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Identification of avian RIG-I responsive genes during influenza infection

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Abstract

Ducks can survive infection with highly pathogenic avian influenza viruses that are lethal to chickens. We showed that the influenza detector, RIG-I, can initiate antiviral responses in ducks, but this gene is absent in chickens. We can reconstitute this pathway by transfecting chicken DF-1 embryonic fibroblast cells with duck RIG-I, which augments their antiviral response to influenza and decreases viral titre. However, the genes downstream of duck RIG-I that mediate this antiviral response to influenza are not known. Using microarrays, we compared the transcriptional profile of chicken embryonic fibroblasts transfected with duck RIG-I or empty vector, and infected with low or highly pathogenic avian influenza viruses. Transfected duck RIG-I expressed in chicken cells was associated with the marked induction of many antiviral innate immune genes upon infection with both viruses. We used real-time PCR to confirm upregulation of a subset of these antiviral genes including *MX1, PKR, IFIT5, OASL, IFNB*, and downregulation of the influenza matrix gene. These results provide some insight into the genes induced by duck RIG-I upon influenza infection, and provide evidence that duck RIG-I can function to elicit an interferon-driven, antiviral response against influenza in chicken embryonic fibroblasts.

1. Introduction

Influenza A viruses cause largely asymptomatic infection in ducks, the natural reservoir (Webster et al., 1992). Most strains replicate in the duck, including 16 hemagglutinin (HA) subtypes and 9 neuraminidase (NA) subtypes (Webster et al., 1992). Because there is seldom disease in ducks, the classification of influenza as highly or low pathogenic refers to infections in chickens (Suarez and Schultz-Cherry, 2000). Highly pathogenic avian influenza can be deadly in poultry in 1–3 days, while low pathogenic influenza causes only mild disease signs (Pantin-Jackwood and Swayne, 2009). Influenza strains of H5 and H7 HA subtypes can replicate in chickens, and can evolve from low to highly pathogenic avian

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influenza (HPAI) (Pasick et al., 2005; Swayne and Suarez, 2000). Since these changes typically happen under immune pressure in alternate hosts, chickens are an important intermediate in the genesis of novel influenza strains.

Circulating H5N1 HPAI strains remain of concern because of their pronounced pathogenicity and transmissibility among chickens, as well as for their ability to occasionally infect humans. Of the 600 human cases in the last 10 years, mortality exceeds 50% http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/. Because these individuals were infected from direct contact with birds, controlling H5N1 strains in poultry is key to prevention of human infection. Recently it was demonstrated that as few as 4 substitutions in H5 from A/Vietnam1203/2004 (H5N1), together with reassortment, can generate a mammalian transmissible virus (Imai et al., 2012). Also, given the fact that some strains circulating in nature already have some of these changes, the possibility exists that a respiratory droplet transmissible strain could evolve in a mammalian host (Russell et al., 2012), as experimentally demonstrated by passaging of A/Indonesia5/2005 (H5N1) in ferrets (Herfst et al., 2012). These recent papers highlight the possibility of a pandemic virus evolving in nature.

The highly pathogenic H5N1 strains are universally lethal to chickens but only certain strains can kill ducks. Typically, ducks are able to limit H5N1-induced pathology (Sturm-Ramirez et al., 2005; Swayne and Pantin-Jackwood, 2006). Because of the acute onset of the infection, we predict that innate immune mechanisms are crucial in this response. The rapid detection of viruses by pattern recognition receptors initiates signalling cascades leading to production of type I interferons (IFN-α/β). Toll-like receptor 7 (TLR7) and retinoic acid-like receptor-I (RIG-I) are the main detectors for influenza viruses. While TLR7 is responsible for IFN-α production by leukocytes, cytosolic detection in the cells initially infected by influenza involves RIG-I. The cells initially infected with H5N1 strains are respiratory tract epithelial cells (Swayne and Pantin-Jackwood, 2006) (Swayne, 2007). We have previously shown that ducks have an intact and functional RIG-I, while chickens appear to lack the gene for the receptor (Barber et al., 2010). Chickens do have the MDA5 receptor, which uses the same signalling pathway as RIG-I, downstream of IPS-1/MAVS/CARDIF. Chicken DF-1 cells cannot respond to the RIG-I ligand, 5' triphosphate RNA (Barber et al., 2010; Karpala et al., 2011b), but transferring the duck receptor to chicken cells reconstitutes recognition (Barber et al., 2010). Chickens do respond to influenza, and cells can respond to *in vitro* transcribed-RNA in a 5' triphophosphate independent manner, involving MDA5 (Liniger et al., 2012). Thus the pathway downstream of IPS-1 is intact in chickens. Using duck RIG-I and the signalling downstream of MDA5, we showed that induction of the immune genes *MX1, IFNB* and *PKR* upon influenza infection was significantly higher in chicken DF-1 cells transfected with duck RIG-I (Barber et al., 2010). Additionally, the presence of duck RIG-I in DF-1 cells reduces the titre of both A/British Columbia 500/2005 (H5N2) and A/Vietnam 1203/2004 (H5N1) influenza strains (Barber et al., 2010). However, the full complement of genes that contribute to this antiviral effect are not known.

Our reconstitution of the RIG-I pathway in chicken cells allows us to exploit the genomic resources available for chickens to identify the avian genes that are altered downstream of RIG-I upon influenza infection. We used 44k Agilent chicken microarrays to compare RIG-I or empty-vector transfectants in a chicken embryonic fibroblast (DF-1) cell line following infection with BC500 or VN1203 viruses. These microarrays showed significantly increased transcripts of innate antiviral genes in chicken cells carrying duck RIG-I, as compared to empty vector following influenza infection. Thus, duck RIG-I is functional in chicken cells and elicits a robust immune response to influenza. The global transcriptional profile reveals important mediators of the RIG-I dependent antiviral response.

2. Methods

2.1. Cloning, transfections and infections

UMNSAH/DF-1 cells, a spontaneously immortalized chicken embryonic fibroblast cell line derived from East Lansing strain eggs (Schaefer-Klein et al., 1998) was obtained from ATCC, and maintained in DMEM supplemented with 10% FBS. Cloning of duck RIG-I into pcDNA3.1, cell transfections and infections were performed as previously described (Barber et al., 2010). Briefly, DF-1 cells were seeded overnight at 1.25×10^5 per well in 24-well plates. Cells were transfected with 1 μg of pcDNA-RIG or empty pcDNA3.1. Twenty-four hours after transfection, cells were infected at a multiplicity of infection (MOI) of 1 with A/mallard/BC/500/05 (H5N2) or reverse genetics A/Vietnam/1203/04 (H5N1) virus. L-(tosylamido-2-phenyl) ethyl chloromethyl ketone–treated trypsin (Worthington Biochemicals) was used for infection with BC500 at a low concentration (0.1 μg/mL). Exogenous trypsin was not added for infection with VN1203. Fifteen hours after infection, RNA was extracted from cells for quantitative RT-PCR (qRT-PCR) and microarrays. This timepoint was selected to identify downstream effectors of the interferon response. Control transfections carried out with 0.5 μg pDsRed-N1 consistently yielded transfection efficiencies of ~ 40–60% for DF-1 cells. Infection with BC500 and VN1203 at an MOI of 1 is expected to infect all cells, and most cells appeared infected by cytopathic effect. We previously observed signalling through RIG-I to our IFNβ luciferase reporter, and a decrease in viral titre in presence of RIG-I by 15 hours post-infection (Barber et al., 2010) in the supernatant of the same experimental samples used for RNA extraction. Influenza virus titer was determined by plaque assay on MDCK cells. Viral titre for BC500 was 5×10^5 in RIG-I transfected cells, and 1.5×10^6 in empty-vector transfected cells, while VN1203 replicated to 2×10^6 in DF-1 cells transfected with empty vector, or 1×10^6 in RIG-I transfected cells (Barber et al., 2010). VN1203 was handled in biosafety level 3+ facilities approved by the United States Department of Agriculture and Center for Disease Control and Prevention.

2.2 Microarray hybridization and analysis

Chicken microarrays from Agilent-015068, Chicken Gene Expression 4×44K (Agilent, Santa Clara, CA) consisting of 42,034 60-mer *in situ* synthesized oligonucleotides were used. Labelled cRNA was prepared from 500 ng of total RNA using the Agilent labelling protocol, and microarray hybridization was performed at 65°C for 18 hours in Agilent's microarray hybridization chambers, followed by wash procedures according to the manufacturer's recommended protocols. Microarrays were carried out using the reference design, whereby RNA from each experimental sample is compared to a single reference RNA sample. Our reference RNA sample was prepared from uninfected, untransfected DF-1 cells. The testing samples (Cy5) were co-hybridized with uninfected, untransfected reference DF-1 samples (Cy3). Three independent experiments were performed with duck RIG-I transfected cells along with empty vector transfected cells, followed by BC500 infection; while two replicate experiments of transfections followed by VN1203 infection were performed. The microarrays were scanned in an Agilent scanner at 3 μm resolution, and the array data was extracted with Agilent feature extraction software (version 10.5.1.1) using the GE2_105_Jan09 protocol. Reproducibility and reliability of each single microarray was assessed using Quality Control report data. Gene expression ratios compared with the reference control (uninfected, untransfected DF-1 cells) were calculated and log2 transformed. Lowess normalization on background-subtracted signal intensity was performed to correct the intensity bias, and additional filtering steps, including valid measurement in at least 50% of the tested samples, were applied to further improve the quality of the data set. The relative differential gene expression between RIG-I transfected and empty vector transfected samples was reported as the difference of log2 ratios between these two groups, and a p-value using t-test was calculated for each gene as statistical

measurement of the replicates. The selection of the genes for Gene Ontology term analysis (DAVID, http://david.abcc.ncifcrf.gov (Huang et al., 2009) was based on a change of at least twofold and a probability of >95% ($P < 0.05$) of differential expression in at least two experiments. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE29596 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSEGSE29596).

2.3 Analysis of immune gene expression by quantitative RT-PCR (qRT-PCR)

RNA was extracted using TRIzol (Invitrogen), followed by purification from the final aqueous phase using the RNeasy Mini Kit (Qiagen). RNA was DNAse treated and first-strand cDNA synthesized using a mix of random primers (Invitrogen) and Oligo$(dT)_{20}$ primer (50 μM) (Invitrogen) using the SuperScript III reverse transcriptase kit (Invitrogen). Gene-specific primers and probe sets (Table 1) were designed using the Primer Express Version 3.0 software and IDT real-time primer design program Sirtool. Primers were obtained from Integrated DNA Technologies and validated for linear amplification, and relative amplification efficiency matched to the *GAPDH* endogenous control. All qRT-PCR assays were performed using Fast Start TaqMan Probe Master Mix (Roche) in the Applied Biosystems 7500 real-time PCR System machine (Applied Biosystems). All experimental samples were assayed in triplicate. Target gene expression was normalized to a constitutively expressed endogenous control gene *GAPDH*. Quantification was carried out using relative quantitation of gene expression (ΔΔCT) and the analysis was performed with the 7500 Fast System software version 1.4 (Applied Biosystems).

3. Results

3.1. Chicken cells expressing duck RIG-I induce immune genes in response to BC500

To define the role of RIG-I in antiviral gene expression during influenza infections, we conducted microarray analyses. Using chicken DF-1 embryonic fibroblast cells, which lack RIG-I, we compared cells transfected with duck RIG-I or empty vector at 15 hours post infection (hpi) with a low pathogenic avian influenza A/mallard/British Columbia/500/2005 (H5N2) (BC500) at a multiplicity of infection (MOI) of 1. Our data show increased expression of a number of genes in the presence of RIG-I, compared to empty vector, following infection with BC500. Of genes that were altered in expression by more than twofold in the presence of RIG-I, the fold-change of 206 of them was statistically significant ($P < 0.05$) across three independent experiments.

Our results show that genes with altered expression in chicken cells expressing duck RIG-I upon infection with BC500 group predominantly within host defense and immune response Gene Ontology (GO) categories (Table 2). Several immune genes appear in multiple hierarchical GO classes. Of the 206 differentially expressed genes (with differences greater than twofold), 21 had known roles in the immune response, with the fold-change of 6 of them being statistically significant across replicates (Table 3). These genes are particularly involved in the innate immune defences to influenza.

3.2. Chicken cells expressing duck RIG-I induce immune genes in response to VN1203

To determine the role of RIG-I in detection and downstream gene expression in response to a highly pathogenic avian influenza, we compared DF-1 cells transfected with duck RIG-I or empty vector, 15 hours post-infection with the highly pathogenic avian influenza A/Vietnam/1203/2004 (H5N1). RNA from two independent experiments were used for microarray hybridization and compared to the RNA reference sample from uninfected, untransfected DF-1 cells. Of the RIG-I responsive genes altered more than twofold by

VN1203 infection, 377 genes had fold changes that were statistically significant (P value of < 0.05) across both replicates.

RIG-I expression and detection of influenza induced the expression of a wide array of genes in different functional groups, including those associated with transcriptional and translational regulation, and the immune response (Table 4). Twenty-eight genes were identified as immune genes and their expression was augmented by the presence of RIG-I, with fold-change of 21 of these genes being statistically significant between biological replicates (Table 5). The presence of RIG-I augmented the expression of several key innate immune genes with known or potential antiviral roles in influenza defense.

3.3. Innate immune genes show increased expression in chicken cells expressing duck RIG-I

Many immune genes were induced by infection with BC500 and VN1203 and their expression is increased in chicken cells expressing duck RIG-I, relative to empty vector (Figure 1). A heatmap of selected immune genes graphically shows their upregulation in the transfected DF-1 cells carrying RIG-I, compared to empty vector. Of these, several have known roles in influenza defense, including *IFIT, MX1, OASL* and *IFIH1* (*MDA5*). Some components of the signalling pathway downstream of RIG-I are also upregulated, including *IRF7* and *IFNB*. All of the differentially expressed immune genes were upregulated by RIG-I, consistent with the role of the gene in eliciting a Type I interferon response upon influenza infection. MyD88 was down regulated in all cells, relative to the untransfected, uninfected DF-1 cells.

To validate the fold-increases indicated by the microarray analyses, we used qRT-PCR to examine the expression of key innate immune genes including, *IFIT5, MX1, OASL, PKR, IFNB* and influenza matrix gene (Figure 2). By qRT-PCR, the presence of RIG-I augmented the expression of *IFNB* following infection with either BC500 and VN1203, by 5-fold and 6-fold respectively. All ISGs showed increased expression in the RIG-I transfected cells, compared to the cells carrying empty vector. *IFIT5* was 36-fold or 12-fold higher, while *MX1* was 48-fold or 12.4 fold higher in BC500 and VN1203 infected cells, respectively. The presence of RIG-I decreased the influenza matrix gene transcript, consistent with the decrease in viral replication we previously measured by plaque assay. Thus the qRT-PCR results were consistent with the fold-changes observed by microarray.

4. Discussion

Through a microarray approach, we have characterized the transcriptional response induced by influenza in chicken cells in the absence or presence of duck RIG-I. We demonstrate that the induction of key innate immune genes by both BC500 and VN1203 was augmented by the presence of duck RIG-I in chicken cells. Many of the immune genes that were induced in cells carrying duck RIG-I correspond to those reported as RIG-I responsive in a microarray comparison of wildtype and RIG-$I^{-/-}$ mouse fibroblasts, including *IFNB, IRF1, IRF7, MDA5, IFIT* and *MX1* (Loo et al., 2008). In the mouse, these genes were defined as the RIG-I bioset, i.e. genes that were induced by influenza infection that were dependent on RIG-I, and expression was absent in RIG-$I^{-/-}$ mouse embryonic fibroblasts. RIG-I stimulates the production of IFNβ, which is essential for protection against influenza (Koerner et al., 2007), because it orchestrates the production of antiviral interferon stimulated genes (ISGs). Thus, duck RIG-I regulates a similar subset of genes in the pathway, as well as downstream effectors. This defines a set of candidate avian antiviral effectors, which can be mined for those with direct antiviral function. We expect that some of these effectors are responsible for our previous observation that duck RIG-I reduces influenza virus titre in chicken DF-1 cells (Barber et al., 2010).

Signalling through duck RIG-I, like mouse RIG-I, also upregulates the genes in its own pathway. Both *IRF7* and *IFNB* are induced by influenza infection in chicken cells transfected with duck RIG-I. In chickens, the gene named *IRF3* more closely resembles *IRF7*, and like *IRF7* is inducible by viral infection and poly (I-C) (Grant et al., 1995). *IRF3* appears to have been selectively lost in avian species (Cormican et al., 2009). It is not known whether IRF7 replaces IRF3 in the signalling pathway downstream of RIG-I, i.e. whether signalling leads to nuclear translocation of chicken IRF7 to drive expression of IFNβ, and the reagents to address this question experimentally in chicken cells do not exist. However, the demonstration of augmentation of expression of downstream ISGs in chicken cells expressing RIG-I implies that this signalling pathway is intact.

DF-1 cells expressing duck RIG-I demonstrated a 33-fold increase in *Mx1* expression in response to infection with BC500, and 21-fold by VN1203. The interferon-inducible Mx proteins confer antiviral function in transfected cells and transgenic animals (Engelhardt et al., 2004; Pavlovic et al., 1995; Pavlovic et al., 1990). In mice, Mx1 is interferon-induced and protects against lethal infection by the 1918 pandemic H1N1 strain and VN1203 (Tumpey et al., 2007). Chickens have a single, polymorphic, interferon-stimulated *Mx1* gene (Schumacher et al., 1994). Although an original report suggested chicken *Mx* lacked antiviral activity (Bernasconi et al., 1995), allelic variants have been identified that have antiviral activity (Ko et al., 2002; Ko et al., 2004b) and recent data showed antiviral effects of some alleles against two different HPAI strains (Ewald et al., 2011). However, siRNA knockdown of Mx in chicken embryonic fibroblasts carrying either allelic variant does not impair the antiviral response to low or highly pathogenic avian influenza, and both variants were shown to lack GTPase activity, which may preclude antiviral function (Schusser et al., 2011).

Protein kinase R (*PKR*) (*EIF2AK2*) was induced in chicken cells transfected with duck RIG-I after both BC500 and VN1203 infection, at a P value < 0.05 for the BC500 but not VN1203 infection. Chicken PKR is a polymorphic protein with demonstrated antiviral function against VSV infection (Ko et al., 2004a). PKR is required for IFNα/β induction in response to several viruses, but not influenza (Schulz et al., 2010). Recent data shows a role for PKR in the formation of antiviral stress granules involved in orchestration of RIG-I and signalling components, and is inhibited by NS1 (Khaperskyy et al., ; Onomoto et al., 2012). The function of avian PKR is unknown.

The gene encoding OAS-like protein (*OASL*) was induced approximately 4-fold by both viruses in the presence of duck RIG-I, compared to empty vector transfected cells. Chicken *OASL*, encodes a domain typical of 2′–5′ oligoadenylate synthase proteins and also two ubiquitin-like (UbL) domains (Tatsumi et al., 2000; Tatsumi et al., 2003). While human OASL lacks oligoadenylate synthetase (OAS) activity, the UbL domains are necessary for antiviral function (Marques et al., 2008). Human OASL inhibits replication of RNA viruses (Marques et al., 2008) (Ishibashi et al., 2010), and is upregulated by influenza through IRF3 (Melchjorsen et al., 2009). *OASL* was upregulated by influenza infection in chicken lung tissues, and recovered in a screen to identify genes involved in survival of chickens to lethal influenza strains (Uchida et al., 2012).

In both BC500 and VN1203 infections, interferon-stimulated genes were significantly induced. *IFIT5*, a member of the IFN-induced proteins with tetratricopeptide repeats (IFIT) family, was induced in cells carrying duck RIG-I by 28-fold upon infection with BC500 or 14-fold by VN1203. IFIT proteins sequester viral RNA transcripts in a multiprotein complex (Pichlmair et al., 2011). *ISG12-2* was induced in RIG-I transfected compared to empty-vector transfected DF-1 cells by 8-fold in response to BC500 and 15-fold by VN1203. *ISG12-2* is induced in chicken lung and tracheal organ culture after influenza infection and

is predicted to have antiviral function through an unknown mechanism (Reemers et al., 2009; Reemers et al., 2010). Human ISG12a has been implicated in sensitizing cells for apoptosis by mitochondrial membrane destabilization (Rosebeck and Leaman, 2008). Apoptotic cell death has been proposed as a mechanism to limit influenza spread, and this effect is much greater in duck than chicken cells (Kuchipudi et al., 2011). RIG-I may be inducing apoptosis upon influenza infection through the pronounced expression of *ISG12-2*.

CCL19 expression is interferon-dependent (Pietila et al., 2007) and was induced by 9-fold by BC500 and 7-fold by VN1203 infection in chicken cells carrying duck RIG-I compared to empty vector, while *CCL20* was induced by 3 to 4-fold. Both CCL19 (Forster et al., 2008; Marsland et al., 2005) and CCL20 (Le Borgne et al., 2006) are involved in recruitment of dendritic cells and naïve lymphocytes to initiate adaptive immune responses. CCL19 was upregulated in VN1203 infected duck lung tissues (Fleming-Canepa et al., 2011).

Interestingly, many of the immune genes that were significantly induced downstream of duck RIG-I in both the BC500 and VN1203 infections were augmented by less in the VN1203-infected samples (*IFIT5, IRF1, MX1* and *ZNFX1*). This contrasts with expression in human primary macrophages, where many innate immune genes were induced more by H5N1, than by H1N1 (Lee et al., 2009). Similarly, our analysis in ducks showed VN1203 infection induced a 200-fold induction of RIG-I, and greater than 1000-fold induction of *IFIT* and *OASL*. In comparison, these genes are induced by BC500 infection 80 and 95-fold, relative to mock infected ducks (Vanderven et al., 2012). In chicken cells expressing RIG-I, the difference between innate immune responses to these viruses was less dramatic. VN1203 replicated to a higher titre in DF-1 cells compared to BC500-infected cells (Barber et al., 2010), and this may have resulted in an earlier peak of innate responses. VN1203 infection also induced higher expression of innate immune genes in cells transfected with empty vector, making the difference due to RIG-I appear less. Differences in response to these viruses may also be due to interference in the pathway by the respective NS1 proteins. The influenza NS1 protein is known to modulate the interferon-mediated antiviral response (Garcia-Sastre, 2011; Garcia-Sastre et al., 1998; Zielecki et al., 2010) and the RIG-I pathway is a known target of NS1 through interaction with TRIM25 (Gack et al., 2009) or binding vRNA (Rehwinkel et al., 2010).

While many ISGs were induced more downstream of RIG-I in BC500-infected DF-1 cells, a notable exception was the expression of Th1 cytokines, where *IFNG* (5.7-fold higher), *IL6* and *IL1*β, were augmented more in the VN1203 infected DF-1 cells. Upregulation of proinflammatory cytokines was also observed in primary human macrophages following infection with A/Vietnam/3212/2004 (H5N1) compared to a seasonal isolate A/HongKong 58/1998 (H1N1) (Lee et al., 2009). However, the profile of upregulated cytokine genes in avian fibroblast cells does not closely mirror human macrophages. *TNF* is missing from the avian genome, and *IL18* and other cytokines (*CCL8, CXCL10*) are not on the microarray, while others (*TNFSF10* and *CCL5*) are expressed more in BC500 infected cells than VN1203. High expression of proinflammatory cytokines was also seen in chicken lung and spleen tissues at 36 hpi with VN1203 (Karpala et al., 2011a). The induction of proinflammatory cytokines is thought to contribute to the pathology of H5N1 infections in humans and chickens.

Our experiment does not address which genes are upregulated by influenza infection in untransfected chicken cells. However, others have used gene expression profiling by microarray to examine the early transcriptional responses to influenza A viruses in chickens (Reemers et al., 2009; Reemers et al., 2010; Sarmento et al., 2008b) (Sarmento et al., 2008a). An examination of the genes expressed in primary chicken embryonic fibroblasts infected with two H5N1 HPAI strains, A/Ck/Hong Kong/220/97 and A/Egret/Hong Kong/

757.2/02 at 4 hpi showed that of 191 genes that demonstrated a twofold induction, 10 were associated with the immune response (Sarmento et al., 2008a). None of these genes appear to be associated with the innate response, and the genes induced in RIG-I transfected chicken cells are notably absent. However, the early 4 hours post-infection timepoint, compared to our later 15 hpi timepoint, is likely to have been too early to detect ISGs. Indeed, in chicken lung tissues at 16 hpi with H9N2, *MX, ISG12-2, OASL* are upregulated (Reemers et al., 2010) albeit less than in RIG-I transfected epithelial cells. This upregulation is likely due to MDA5 and TLR recognition of influenza. Our experiment also cannot rule out that some genes are upregulated due to the higher expression of TLR3 and MDA5 in chicken DF-1 cells expressing RIG-I. MyD88 was downregulated in all cells relative to uninfected, untransfected control DF-1 cells, therefore it is unlikely that TLR7 is contributing to gene induction observed in our microarrays.

The genes identified by comparison of influenza-infected DF-1 cells expressing RIG-I or empty vector are a subset of the genes identified in the RIG-I knockout mouse compared to wildtype. Of the RIG-I bioset genes, or genes downstream of RIG-I (Loo et al., 2008), some were not observed in our microarrays. Some, like RIG-I itself, appear to be missing from the chicken genome (*IRF3, ISG15* and other *IFITs*) while others were not included in the microarray (*RSAD2* and *OAS*). Among the genes downstream of RIG-I and TNF pathways in human macrophages that are upregulated more than 10-fold by infection with H5N1 (*IFIT, OASL, PTGS2*) (Lee et al., 2009) these are also upregulated in DF-1 cells with RIG-I. Other genes (*PMAIP, GBP4, CCL8, CXCL10*) were not on the chicken microarray. Finally, increased expression of *IFITM5* was unexpected since it does not inhibit influenza (Huang et al., 2011) and expression is restricted to developing bone in mammals (Moffatt et al., 2008). *IFITM1*, which has antiviral function, is not on the microarray.

It is not clear how the loss of RIG-I in chickens contributes to the inability of chickens to defend against highly pathogenic influenza strains. Chickens certainly have an interferon response to influenza, and induce an inflammatory response upon infection with VN1203 (Karpala et al., 2011a). Chickens also have a strong innate immune response to other RNA viruses such as Newcastle Disease Virus (NDV) upregulating many innate immune genes including *IFIT5, MX1, ISG12-2* and *CCL19* in spleen (Rue et al., 2011). Furthermore, influenza-infected chicken trachea and tracheal organ cultures express numerous innate immune genes (Reemers et al., 2009). Also contributing to the overall response, infection with highly pathogenic influenza consistently induced high levels of Type I interferon in chicken splenocytes, likely through the TLR7 pathway (Moulin et al., 2011). Chicken MDA5 also contributes to influenza detection in chicken epithelial cells (Liniger et al., 2012), but knockdown does not affect the titre of influenza strain A/Wyoming/3/03 (H3N2) (Karpala et al., 2011b), thus it is not completely clear what contribution MDA5 makes to host defense in the chicken. Lack of protection by RIG-I in chicken epithelial cells is undoubtedly detrimental in an acute influenza infection, given that these cells are on the frontline of defense against influenza viruses.

The possibility of augmenting influenza defense in chickens through transgenesis is compelling. Lyall and colleagues successfully used such an approach by creating a transgenic chicken expressing a short-hairpin RNA that inhibits the influenza polymerase. While the chickens still succumbed to infection, importantly, transmission of influenza to uninfected chickens was reduced (Lyall et al., 2011). Because duck RIG-I can induce many known antiviral mediators in chicken cells, a transgenic chicken expressing duck RIG-I could theoretically translate to increased protection to influenza *in vivo*. Chickens expressing duck RIG-I in tracheal epithelial cells might be expected to control influenza virus replication more efficiently. There are certainly concerns to creating a transgenic chicken with RIG-I, such as creating a chicken that can replicate HPAI strains without

symptoms. However, a superior innate immune response may limit viral replication and thus reduce the emergence of highly pathogenic strains.

Here we compare global gene expression by microarray for chicken DF-1 cells infected with low or highly pathogenic strains of influenza in the presence or absence of duck RIG-I. Overall, the microarray results demonstrate that signalling through duck RIG-I can induce important antiviral genes.

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Research Highlights

- 44K Agilent microarray analyses of chicken DF-1 cells with duck RIG-I or empty vector
- transcriptome of H5N2 infected cells with duck RIG-I or empty vector
- transcriptome of H5N1 infected cells with duck RIG-I or empty vector
- identification of genes augmented in expression by duck RIG-I
- quantitative real-time PCR confirms upregulation of key innate immune genes

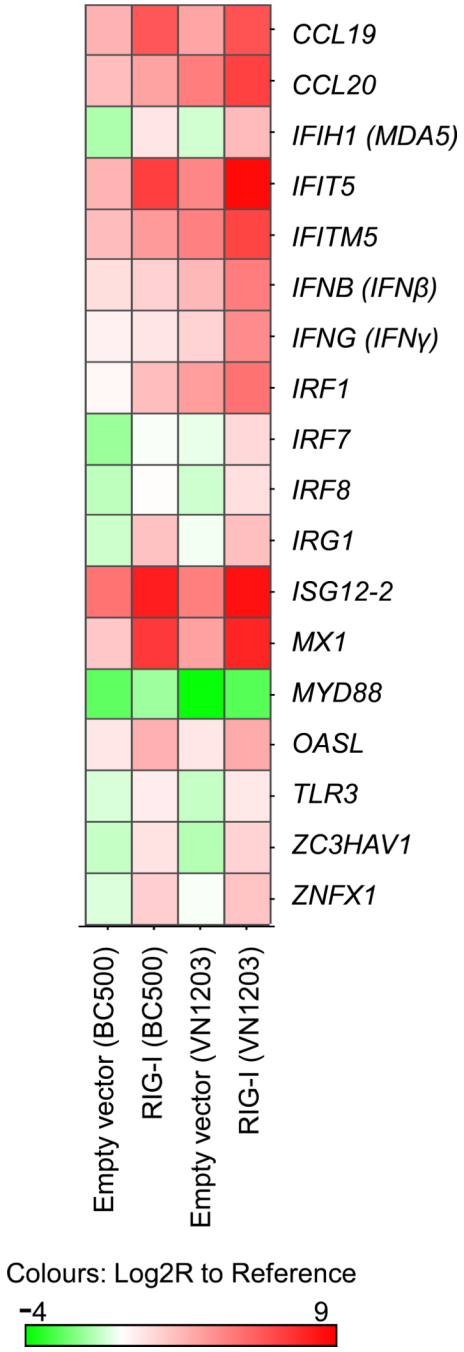

Figure 1. Upregulation of RIG-I-responsive innate immune genes in influenza infected chicken cells expressing duck RIG-I

A heat map shows the differential expression of RIG-I-responsive immune genes following influenza infection. Chicken embryonic fibroblasts (DF-1 cells) were transfected with duck RIG-I or empty vector. 24 hours post-transfection, the DF-1 cells were infected with A/mallard/BC/500/2005 (H5N2) (BC500) or A/Vietnam/2004 (H5N1) (VN1203) at a multiplicity of infection (MOI) of 1. At 15 hpi, cellular RNA was extracted for microarray analysis. Micorarray hybridizations were normalized to a reference RNA sample (untransfected, uninfected DF-1 cell RNA). Heatmap is generated from three independent

experiments of RIG-I (6 arrays) and empty vector for BC500, or two independent experiments VN1203 (4 arrays).

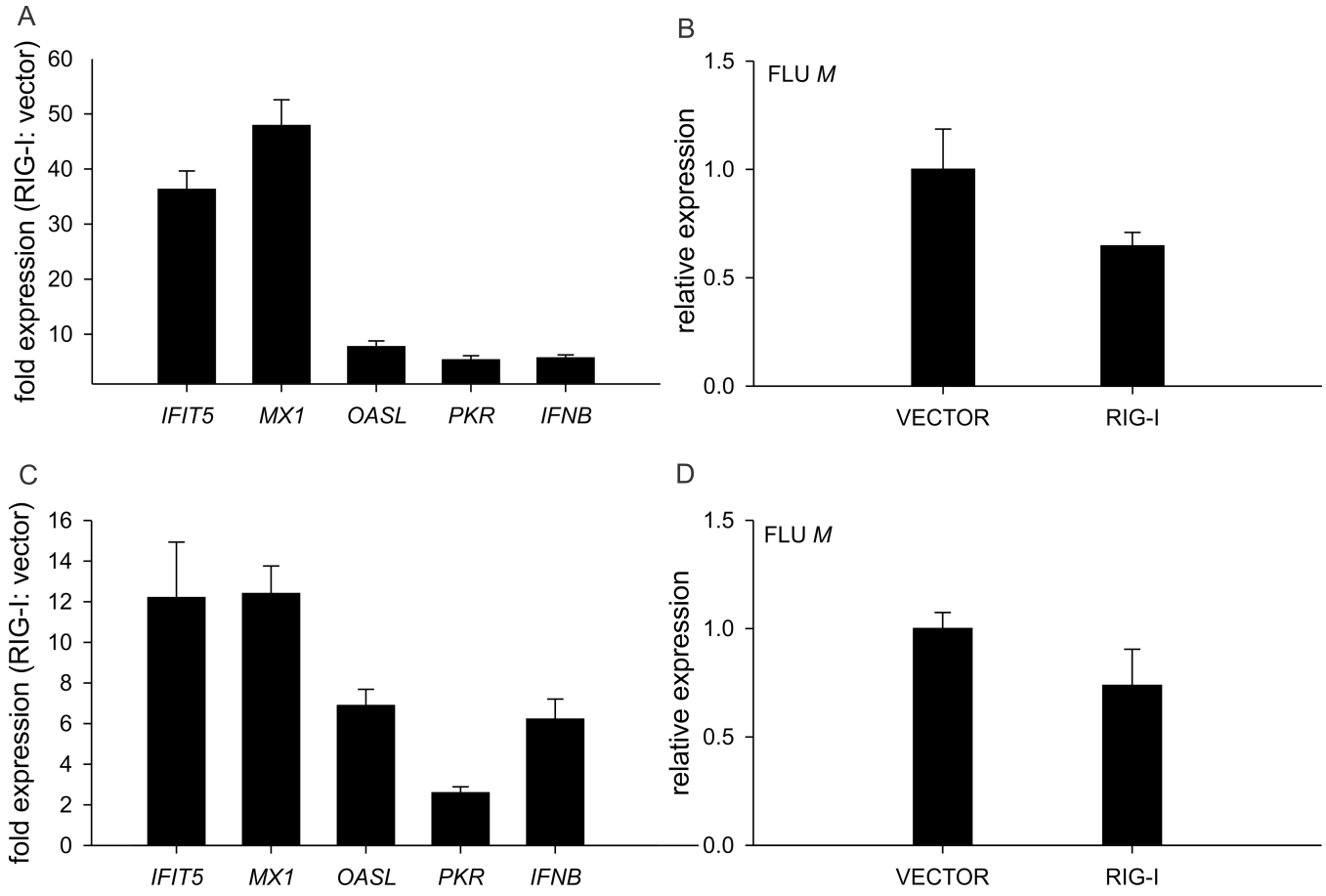

Figure 2. Upregulation of key innate immune genes in influenza infected chicken cells expressing duck RIG-I

RIG-I–transfected DF-1 cells respond to BC500 infection (MOI, 1) (A) or VN1203 infection (C) with increased expression of chicken *IFN*β and the IFN-stimulated genes *IFIT, MX1, OASL* and *PKR*, and decreased influenza matrix gene expression (B and D), compared to empty vector transfected cells. RNA was extracted from cells for qRT-PCR 15 hpi, and fold difference in gene expression calculated for RIG-I and empty-vector transfected DF-1 cells. A representative of three independent experiments (BC500) or two independent experiments (VN1203) is shown and error bars show $RQ_{Min/Max}$ at a 95% confidence level.

Table 1

Primer and probe sequences for qRT-PCR.

Gene	Primer and Probe Sequence (5'→3')
InfA *M*	Primer 1: GACCRATCCTGTCACCTCTGAC
	Primer 2: AGGGCATTYTGGACAAAKCGTCTA
	Probe:/56-FAM/ TGCAGTCCT/Zen/CGCTCACTGGGCACG/3IABkFQ
IFIT5	Primer 1: CAGAATTTAATGCCGGCTATGC
	Primer 2: TGCAAGTAAAGCCAAAAGATAAGTGT
	Probe:/56- FAM-TCTGAAGCG/Zen/TGCACTGAAACTGAATCCAA-3IABkFQ
MX1	Primer 1: GGACTTCTGCAACGAATTG
	Primer 2: TCCCACAAGTTCATCTGTAAG
	Probe:/56-FAM/ CTTCACCTC/Zen/CGCAATCCAGCAAGA/3IABkFQ
OASL	Primer 1:ACATCCTCGCCATCATCGA
	Primer 2: GCGGACTGGTGATGCTGACT
	Probe:/56-FAM/ AGTGCCTCC/Zen/CGACGCTGTCCTTC/3IABkFQ
PKR	Primer 1: CATTGAGGCACATTTCACAGATTATA
	Primer 2: GGTCAATCAGTTCATACCTTATGTTGA
	Probe:/56-FAM/ CTGAACACC/Zen/TCTGCTGGCCTTACTGTCA/3IABkFQ
IFNB	Primer 1: TCCAACACCTCTTCAACATGCT
	Primer 2: TGGCGTGTGCGGTCAAT
	Probe:/56-FAM/AGCAGCCCA/Zen/CACACTCCAAAACACTG/3IABkFQ
GAPDH	Primer 1:GGTGCTAAGCGTGTTATCATCTCA
	Primer 2: CATGGTTGACACCCATCACAA
	Probe:/56-FAM/ CTCCCTCAG/Zen/CTGATGCCCCCATG/3IABkFQ

Table 2

Categories of upregulated genes in RIG-I transfected compared to empty vector transfected DF-1 cells, 15 hpi with BC500.

Gene Ontology Term	P-value	Genes
GO:0006955~immune response	1.05E-04	*OASL, TNFSF10, CCL20, TLR15, IRF7, IRF8, TNFSF15, CCL19, TLR3*
GO:0006952~defense response	0.0799	*NFKBIZ, TLR15, TLR3, GAL12*
GO:0042107~cytokine metabolic process	0.0851	*IRF7, TNFSF15*

Table 3

A selection of immune genes upregulated more than twofold in RIG-I transfected DF-1 cells, 15 hpi with BC500.

	Gene Name	Accession Number	Fold Change	P-value
CCL19	chemokine (C-C motif) ligand 19	BX929857	9.25	0.0899
CCL20	chemokine (C-C motif) ligand 20	AB101005	3.34	0.398
DHX58 (LGP2)	probable ATP-dependent RNA helicase DHX58	AM070728	3.67	0.0724
EIF2AK2 (PKR)	**eukaryotic translation initiation factor 2-alpha kinase 2**	**AB125660**	**3.22**	**0.0269**
GAL12	beta-defensin 12	AY534898	2.48	0.0678
IFIH1 (MDA5)	**interferon-induced helicase C domain-containing protein 1**	**CR385175**	**7.33**	**0.0461**
IFIT5	**interferon-induced protein with tetratricopeptide repeats 5**	**XM_421662**	**27.5**	**0.0200**
IFITM5	interferon induced transmembrane protein 5	XM_420924	2.31	0.0686
IRF1	**interferon regulatory factor 1**	**L39766**	**3.98**	**0.0364**
IRF7	interferon regulatory factor 7	U20338	2.81	0.264
IRF8	interferon regulatory factor 8	L39767	2.15	0.0805
ISG12-2	interferon stimulated gene 12-2 protein-like	NM_001001296	8.23	0.0660
MX1	**myxovirus (influenza virus) resistance 1, interferon-inducible protein p78**	**AB088533**	**32.6**	**0.0096**
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	AJ721113	2.36	0.406
OASL	2'–5'-oligoadenylate synthetase-like	NM_205041	3.81	0.0563
TLR15	toll-like receptor 15	DQ267901	2.76	0.557
TLR3	toll-like receptor 3	AY633575	2.37	0.457
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	AJ720191	2.16	0.507
TNFSF15	tumor necrosis factor (ligand) superfamily, member 15	BX930081	2.79	0.101
ZC3HAV1	zinc finger CCCH-type, antiviral 1	CR524013	3.73	0.0910
ZNFX1	**zinc finger, NFX1-type containing 1**	**XM_417395**	**7.96**	**0.0448**

Genes with statistically significant ($P < 0.05$) fold-differences across three replicates are indicated in bold.

Table 4

Categories of upregulated genes in RIG-I transfected compared to empty vector transfected DF-1 cells, 15 hpi with VN1203.

Gene Ontology Term	P-value	Genes
GO:0009615~response to virus	2.14E-05	*IFNB, MYD88, IRF7, IFNG, TLR3*
GO:0006955~immune response	1.90E-04	*OASL, MYD88, CCL20, IRF7, IRF8, IFNG, ZAP70, CCL19, TLR3, IL10*
GO:0005125~cytokine activity	5.48E-04	*IFNB, CCL20, IFNG, CCL19, IL10, THPO*
GO:0003950~NAD+ ADP-ribosyltransferase activity	8.11E-04	*PARP9, PARP12, ZC3HAV1, PARP14*
GO:0045080~positive regulation of chemokine biosynthetic process	0.00131	*MYD88, IFNG, TLR3*
GO:0006952~defense response	0.00218	*IFNB, LSP1, LIPA, MYD88, IFNG, TLR3, IL10*
GO:0001816~cytokine production	0.0233	*LIPA, MYD88, IRF7*
GO:0045348~positive regulation of MHC class II biosynthetic process	0.0298	*IFNG, IL10*

Table 5

A selection of immune genes upregulated more than twofold in RIG-I transfected DF-1 cells 15 hpi with VN1203 HPAI.

	Gene Name	Accession Number	Fold Change	P-value
CCL19	**chemokine (C-C motif) ligand 19**	**BX929857**	**7.48**	**0.0201**
CCL20	**chemokine (C-C motif) ligand 20**	**AB101005**	**4.33**	**0.00970**
EIF2AK2 (PKR)	eukaryotic translation initiation factor 2-alpha kinase 2	AB125660	2.44	0.0698
EPAS1	endothelial PAS domain protein 1	AF129813	2.88	0.376
GDNF	glial cell derived neurotrophic factor	AF176017	2.20	0.378
IFIH1 (MDA5)	**interferon-induced helicase C domain-containing protein 1**	**CR385175**	**18.7**	**0.0159**
IFIT5	**interferon-induced protein with tetratricopeptide repeats 5**	**XM_421662**	**13.8**	**0.0138**
IFITM5	**interferon induced transmembrane protein 5**	**XM_420924**	**4.39**	**0.0456**
IFNB	**interferon beta**	**AY831397**	**4.21**	**0.00862**
IFNG	**interferon, gamma**	**AY705909**	**5.66**	**0.0309**
IL10	interleukin 10	AJ621254	2.38	0.245
IRF1	**interferon regulatory factor 1**	**L39766**	**2.91**	**0.0474**
IRF7	**interferon regulatory factor 7**	**U20338**	**3.12**	**0.0314**
IRF8	**interferon regulatory factor 8**	**L39767**	**3.70**	**0.00949**
IRG1	**immunoresponsive 1 homolog (mouse)**	**AJ720739**	**5.44**	**0.00689**
ISG12-2	**ISG12-2 protein-like**	**NM_001001296**	**14.8**	**0.00209**
LIPA	**lipase A, lysosomal acid, cholesterol esterase**	**XM_421661**	**2.73**	**0.0140**
LSP1	lymphocyte-specific protein 1	AB101641	4.36	0.171
MAFB	**v-maf musculoaponeurotic fibrosarcoma oncogene homolog B**	**NM_001030852**	**2.81**	**0.0282**
MX1	**myxovirus (influenza virus) resistance 1, interferon-inducible protein p78**	**AB088533**	**20.8**	**0.0209**
MYD88	**myeloid differentiation primary response gene (88)**	**CR389938**	**2.34**	**0.00948**
OASL	**2'–5'-oligoadenylate synthetase-like**	**NM_205041**	**4.43**	**0.0226**
THPO	thrombopoietin	AY613434	2.06	0.124
TLR3	**toll-like receptor 3**	**AY633575**	**3.23**	**0.0137**
TLX1	**t-cell leukemia homeobox 1**	**AF071874**	**2.44**	**0.0156**
ZAP70	zeta-chain-associated protein kinase 70	XM_418206	2.11	0.120
ZC3HAV1	**zinc finger CCCH-type, antiviral 1**	**CR524013**	**6.43**	**0.0113**
ZNFX1	**zinc finger, NFX1-type containing 1**	**XM_417395**	**4.51**	**0.0145**

Genes with fold-changes that are statistically significant ($P < 0.05$) across two replicates are indicated in bold.