in galliformes (Barber, 2011). Furthermore, we showed that chicken DF-1 cells cannot detect RIG-I ligand, but if we transfect the cells with duck RIG-I we can reconstitute the pathway (Barber et al., 2010). The loss of RIG-I likely contributes to the susceptibility of chickens to infection compared to ducks to a variety of single-strand RNA viruses, including influenza A virus and Newcastle disease virus, both of which cause more harm in chickens than ducks. The related RNA detector, MDA5, can partially compensate and detect avian influenza in chicken cells to generate an interferon response (Karpala et al., 2011; Liniger et al., 2012).

It is difficult to speculate on the selective forces resulting in loss of RIG-I in some birds. Some have suggested the possible existence of a compensatory yet-to-be identified alternate receptor (Karpala et al., 2011), which could certainly facilitate the loss from the genome. RIG-I was initially identified in a leukemia cell line upregulated by retinoic acid (Liu et al., 2000), and indeed it is upregulated by a variety of stress inducers. RIG-I is implicated in a number of other biological events including cell proliferation, apoptosis, senescence, and acute and chronic inflammatory diseases (Liu and Gu, 2011). It is possible that selection to eliminate RIG-I from aberrant activation in one of these alternate roles had resulted in the loss of RIG-I in galliform birds. Finally, there remains the intriguing possibility that the RIG-I receptor was the prey of one of the many single-strand RNA viruses that infect birds, including influenza virus, Newcastle disease virus, West Nile virus, and coronaviruses, and was usurped for the virus' advantage. The TLR and RLR signaling pathways are targets for viral subversion (reviewed by Es-Saad et al., 2012; Ramos and Gale, 2011). Influenza virus interferes in the mammalian RIG-I pathway in several places, through the action of NS1 protein (Gack et al., 2009). In a similar manner, paramyxoviruses make the V protein that interferes with signaling by the chicken MDA5 receptor (Childs et al., 2007) involving direct protein–protein interaction and preventing interaction with the RNA ligand (Motz et al., 2013). While this interference renders the receptor non-functional during an infection, it would not necessarily lead to selection to eliminate the receptor. However, we can envision interactions with the RIG-I receptor where regulation is aberrant, or excessive activation leads to death. In this scenario, loss of RIG-I could provide a selective advantage to survive a lethal infection with an unknown pathogen. Suggesting that *RIG-I* is also involved in development in some capacity, RIG-I knockout mice are embryonic lethal due to liver damage (Kato et al., 2005). Aberrant or loss of expression of RIG-I during development due to pathogen subversion, and associated embryonic lethality, could result in selective loss of this receptor. While this raises the question of how the birds without RIG-I survived, we note that *RIG-I* is not absolutely essential for development, since RIG-I knockout mice have since been made on a different genetic background which are fertile and viable (Wang et al., 2007). Given that RIG-I expression is impaired in lethal infections (Kobasa et al., 2007), it is possible to envision a scenario by which aberrant expression of RIG-I leads to death, and the gene is selectively lost in a common ancestor of chickens and turkeys.

Human RIG-I is regulated through polyubiquitinylation, ISGylation, sumolyation, and phosphorylation and alternate splicing (Eisenacher and Krug, 2012; Loo and Gale, 2011; Maelfait and Beyaert, 2012; Oshiumi et al., 2012; Wang et al., 2011). A major on–off switch for human RIG-I upon viral infection is polyubiquitination by host E3 ubiquitin ligase, tripartite motif protein 25 (TRIM25) (Gack et al., 2007). Sequences at the T55 residue implicated in interaction with TRIM25 and the site of attachment of polyubiquitin chains, K172, are not conserved in duck or zebra finch RIG-I (Barber et al., 2010) or goose RIG-I (Sun et al., 2013). Thus activation of avian RIG-I involves ubiquitination at alternate residues, or interaction with unanchored polyubiquitin chains, not attached to any protein, can activate RIG-I (Zeng et al., 2010).

Given the lack of RIG-I in chickens, the recent observation that knockdown of chicken TRIM25 impairs the interferon response of chicken cells is intriguing (Rajsbaum et al., 2012). Perhaps TRIM25 is involved in the activation of chMDA5. The binding of unanchored K63 polyubiquitin chains can activate human MDA5 *in vitro* (Jiang et al., 2012). A role for TRIM25 in generating or attaching ubiquitin chains to MDA5 could explain the importance of chTRIM25 in the interferon response of chicken cells.

3.2. RLR modifier Riplet is missing in chickens

Riplet/RNF135 is a cytoplasmic E3-ligase identified by yeast two-hybrid as one of the proteins binding RIG-I, and is essential for RIG-I activation in human cell lines upon infection with an RNA virus (Oshiumi et al., 2009; Oshiumi et al., 2010). Riplet shares 60.8% identity with TRIM25 in humans (Oshiumi et al., 2009), and also has an N-terminal RING domain and C-terminal PRY/SPRY domain. The RING domain confers ubiquitin E3 ligase activity (Nisole et al., 2005) and also contributes to other protein–protein interactions (Borden, 2000). Riplet also mediates K63-polyubiquitination of RIG-I (Gao et al., 2009; Oshiumi et al., 2010). However, there is debate as to whether Riplet interacts with the CARD domains of RIG-I (Gao et al., 2009) the C-terminal repressor domain of RIG-I or both (Oshiumi et al., 2010). Riplet is crucial for RIG-I activation in cells regardless of expression of TRIM25 (Oshiumi et al., 2010). Knockout of *Riplet* (Oshiumi et al., 2010) or TRIM25 (Gack et al., 2007) impaired the RIG-I dependent innate immune response, suggesting that both are required. Knockout of *Riplet* resulted in animals that were deficient in the production of interferon in response to RNA, but not DNA viruses (Oshiumi et al., 2010).

Riplet is present in zebra finch (*Taeniopygia guttata*), but we were unable to find the ortholog in the chicken (*Gallus gallus*) genome. In the duck, we have located a putative *Riplet* coding region, but it lacks exon 1. In repeated 5′ RACE experiments, all clones recovered contain sequences that correspond to an intact open reading frame, but lack the expected RING domain. In mice, deletion of the RING domain prevents RIG-I activation (Oshiumi et al., 2010) therefore we hypothesize that deletion of the RING domain in ducks may render it functionally inactive. Nonetheless, we saw upregulation of *Riplet* and *TRIM25* in duck lung at 1dpi with highly pathogenic avian influenza virus (Fleming-Canepa X. et al., unpublished data). Because Riplet may not be functional in ducks, but still highly upregulated during influenza infection, we speculate that Riplet is acting as a decoy for the viral NS1. Influenza A NS1 protein interacts with TRIM25 (Gack et al., 2009) and Riplet (Rajsbaum et al., 2012) causing inhibition of innate immune signaling. Alternatively, Riplet may dimerize with other E3 ligases to function, as recently shown for TRIM16 (Bell et al., 2012).

Comparison of embryonic fibroblast cells from RIG-I knockout and wild-type mice upon influenza infection, reveal genes that are downstream of RIG-I signaling. These genes have been referred to as the RIG-I bioset, the genes induced by influenza infection in a RIG-I dependent manner. In mouse fibroblast cells, the genes include *IFNB*, *IRF3*, *IRF7*, *STAT1*, *STAT2*, *PKR*, *OAS*, *MX1*, *IFIT2* (*ISG54*),

IFIT1 (*ISG56*) and *RSAD2* (viperin) (Loo et al., 2008). While the overlap between RIG-I and MDA5 inducible genes downstream of MAVS signaling in chicken cells is unknown, we used a microarray approach to examine the genes turned on by RIG-I in chicken cells. Using chicken DF-1 cells, transfected with duck RIG-I, the expression of the RIG-I gene bioset in avian species is augmented (Barber et al., 2013). We noted that some essential genes of the mouse RIG-I bioset are missing in avian species, including *IRF3*, *ISG15*, and *IFIT2* (*ISG54)* and *IFIT1 (ISG56)*.

3.3. IRF3 is missing in birds

Interferon regulatory factor-3 is a critical player in the induction of type I IFNs following virus infection (Au et al., 1995). IRF3 and IRF7 have different and crucial roles in the induction of INF-α/β (Honda et al., 2006; Honda and Taniguchi, 2006). IRF3 is constitutively expressed, and is activated by C-terminal phosphorylation that allows dimerization and nuclear localization (Lin et al., 1998). This led to the suggestion that IRF3 was responsible for the initial upregulation of the *IFNB* gene, followed by interferon dependent induction of IRF7. However, *IRF7*−/− knockout mice are severely impaired in interferon production upon infection with ssRNA viruses (Honda et al., 2005), suggesting the contribution of IRF3 is minor. Although a gene has been named *IRF3* in chickens (Grant et al., 1995), it is interferon inducible and more similar to *IRF7*. Others have noted the absence of *IRF3* in chickens (Huang et al., 2010) and in avian species (Cormican et al., 2009). It is not known which IRF is translocating to the nucleus to activate interferon in the RIG-I/MDA5 pathway in avian species. We speculate that IRF7 fulfills the nuclear translocation and activation of type I IFNs in both TLR and RLR signaling, but this has not been experimentally examined.

3.4. ISG15 is missing in birds

Interferon stimulated gene 15 (*ISG15*) is highly up regulated by interferon treatment and was the first ubiquitin-like modifier identified. The amino acid sequence of ISG15 is similar to a linear ubiquitin dimer (reviewed in Zhang and Zhang, 2011). ISG15 is conjugated to proteins like ubiquitin, through a process called ISGylation. Among the identified ISGylated substrates are interferon-induced proteins like PKR, RIG-I, MXA (Zhao et al., 2005). IRF3 ISGylation by HERC5 (the main ISG15 E3 ligase in human) increases stability of IRF3, exerting a positive regulation in the RIG-I pathway (Shi et al., 2010). Negative feedback on RIG-I expression and signaling is mediated by ISG15 conjugation to RIG-I (Kim et al., 2008). ISG15 is also involved in a direct antiviral mechanism where ISGylation of influenza A virus NS1 protein impairs viral replication (Zhao et al., 2010).

No genes homologous to human *ISG15* have been annotated in any of the available avian genomes. In the chicken, genes located adjacent to human *ISG15* were predicted; including *HES1* (homologous to human *HES4*) and *AGRN*. Within this syntenic region of the chicken genome no ubiquitin-like gene was present. Similarly, homologs of *HES1* and *ARGN* genes were found in the duck scaffolds (scaffold 1197 and 2665, respectively) but synteny analysis cannot be performed because the scaffolds do not overlap.

Enzymes involved in the ISGylation system (including UbE1L, UbCH8, HERC5 and USP18) are present in the chicken and duck genomes, but there is not yet any functional evidence of ISGylation in these species. USP18, which is responsible for cleavage of ISG15 from ISGylated substrates, correlates with survival of influenza-infected chickens, indirectly suggesting some functionality of the ISGylation system (Uchida et al., 2012).

ISG15 conjugation plays many roles in mammalian antiviral immunity, including ISGylation of MX, PKR, RIG-I, and IRF3, and influenza NS1 protein (reviewed in Skaug and Chen, 2010). However, given the absence of several ISG15 targets, including RIG-I in chickens, IRF3 in birds, and evidence that MX is non-functional in chickens (Schusser et al., 2011) and ducks (Bazzigher et al., 1993), the absence of this ubiquitin modifier in birds would be less significant. It is not known whether NS1 is modified by ISG15 in avian hosts. We cannot rule out the possibility that we have failed to identify the avian ISG15 homolog because of low sequence conservation with human ISG15. It is also possible that another unknown ubiquitin-like modifier in birds plays the role of ISG15 within the ISGylation system. Intriguingly, the most similar sequence to ISG15 in the duck and chicken genome lies within the C-terminal end of 2′,5′-oligoadenylate synthetase-like (*OASL*) gene. OASL has two tandem ubiquitin-like domains that share 37% amino acid identity to human ISG15. The antiviral activity of the human P59 protein, encoded by human OASL, is dependent on the C-terminal ubiquitin-like domain (Marques et al., 2008). However, the biological function of the OASL ubiquitin-like domains is not yet clear and its role as ubiquitin-like modifier has not been described.

3.5. Birds have a single IFIT gene

The interferon-induced proteins with tetratricopeptide repeats (*IFIT*) genes are highly upregulated by type I IFNs or by viral infection (Bluyssen et al., 1994; Levy et al., 1986; Wathelet et al., 1986). The human *IFIT* gene family consists of *IFIT1* (*ISG56*), *IFIT2* (*ISG54*), *IFIT3* (*ISG60*), and *IFIT5* (*ISG58*), while the mouse *IFIT* family lacks *IFIT5* and contains *IFIT1*, *IFIT2*, and *IFIT3* (Bluyssen et al., 1994). The *IFIT* family appears to be limited to a single gene in marsupials, birds, frogs and fish (reviewed by Zhou et al., 2013). While these proteins have served as markers of viral infection, only recently have their functions in the innate antiviral response been elucidated (Daffis et al., 2010; Fensterl et al., 2012; McDermott et al., 2012; Pichlmair et al., 2011; Schmeisser et al., 2010). IFIT proteins reside within the cytoplasm of cells, and all contain multiple tetratricopeptide repeats (TPRs) (Lamb et al., 1995). The TPRs within these proteins consist of a helix-turn-helix motif and facilitate protein–protein interactions (Blatch and Lassle, 1999). IFIT1 and IFIT2 mediate their antiviral activity by a disruption of translation via an interaction with eukaryotic initiation factor 3 (eIF3) (Li et al., 2010). IFIT1 and IFIT2 also inhibit the translation of viral mRNAs lacking a 2′-O methylation cap structure (Daffis et al., 2010). IFIT1 and IFIT5 have the ability to bind to, and sequester viral 5′-triphosphate RNA (Pichlmair et al., 2011). The crystal structure of IFIT2 has revealed an RNA binding domain that also may function in an antiviral context (Yang et al., 2012). The multi-functional IFIT1 protein can also restrict the replication of human papilloma virus (HPV) by binding the viral helicase E1, and restricting its function in viral replication (Saikia et al., 2010). Interestingly, IFIT1 has also been associated with negative feedback regulation of genes upregulated during viral infection, further demonstrating the diverse function of these genes (Li et al., 2009). The *IFIT* gene family represents a significant contributor to the broad-ranged antiviral activity of interferons, and plays an important role in the cellular, innate antiviral response.

In avian species, the only identifiable *IFIT* gene encodes a protein that aligns with other IFIT5 proteins in a phylogenetic tree (Fig. 2). The upregulation of *IFIT5* following viral infection of chicken cells expressing duck RIG-I (Barber et al., 2013) or infection of ducks (Vanderven et al., 2012) suggests IFIT5 is an important antiviral effector in avian species. The apparent absence of an expanded *IFIT* gene family in avian species suggests that several of the functions attributed to IFIT proteins will be missing. Indeed, the specific role of avian IFIT5 during a viral infection is unknown.

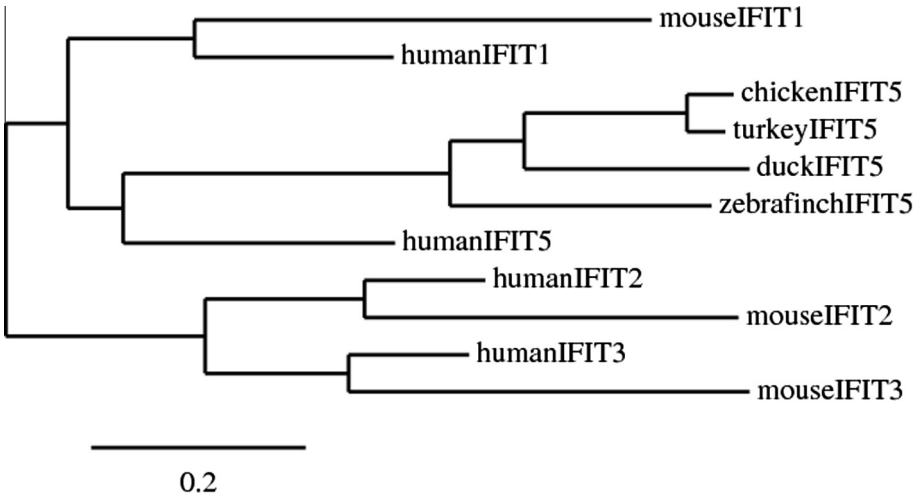

Fig. 2. A phylogenetic tree showing similarity of avian and mammalian IFIT sequences. Sequences were aligned and phylogenetic tree generated using a maximum likelihood estimation using a program called PhyML using www.phylogeny.fr. (Dereeper et al., 2008). Accession numbers for the IFIT sequences were: chicken IFIT5 (XM_421662.3), turkey IFIT5 (XM_003208028.1), zebra finch IFIT5 (XM_002188552.1), human IFIT1 (NM_001270927.1), mouse IFIT1(NM_008331.3), human IFIT2 (NM_001547.4), mouse IFIT2 (NM_008332.3), human IFIT3 (NM_001031683.2), mouse IFIT3 (NM_010501.2), human IFIT5 (NM_012420.2). Note the duck IFIT sequence is a partial sequence.

4. Birds have a minimal immunoglobulin locus

Birds have only three antibody isotypes, IgM, IgA and IgY. IgY, the avian serum Ig most similar to mammalian IgG, is a precursor to IgG and IgE that has composite function of both isotypes (Warr et al., 1995). Ducks make a truncated version of IgY (Magor et al., 1992). In addition, birds use a single light chain gene of the λ type (Magor et al., 1994a; Reynaud et al., 1983).

4.1. IgD is missing from the duck IgH locus

Ducks have three immunoglobulin heavy chain genes arranged in the gene order *IGHM*, *IGHA* and *IGHY* encoding the mu, alpha and upsilon chains for IgM, IgA and IgY, respectively (Lundqvist et al., 2001; Magor et al., 1999). The *IGHA* gene, encoding alpha is inverted in the locus, and *IGHD* (delta) is absent. Despite availability of chicken, zebrafinch and turkey genomes, no other avian immunoglobulin heavy chain locus has yet been assembled. From the limited analysis that has been published for chicken IgH (Zhao et al., 2000), it shares the same organization. The transposition of *IGHA* from the 3′ most position in the locus, to an inverted position downstream of *IGHM*, may have also resulted in the loss of *IGHD*. Lack of *IGHD* is evident from genomic sequencing for ducks. Early studies reported a δ chain in chickens (Chen et al., 1982), but it is generally accepted that there is no avian homologue of IgD. Since IgD has been identified in teleosts (Bengten et al., 2002; Wilson et al., 1997) frogs (Zhao et al., 2006) and reptiles (Cheng et al., 2013; Wei et al., 2009) the *IGHD* gene was lost in birds.

IgD is an enigmatic antibody that exists in a wide variety of forms in different species, except birds. The function of IgD is beginning to emerge from observations first made for fish, and subsequently for human IgD. IgD functions at the interface of innate and adaptive immune responses. In fish, IgD specific B cells have been identified, and secreted IgD lacks the variable region suggesting it functions more like a pattern recognition receptor (Edholm et al., 2010). IgD is found on the surface of granulocytes in fish, which do not make the IgD transcript, and involves a specific receptor (Edholm et al., 2010). In humans, circulating IgD binds to basophils and activates antimicrobial and inflammatory factors (Chen et al., 2009). IgD from IgD+ IgM- B cells binds to basophils, and can also bind to certain bacteria in the respiratory tract. The basophil binds IgD through a specific receptor, and cross-linking of IgD leads to the production of B cell activating factors (BAFF) and pro-inflammatory cytokines. Serum IgD is elevated in patients with chronic infections, and specific IgD antibodies could be demonstrated in a number of these infections (reviewed by Chen and Cerutti, 2010). This ancient surveillance system serves to instruct the B cells of the type of pathogens in the respiratory tract. As the specific functions of IgD are elucidated, the consequences of the lack of IgD antibody in birds will become evident. Birds have basophils, but it is unclear whether a different Ig isotype can bind to the basophil IgD receptor to compensate, or whether the receptor exists in birds. Indeed, it remains to be demonstrated that a homologous receptor is involved in the IgD binding by basophils of humans and fish.

4.2. Duck IgY lacks the Fc domain

Duck IgY is made in two secreted forms, a full-length form and a truncated form. The truncated form, called IgYΔFc, lacks the Fc region entirely. It arises from alternate splicing that adds an exon encoding just two amino acids after the CH1 and CH2 domains, and uses an alternate polyadenylation site (Magor et al., 1992) (Magor et al., 1994b). What controls the alternate splicing is unknown, but the truncated form predominates later in the immune response. The IgYΔFc antibodies would be expected to be defective in several processes such as antigen internalization, which is required for appropriate presentation of antigens needed to generate T cell help. The truncated IgY also does not participate in complement fixation, opsonization, precipitation reactions, and reportedly also cannot participate in hemagglutination inhibition (HI) (Higgins et al., 1987). Of benefit to ducks, perhaps the truncated IgY helps prevent viral internalization through receptor-mediated endocytosis and subsequent infection of macrophages and other leukocytes (Magor, 2011).

5. Expansion of the leukocyte immunoglobulin-like receptor family in chickens

The chicken Ig-like receptor (*CHIR*) genes (Dennis et al., 2000) are counterparts of the leukocyte immunoglobulin-like receptor family (*LILR*). The *CHIR* genes constitute a large and diverse family of genes in the chicken, with more than 100 members located in a region syntenic to the mammalian leukocyte receptor complex

(LRC) (Laun et al., 2006; Nikolaidis et al., 2005; Viertlboeck and Gobel, 2011; Viertlboeck et al., 2005) and vast diversity within an individual (Viertlboeck et al., 2010). CHIR are expressed in a variety of myeloid and lymphoid cells, with individual receptors expressed in a cell-type restricted manner (Viertlboeck et al., 2005). Receptor diversity includes variation within a hypervariable region, the putative binding region, alternate transcript splicing, and presence or absence of functional activation or inhibitory motif. The extensive expansion and diversification of this family in chickens, and leukocyte expression, suggests their evolution is in response to the pressure of pathogens, as suggested for human LILR and mouse PIR genes (Barclay and Hatherley, 2008). Human LILR receptors are involved in self/non-self recognition and some engage MHC class I targets, as well as pathogen mimics of MHC proteins (Anderson and Allen, 2009; Brown et al., 2004). *Staphylococcus aureus* targets the mouse inhibitory receptor PIR-B for increased virulence (Nakayama et al., 2012). In turn, activating PIR-A receptors may have evolved in response to the selective pressure from pathogens, as indicated by the relict ITIMs in the PIR-A gene suggesting it is derived from a PIR-B ancestor (Nakayama et al., 2012). Thus, counterbalance through inhibitory and activating CHIR proteins may have evolved in response to pathogen manipulation of immune signaling through these receptors.

5.1. LILR receptors are missing in ducks

We have searched unsuccessfully for immunoglobulin superfamily members homologous to the chicken CHIR receptors in ducks. In high and low stringency Southern blots, genomic DNA from chickens shows an extensive pattern of hybridization, while DNA from ducks shows no significant hybridization (MacDonald et al., 2007). All efforts to amplify these genes by polymerase chain reaction using several sets of degenerate primers are completely unsuccessful. Our searches of the draft assembly of the duck genome, and 70,000 expressed tag sequences generated by 454 sequencing, also find no evidence of CHIR homologues. We cannot rule out the possibility that we have simply missed the *CHIR* genes due to weak homology, if they have evolved to be quite different in ducks. This in itself is quite intriguing. The rapid species-specific divergence of primate LILR genes, with only some genes showing clear orthologous relationships between species (Canavez et al., 2001), while others have evolved to be unique in each species is thought to reflect their species-specific interactions with pathogens. Alternatively, these genes are truly absent from ducks, despite their presence in chickens. There are several examples where different vertebrates have employed different families of leukocyte receptors (Parham and Moffett, 2013). For example, cattle use KIR as NK cell receptors, which have undergone expansion (McQueen et al., 2002) while horses use Ly49 (Takahashi et al., 2004).

5.2. CHIR-AB1, the chicken Fc receptor for IgY, is missing in ducks

Although there are hundreds of CHIR genes, the only CHIR with a known ligand is CHIR-AB1, which functions as the chicken IgY Fc receptor (Viertlboeck et al., 2007). Chickens have a large number of CHIR-AB1 genes that have varying specificities for IgY (Viertlboeck et al., 2009). Remarkably, we cannot find identifiable homologues of the CHIR-AB1 in ducks. Duck full-length IgY does not bind the chicken Fc receptor (Viertlboeck et al., 2007). As noted above ducks also make a truncated IgYΔFc that would be expected to not to bind Fc receptor. Göbel speculated that the loss of the IgY Fc fragment, and the duplication and divergence of the chicken CHIR-AB1 (Fc receptor) family were both strategies to evade a pathogen interfering with the IgY-Fc receptor interaction in birds (Purzel et al., 2009). Ducks evade this pathogen by production of an antibody lacking the Fc region, retaining the specificity for the antigen, as this truncated form predominates in the later immune response. In chickens, selection favored the duplication of the CHIR receptor family to make a large number of potential 'decoy receptors' for IgY. While no known pathogen targets the Fc-IgY interaction in birds, this is a very interesting hypothesis. Chickens may elude this pathogen through the binding of decoy receptors, while ducks may avoid the internalization of an intracellular pathogen through the production of the IgYΔFc.

6. Birds have a 'minimal MHC'

The vertebrate MHC is the most dynamic part of the genome, showing repeated cycles of 'birth-and-death' evolution (Kelley et al., 2005). Polygeny and polymorphism are hallmarks of the region, with varying numbers of genes between species (and sometimes between individuals). Usually it is not possible to identify orthologous genes between species. The MHC class I and class II genes are the most polymorphic genes in the vertebrate genome.

The MHC of the chicken has been referred to as the 'minimal MHC' (Kaufman et al., 1995), fulfilling all the requirements of an MHC region, with a limited set of genes. The B locus, or genomic MHC region, contains just 19 genes within 92 kb (Kaufman et al., 1999). MHC class I genes flank either side of the transporters for antigen processing (*TAP*) genes. They are referred to as the major (*BF2*) and minor (*BF1*) MHC class I loci. Similarly, the MHC class II genes (*BLB1* and *BLB2*) are located on either side of tapasin (*TAPBP*), and in close proximity to the chaperones involved in MHC class II loading (*DMA* and *DMB*). Several genes are notably absent, including the proteasome genes *LMP2* and *LMP7*, as well as genes encoding TNF alpha, and lymphotoxin alpha and beta.

Kaufman argues the MHC organization critically affects function because proximity of the genes involved in antigen transport and presentation, allows their encoded proteins to evolve to work together. Indeed, *TAP1* and *TAP2* genes are also polymorphic, and using a peptide translocation assay, Kaufman recently showed that TAP determines specificity for the linked dominant MHC class I gene (Walker et al., 2011). The limitation to one MHC class I gene in chickens, impairs defense against viral pathogens, as ability to defend against a particular pathogen is completely dependent on whether or not it can load peptides from that pathogen. The best illustration of the consequences of limited MHC class I presentation is the ability of chickens of one genotype to defend against Rous sarcoma virus, while other strains cannot (Wallny et al., 2006).

The duck has a functionally similar MHC class I region, with 5 MHC class I genes encoded adjacent to the TAP genes (Moon et al., 2005). Ducks predominantly express one gene, which is adjacent to the *TAP2* gene, which is also polymorphic (Mesa et al., 2004). In ducks, as in chickens, this is expected to have functional consequences for the defense against viruses. Viruses can easily change the one or two epitopes that can be presented by alleles encoded by one MHC class I gene, and thus escape the cytotoxic T cells focused on these epitopes.

6.1. Tapasin is missing in duck MHC

We have been unable to identify the tapasin (*TAPBP*) gene in the duck MHC. Tapasin bridges the gap between the TAP transporter and empty MHC class I molecules, bringing them into close proximity to the translocation core where peptides are loaded (Sadasivan et al., 1996). In the absence of tapasin, empty MHC class I molecules weakly associate with TAP leading to binding and cell surface expression of less than optimal peptides (Grandea et al., 1995). Tapasin has been identified adjacent to MHC class II in other

birds, including chicken (Frangoulis et al., 1999), quail (Shiina et al., 1999), turkey (Chaves et al., 2009), pheasant (Ye et al., 2012) and zebra finch (Balakrishnan et al., 2010) and black grouse (Wang et al., 2012). Also, the *tapasin* gene is polymorphic in chicken, turkey and pheasant (Sironi et al., 2006). Through analysis of the unannotated duck genome (PreEnsemble) we can identify the location of the duck MHC class II genes, but a search for *tapasin* within proximity is unsuccessful. Using primers based on the chicken sequence, or conserved regions identified in aligned avian tapasin sequences, our attempts to amplify *tapasin* from mallards or a domestic duck by RT-PCR or from genomic DNA fail to yield a *tapasin* product. It is possible that failure to amplify *tapasin* from ducks is due to sequence divergence of tapasin between avian species. The galliform tapasin proteins are about 90% identical (Wang et al., 2012), but the human and chicken tapasin share only 36% amino acid identity (Frangoulis et al., 1999). In low stringency southern blot analysis, distinctive bands could be detected in chicken, however the probe does not hybridize to duck genomic DNA (Petkau, 2012).

Although tapasin seems to play an important role in antigen presentation, certain human MHC class I alleles can function in a tapasin-independent manner (Park et al., 2003; Lewis et al., 1998). A single amino acid substitution in HLA-B from Asp116 to Tyr116 allows the latter to function in a completely tapasin independent manner (Sieker et al., 2007). It also appears that tapasin influences the peptide repertoire presented to MHC class I, favoring certain peptides over others. In the absence of tapasin antigen presentation is altered, rather than deficient, and is still sufficient to induce in an immune response (Boulanger et al., 2010). Similarly, the absence of immunoproteasomes in mice, results in a change in 50% of the loaded peptide repertoire (Kincaid et al., 2012).

We could speculate that loss of tapasin was advantageous in ducks. We presume that ducks, like chickens, already had constraints on presentation of antigens by MHC class I due to potential co-evolution of the TAP transporters and adjacent MHC class I genes. Evidence for this is that both *TAP1* and *TAP2* are polymorphic in ducks, and they express one dominant MHC class I gene (Mesa et al., 2004). Perhaps the loss of tapasin permits closer interaction of the TAP transporter and the specific MHC class I molecule intended for loading. Proteins encoded by genes co-evolving along a haplotype reach a best fit. Tapasin as the bridge between TAP and MHC class I molecules could serve to bring the incorrect MHC class I into proximity of the TAP transporters from the other haplotype, unless it was also evolving to keep step.

6.2. Lymphotoxin-alpha and beta are missing from the avian MHC

The genes encoding TNF-alpha (TNF-α), and lymphotoxin-alpha and beta (LTα and LTβ) are missing from the avian MHC. Extensive efforts by PCR, EST mining, and hybridization to identify TNF-α in chickens and ducks have failed. Similarly, the two genes encoding LTα and LTβ are missing in chickens (Kaiser, 2012), and a scan of the genome sequence shows they are also absent in ducks. Lymphotoxin-alpha knockout mice lack lymph nodes (De Togni et al., 1994), and similarly chickens have no lymph nodes. Primitive lymph nodes were previously described in ducks (Berens von Rautenfeld and Burdras, 1983) and immunoglobulin transcripts were analyzed in lymphatic tissues isolated from ducks (Bando and Higgins, 1996; Magor et al., 1994a). However, we question whether these tissues contain recognizable lymph nodes containing secondary lymphoid tissue, as we are unable to identify anything in the lymphatic tissue that resembles a lymph node, even tracking with injected India ink. We examined isolated lymphatic tissues in ducks for mRNA expression of CCL19 and CCL21, the two chemokines which are responsible for recruiting naïve T cells and dendritic cells to lymph nodes. We showed expression of these chemokines was negligible in lymphatic tissues, and abundant in spleen and influenza-infected lung tissues (Fleming-Canepa et al., 2011). Clearly, the lymphatic tissues of ducks are not sites of recruitment of lymphocytes and dendritic cells as expected for secondary lymphoid tissues. Thus, ducks and chickens, like all other non-mammalian vertebrates (Hofmann et al., 2010), lack true lymph nodes.

7. Comparative genomics going forward

Through comparison of the immune arsenal of ducks and chickens we highlight several immune genes that are 'MIA or missing in action' from the flight divisions. We refer to these genes as MIA, because we cannot say with certainty that these genes are not present, at least until the sequencing and annotation of avian genomes is more complete. If these genes are truly absent, what emerges is a picture in which chickens, missing TLR8 and RIG-I, have less ability to detect RNA viruses and intracellular bacteria than ducks, which lack only TLR8. In addition, birds are missing components in the RIG-I pathway, and interferon-responsive antiviral effectors. Chickens have a large expanded family of leukocyte receptors, which are apparently missing in ducks, which include the IgY Fc receptors. Notably, ducks make a truncated IgY that is lacking the Fc region as their most abundant serum antibody. By similarity to their human homologues, the LILR receptors, other members of the CHIR family are presumed to be involved in self/non-self recognition, and their expansion may somehow compensate for the deficit due to the minimal avian MHC. In contrast, ducks may have lost tapasin, and with it lost some of the constraint on antigen presentation due to co-evolving linked MHC class I and TAP transporter genes. Whether the host-pathogen arms race is cause or consequence of these gene losses, birds have had a long evolutionary relationship with RNA viruses, including those causing zoonoses.

Acknowledgements

The idea for this review came from a conversation with Martin Flajnik in the beer tent at the Comparative Immunology Workshop, held in Waterloo, Ontario in 2010.

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