Association of RIG-I with innate immunity of ducks to influenza

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Ducks and wild waterfowl perpetuate all strains of influenza viruses in nature. In their natural host, influenza viruses typically cause asymptomatic infection and little pathology. Ducks are often resistant to influenza viruses capable of killing chickens. Here, we show that the influenza virus sensor, RIG-I, is present in ducks and plays a role in clearing an influenza infection. We show evidence suggesting that RIG-I may be absent in chickens, providing a plausible explanation for their increased susceptibility to influenza viruses compared with ducks. RIG-I detects RNA ligands derived from uncapped viral transcripts and initiates the IFN response. In this study, we show that the chicken embryonic fibroblast cell line, DF-1, cannot respond to a RIG-I ligand. However, transfection of duck RIG-I into DF-1 cells rescues the detection of ligand and induces IFN-β promoter activity. Additionally, DF-1 cells expressing duck RIG-I have an augmented IFN response resulting in decreased influenza replication after challenge with either low or highly pathogenic avian influenza virus. Implicating RIG-I in the antiviral response to an infection in vivo, we found that RIG-I expression is induced 200 fold, early in an innate immune response in ducks challenged with the H5N1 virus A/Vietnam/1203/04. Finding this natural disease resistance gene in ducks opens the possibility of increasing influenza resistance through creation of a transgenic chicken.

comparative immunology | evolution | H5N1 avian influenza | animal reservoir | chicken

All strains of influenza A virus are perpetuated in the duck reservoir (1). Although rare, when influenza viruses cross over from the avian reservoir into humans they may evolve into pandemic strains. After emerging in 1996, the highly pathogenic avian influenza (HPAI) H5N1 viruses have evolved into multiple clades and subclades and have spread to Europe and Africa, probably by wild birds (2). Although H5N1 viruses do not frequently infect humans they are often highly pathogenic and cause death in otherwise healthy individuals. Among 445 laboratory-confirmed human H5N1 infections, the mortality rate is 60% (3).

In 2002, H5N1 viruses began killing wild waterfowl (4, 5), including ducks, which is highly unusual and a disruption of the normal ecology (1). Typically, ducks do not show signs of disease upon infection with influenza. H5N1 viruses may rapidly adapt, becoming less pathogenic to ducks (6, 7), suggesting that virus evolution helps maintain the balance between virus and host. Many strains of highly pathogenic H5N1 now cause asymptomatic infection in ducks, making them a "Trojan horse" in the spread of these influenza viruses (6–9). In contrast, many of these same H5N1 viruses cause 100% mortality in chickens within hours or days. We therefore reasoned that superior innate immunity might protect the duck during this critical period.

The molecular basis of the natural resistance of ducks to influenza infection is unresolved. A successful innate immune response to influenza infection involves a robust, yet transient induction of IFN-stimulated antiviral genes. RIG-I is a cytoplasmic RNA sensor (10), and triggering by influenza virus leads to production of IFN-β and expression of downstream IFN-stimulated antiviral genes (11). A hallmark of lethal influenza virus infection is interference with expression of RIG-I and

downstream genes (12). Further, RNA viruses have been shown to be more virulent and replicate to higher levels in mice lacking RIG-I (13). Considering this, we speculated that the outcome of influenza infection in birds may also be determined by RIG-I and the downstream subset of IFN-stimulated genes. Here we show that the duck, the natural host of influenza, has an intact and functional RIG-I that is induced upon infection with H5N1 A/Vietnam/1203/04 (VN1203), whereas chickens appear to lack RIG-I. However, we demonstrate that transfection of duck RIG-I into a chicken embryonic fibroblast cell line can restore recognition of RIG-I ligand and initiate an antiviral IFN response.

Results

RIG-I Is Present in Ducks and Apparently Absent in Chickens. Given its role in antiviral defense in mammals, we searched for avian homologues of RIG-I. We identified a duck (*Anas platyrhynchos*) RIG-I homologue with 53% amino acid identity to human and 78% identity to zebra finch RIG-I (Fig. 1). Remarkably, we are unable to identify a chicken homologue of *RIG-I*, even though we used a variety of approaches to identify one. Searches of the chicken (*Gallus gallus*) genome (14) with the duck or finch *RIG-I* sequence do not reveal a match. In addition, a phylogenetic analysis of RIG-like receptors also noted that RIG-I was absent in chickens (15). However, the related melanoma differentiation associated gene–5 (*MDA5*) is present in the chicken genome (16). RIG-I and MDA5 initiate signaling cascades that converge on the same pathway at IPS-1 and lead to induction of IFN-β and expression of downstream IFN-stimulated antiviral genes (Fig. S1). MDA5 is a detector of long double-stranded RNA, poly-inosinic–polycytidylic acid [poly (I:C)], and picornaviruses (13). To ensure that the helicase we isolated was *RIG-I* and distinct from *MDA5*, we amplified a large fragment of duck *MDA5*. The fragment of MDA5 shared 91% amino acid identity with chicken MDA5 (Fig. S2), yet only 33% identity with duck RIG-I.

We identified the RIG-I syntenic region on the chicken Z chromosome where *acontinase I* is encoded; this is a gene flanking the mammalian *RIG-I* homologue. A local BLAST search of the adjacent 4 Mb reveals no match to *RIG-I*, although there are sequence ambiguities in this region. Confirming the syntenic region is conserved in other birds, we identified a *RIG-I* homologue in the recently released draft genome for zebra finch (*Taeniopygia guttata*) on chromosome Z and flanked by *acontinase I*. A search of the finch expressed sequence tag (EST) database revealed two *RIG-I* transcripts among the 92,000 sequences. In contrast, no *RIG-I* sequences are present among the 600,000 ESTs

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Duck    1   MTADEKRSLQCYRRYIERSLNPVYVLGNMTDWLPDELRERIRKEEE-RGVSGAAALFLDAVLQLEARGWFRGMLDAMLAAGYTGLAEAIENWDFSKLEKL
Finch   1   MTAEEKQNLRFYRRYIERSLNPVYILSNMTEWLSDEMEERVRKEEE-KGVTAAAALFLDAVLLEAEGWFRGFQDALAAAGYTGLAEAIENWDFGKLEKL
Human   1   MTTEQRRSLQAFQDYIRKTLDPTYILSYMAPWFREEEVQYIQAEKNNKGPMEAATLFLKFLLELQEEGWFRGFLDALDHAGYSGLYEAISWDFKKIEKL

Duck  100   ELHRQLLKRIBATMLE-VDPVALIPYISTCLIDRECEEIQQISENRSKAAGITKLIECLRSDKEHWPKSLQLALDTTGYYRASELWDIREDNAKDVDSE
Finch 100   ELHRQLLKRIBSTMLE-IDPVAIMPYINTCLIERECDEILQISEYRSKAAGITKLIECLRSDKENWPKSLQLALDSAGYYNASELWNIREDNGKDVDGE
Human 101   EEYRLLLKRLQPEFKTRIIPTDIISDLSECLINQECEEILQICSTKGMMAGAEKLVECLLRSDKENWPKTLKLALEKE-RNKFSELW-IVEKGIKDVETE

Duck  199   MTDASEDCLEASMTYSEEAEPDDNLSENLGSAAEGIGKPPPVYETKKARSYQIELAQPAINGKNALICAPTGSGKTFVSILICEHHFQNMPAGRKAKVVF
Finch 199   MTDASENNFETIMTFSEEAECD-NLSKNLSSVSECICESSSVYEPKKARSYQIELAQPAIDGKNTLICAPTGSGKTFVAVLICEHHFQNIPSGRKAKVVF
Human 199   DLEDKMETSDIQIFYQEDPECQ-NLSENSCPPSE--VSDTNLYSPFKPRNYQLELALPAMKGKNTIICAPTGCGKTFVSLLICEHHLKKFPQGQKGKVVF

Duck  299   LATKVPVYEQQKNVFKHHFERQGYSVQGISGENFSNVSVEKVIEDSDIIVVTPQILVNSFEDGTLTSLSIFTLMIFDECHNTTGNHPYNVLMTRYLEQKF
Finch 298   LATKMPVYEQQKNVFRQHFERSGYFVQGICGETVANISVENVIQDSDIIVVIMDKGILSSLSIFTLMIFDECHNTAGNHPYNVLMTRYLDQKF
Human 296   FANQIPVYEQQKSVFSKYFERHGYRVTGISGATAENVPVEQIVENNDIIILTPQILVNNLKKGTIPSLSIFTLMIFDECHNTSKQHPYNMFNYLDQKL

Duck  399   N-SASQLPQILGLTASVGVGNAKNIEETIEHICSLCSYLDIQAISTVRENIQELQRFMNKPEIDVRLVKRRIHNPFAAIISNLMSETEALMRTIYS-VDT
Finch 398   DSSAKQLPQIVGLTASVGVGNAKSTKETVEHICLCSYLDIQAISTVRENKQDLQRFANKPEIHIRWVKMRAQNRFADIISGLMSETEVLMRKIYS-VDT
Human 396   GGSSGPLPQVIGLTASVGVGDAKNTDEALDYICKLCASLDASVIATVKHNLEELEQVVYKPQKFFRKVESRISDKFKYIIAQLMRDTESLAKRICKDLEN

Duck  497   ISQNSKKDFGTQNYEHWIVVTQRKCRLLQLEDKEEESRICRALFICTEHLRKYNDALIISEDARIIDALSYLTEFFTNVKNGPYTELEQHLTAKFQEKEP
Finch 497   ISQINKIYFGTQRYEHWIVSTQKKCRLLQLEDKEKESSICRDLFICTEHLRKFNDALIISEDARIEDALAYLNEFFTNVKNGPYTELEKQLTEKFQEIEQ
Human 496   ISQIQNREFGTQKYEQWIVTVQKACMVFQMPDKDEESRICKALFLYTSHLRKYNDALIISEHARMKDALDYLKDFFSNVRAAGFEEIEQDLTQRFEEKLQ

Duck  597   ELIIALSKDETNENPKLEELVCILDDAYRYNPQTRTLLFAKTRALVSALKKCMEENPILNYIKPGVLMGRGRRDQTTGMTLPSQKGVLDAFKTSKDNRLLI
Finch 597   ELTALSKDESNENPKLEELACILDEAYHYNPQTRTILFAKTRALVAALKKWVEANPLLSHIKPDVMMGKGRRDRTVGMTLPMQKGVLDAFRNDKDIRLLI
Human 596   ELESVSRDPSNENPKLEDLCFILQEEYHLNPETITILFVKTRALVDALKNWIEGNPKLSFLKPGILTGRGKTNQNTGMTLPAQKCILDAFKASGDHNILI

Duck  697   ATSVADEGIDIVQCNLVVLYEYSGNVTKMIQVRGRGRAAGSKCILVTSKTEVVENEKCNRYKEEMMNKAVEKIQKWDEETFAKKIHNLQMKERVLRDSRR
Finch 697   ATSVADEGIDITECNLVVLYEYFGNVTKMIQVRGRGRAKDSKCILVTSKTEVVENEKLNTYKEEMMNAAIEKLQNWDETTFARKIRGLQMKEKMLRDSRK
Human 696   ATSVADEGIDIAQCNLVILYEYVGNVTKMIQTRGRGRARGSKCFLLTSNAGVIEKEQINMYKEKMMNDSILRLQTWDEAVFREKILHIQTHEKFIRDSQE

Duck  797   KEIKPKVVEGQKNLLCGKCKAYACSTDDIRIIKDSHHIVLGEAFKERYTTKPHKKPMQFDGFEKKSKMYCRNNNCQHDWGITVKYLTFDNLPVIKIKSFV
Finch 797   NETKHELVEGKKKLYCGKCKAYACSTDDIRIIKGSHHIVLGNAFQERYTTKPHRKPVQFDDFVKKSKMHCRNTECQHDWGIIVKYKIFDNLPVIKIRSFV
Human 796   K-PKPVPDKENKKLLCRKCKALACYTADVRVIEECHYTVLGDAFKECFVSRPHPKPKQFSSFEKRAKIFCARQNCSHDWGIHVKYKTFE-IPVIKIESFV

Duck  897   MESTATGTQMDFQKWKSINSSLKNFDVEEMSNLYPPF
Finch 897   LEDVESGSQMDFQKWRSINLSLKNFDEETCS------
Human 894   VEDIATGVQTLYSKWKDFHFEKIPFDPAEMSK-----

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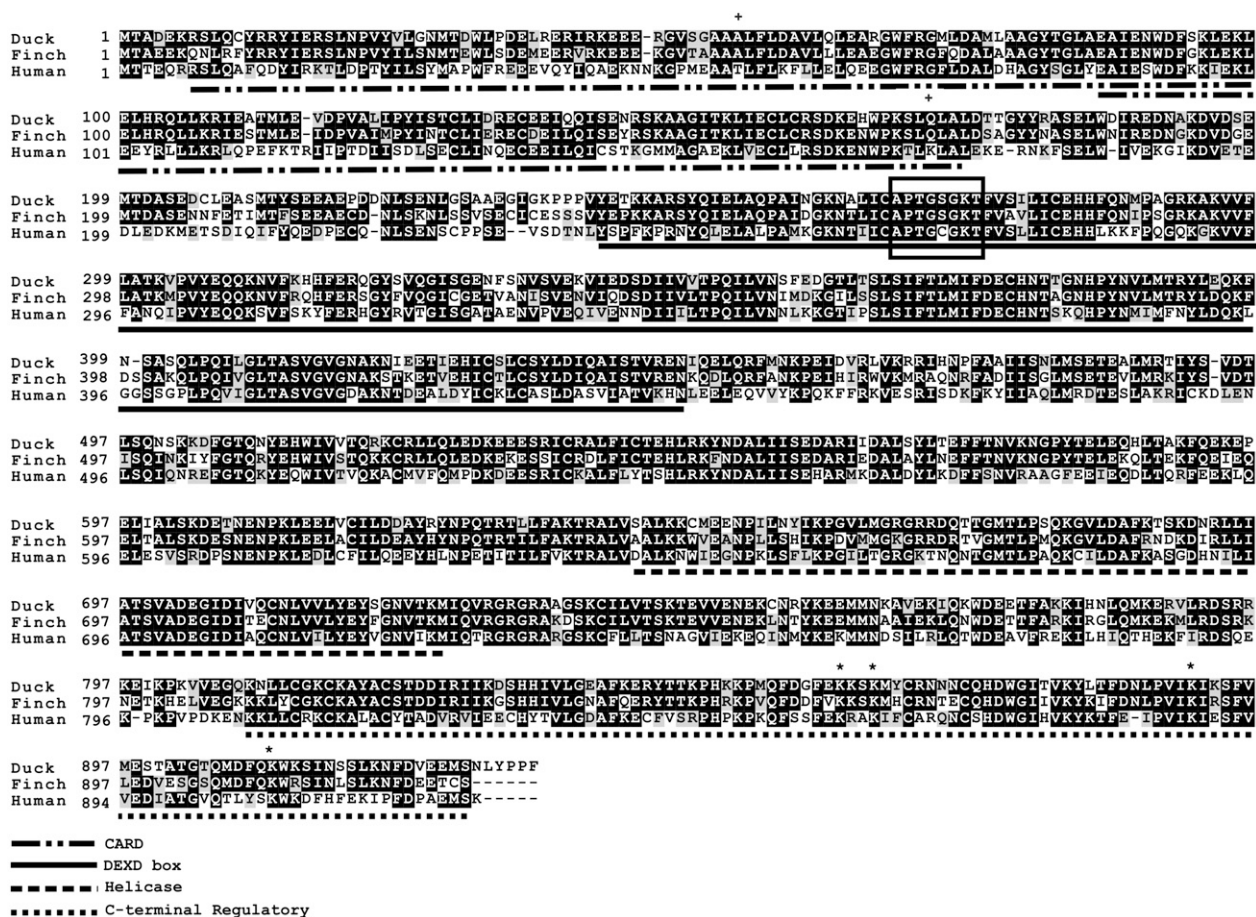
Fig. 1. Amino acid alignment of duck, zebra finch, and human RIG-I. Alignment of duck RIG-I (accession no. EU363349), zebra finch (accession no. XM_002194524), and human RIG-I (accession no. AF038963) was performed using the ClustalW program and edited with Boxshade. Black shading indicates amino acid identity and gray shading indicates similarity (50% threshold). Plus signs indicate human residues involved in polyubiquitination and asterisks indicate residues involved in ligand binding. The ATP binding motif is boxed.

from chicken. Notably, *MDA5* transcripts are present. Thus, *RIG-I* appears to be absent from the chicken genome sequence derived from the Red Jungle Fowl, which resembles the ancestral chicken, as well as the modern chicken lines represented in the EST sequences. Combined, these data suggest that chickens may have lost *RIG-I* before their domestication.

Avian RIG-I proteins have features in common with mammalian RIG-I. Duck RIG-I is 933 amino acids, and zebra finch RIG-I is 927 amino acids. Domain prediction reveals the expected tandem N-terminal CARD domains, a helicase domain and a DeXD/H box helicase domain, consistent with the mammalian structure (10, 17). RIG-I is a ligand-dependent ATPase, and the Walker A ATP-binding motif is conserved. The hydrophobic core and the four lysine residues implicated in ligand-binding, K858/861/888/907 (17), are completely conserved within the C-terminal regulatory domain. However, residues T55 and K172, critical for interaction and polyubiquitination by TRIM25 needed for IPS-1 binding and signal induction (18, 19), are not conserved, suggesting this pathway does not function or involves different residues in birds.

To provide further evidence for the absence of RIG-I in chickens, we hybridized a Southern blot of genomic DNA from ducks and chickens with a probe amplified from the helicase region of duck RIG-I (Fig. 2*A*). Although we observed a polymorphic pattern of hybridization to duck DNA, we did not detect cross-hybridization to chicken DNA. However, the duck probe cross-hybridizes with *RIG-I* from the more phylogenetically dis-

tant pigeon (Fig. 2*B*), providing evidence that the probe can recognize *RIG-I* from other avian species. The duck RIG-I probe hybridized to pigeon DNA in only the PstI digestion, although this may be because the NdeI and SpeI digestions produced hybridizing fragments of DNA too small to be visualized. Hybridization of our duck probe to pigeon DNA was observed on several other blots, whereas hybridization to chicken DNA was never observed. It is noteworthy that pigeons are remarkably resistant to influenza viruses, including HPAI H5N1 (20). In comparison, strong crosshybridization of a duck *MDA5* probe with DNA from pigeon and chicken (Fig. 2*C*) suggests it has diverged considerably less than *RIG-I* and is presumably under less selective pressure in avian species. Demonstrating that our duck *RIG-I* probe hybridizes to pigeon but not to chicken genomic DNA further supports the absence of *RIG-I* in chickens. However, we cannot rule out that chicken *RIG-I* has diverged to an extent that it is not detectable through bioinformatics or hybridization approaches, which may preclude function in any case.

Duck RIG-I Detects in Vitro Transcribed RNA and Activates the Chicken IFN-β Promoter. The apparent absence of RIG-I in chickens led us to investigate whether the DF-1 chicken cell line (chicken embryonic fibroblasts) can respond to a RIG-I ligand. RIG-I signaling is activated by 5′triphosphate RNA (5′ppp RNA) containing short regions with double-stranded conformation, such as that derived from viral RNA with panhandle structures (21, 22), and from in vitro transcribed products (23). With this in mind, we challenged

Fig. 2. *RIG-I* is present in ducks and pigeons, but apparently absent in chickens. (*A*) Hybridization of a multiple exon duck *RIG-I* probe to HindIII and XbaI-digested genomic DNA from four White Pekin ducks and two White Leghorn chickens. (*B*) Hybridization of a single exon duck *RIG-I* probe to PstI, NdeI, and SacI digested genomic DNA from duck, chicken, and pigeon. (*C*) Hybridization of a single exon duck *MDA5* probe to same blot.

DF-1 cells with an in vitro transcribed 21mer 5′ppp RNA shown to activate mammalian RIG-I (23). Transfection of this ligand failed to activate a chicken IFN-β promoter-luciferase reporter (Fig. 3*A*). Poly (I:C), known to drive the chicken IFN-β promoter in this cell line (24), showed a 2.4-fold increase in IFN-β promoter activity compared with unstimulated cells. Poly (I:C) triggers the cytoplasmic receptor MDA5 and the endosomal dsRNA receptor TLR3. However, the response to poly (I:C) in DF-1 cells is largely through MDA5, as viral inhibition of MDA5 signaling nearly abrogates IFN-β promoter activity (23). Thus

DF-1 cells respond to poly (I:C) mostly through MDA5, demonstrating that they possess the downstream components of the shared RIG-I/MDA5 pathway.

We next investigated whether duck RIG-I could confer recognition of a RIG-I ligand and signal through these downstream components. DF-1 cells were transfected with duck RIG-I before stimulation with 5′ppp RNA. DF-1 cells expressing duck RIG-I responded to 5′ppp RNA with a twofold induction of the IFN-β promoter compared with mock-transfected cells. However, phosphatase removal of the 5′ triphosphate abrogated the response, similar to the previous report in mammalian cells (23). Thus duck RIG-I is functional and can induce IFN-β promoter activity in the DF-1 chicken cell line. Although the twofold induction of the IFN-β promoter is modest, this is consistent with the up-regulation by poly (I:C) stimulation downstream of chicken MDA5. Given the estimated 90 My of divergence between chicken and duck (25), it is remarkable that the duck RIG-I even binds to chicken IPS-I to connect to downstream signaling components.

Transfected Duck RIG-I Detects Influenza and Induces an Antiviral Response in Chicken Cells. To determine if duck RIG-I could detect influenza virus and induce an antiviral response in the chicken DF-1 cells, we transfected the cells with duck RIG-I or vector only, followed by infection with influenza viruses. We chose H5N2 A/mallard/British Columbia/500/2005 (BC500), a low pathogenic avian influenza (LPAI) isolated from wild ducks that causes no pathology in its natural host and VN1203, an HPAI isolated from a fatal human infection and known to be lethal to ducks and chickens (7). After a 15 h infection with BC500, there was increased expression of *IFN-β* as well as the antiviral IFN-stimulated genes *Mx1* and *PKR*, known to be RIG-I responsive in mouse fibroblasts (11) (Fig. 3*B*). Although *IFN-β* and *PKR* were only slightly up-regulated, the IFN-stimulated gene *Mx1* was induced approximately 30 fold. Influenza A matrix gene expression was significantly reduced in RIG-I–transfected DF-1 cells compared with vector-transfected control cells (Fig. 3*B*). Furthermore, the IFN response initiated by duck RIG-I resulted in significant reduction in viral titer for both BC500 (Fig. 3*C*) and VN1203 (Fig. 3*D*) infection, indicating that

Fig. 3. Duck RIG-I rescues detection of 5′ppp RNA and induces an antiviral response in DF-1 chicken embryonic fibroblast cells. (*A*) IFN-β promoter activity in RIG-I or empty vector–transfected DF-1 cells following 15 h of ligand stimulation compared to mock-treated cells, shown as mean fold induction (±SD). Results are representative of three independent experiments and were analyzed using a single-factor ANOVA and Tukey post hoc test (different letters, $P < 0.05$). (*B*) RIG-I–transfected DF-1 cells respond to BC500 infection (MOI, 1) with increased expression of chicken *IFN-β* and the IFN-stimulated genes *Mx1* and *PKR*, and decreased influenza matrix gene expression, relative to empty vector–transfected cells. RNA was extracted from cells for qRT-PCR 15 h PI, and fold difference in gene expression calculated for RIG-I and vector-only–transfected DF-1 cells. Results are representative of three independent experiments and error bars show RQ$_{Min/Max}$ at a 95% confidence level and represent SE ($n = 3$). (*C and D*) RIG-I–transfected DF-1 cells had significantly lower influenza virus titers compared with empty vector–transfected cells for BC500 (*C*) or VN1203 (*D*). Both infections were performed 24 h after transfection at an MOI of 1. After 15 h, titer was determined by plaque assay from triplicate wells and results were analyzed with the two-tailed Student's *t* test ($n = 3$; $P = 0.002$).

duck RIG-I is capable of reducing influenza replication in chicken cells.

RIG-I Is Highly Up-Regulated in Ducks Infected with VN1203. To determine whether RIG-I contributes to the antiviral response to influenza infection in ducks, we measured the expression of *RIG-I* in 6-week-old White Pekin duck tissues following infections with BC500 (H5N2) or VN1203 (H5N1). Infection with VN1203 induced significant up-regulation of *RIG-I* gene expression in the infected lung (Fig. 4*A*). *RIG-I* expression was induced more than 200-fold by d 1 postinfection (PI), whereas by d 3 PI, *RIG-I* was only modestly expressed, suggesting the induction is early and transient (Fig. 4*B*). In comparison, infection with BC500 induced only slight up-regulation of *RIG-I* in lung tissue. Because LPAI strains predominately replicate within the intestinal epithelium of ducks (26), we also assessed *RIG-I* expression in BC500 infected duck intestine (Fig. 4 *C* and *D*). *RIG-I* expression was not significantly induced by BC500 infection in duck intestine on d 1 or d 3 PI. Duck RIG-I is thus expressed early during an innate immune response to a highly pathogenic influenza virus. However, it is not clear why VN1203 infection results in tremendous up-regulation of duck RIG-I whereas BC500 does not. Considering that RIG-I expression is itself regulated by IFN, high expression in the lungs following VN1203 infection may reflect IFN levels. Additionally, influenza viruses vary in their ability to actively inhibit IFN induction in birds and mammals (27, 28), a function that is dependent on the viral protein NS1 (29, 30) that directly targets the RIG-I pathway preventing activation of IFN-β (31). One possibility is that the NS1 protein of BC500 interferes with viral activation of the IFN pathway more efficiently than the NS1 of VN1203. Although VN1203 is potentially lethal in ducks (7), none of the ducks showed severe symptoms or died within 3 d of the experiment. We show duck RIG-I is functional and initiates antiviral gene expression in chicken cells. Thus, we speculate that the robust and transient up-regulation of *RIG-I* contributes to the successful innate response of ducks.

Discussion

This study establishes the presence of RIG-I in ducks—the natural reservoir of influenza viruses—but its apparent absence in chickens. This may reflect the differential susceptibility of ducks and chickens to influenza-induced pathology. We found that RIG-I is expressed during the innate immune response to influenza infection in ducks, providing evidence for the antiviral relevance of RIG-I in the natural host of the virus. RIG-I is highly up-regulated early in an innate immune response to VN1203 infection in ducks, likely contributing to antiviral defense against this potentially lethal virus. In contrast, RIG-I appears to be absent in chickens and a chicken embryonic fibroblast cell line fails to respond to 5′ppp RNA, a function that can be conferred to the cells by transfection with duck RIG-I. Expression of duck RIG-I augments the antiviral IFN response and reduces influenza replication in chicken cells.

Despite the lack of RIG-I, chicken cells do produce IFNs. Indeed, IFNs were initially discovered in chicken cells treated with heat-inactivated influenza virus (32). Although other pathways can produce IFN-α, IFN-β expression upon influenza infection is largely dependent on RIG-I. Embryonic fibroblasts from a RIG-I–KO mouse fail to induce IFN-β and a subset of genes involved in innate immunity following influenza infection (11). siRNA knockdown of RIG-I (33) or introduction of a dominant-negative RIG-I (34) has been demonstrated to significantly reduce the influenza-induced IFN-β production in human cell lines. Additionally, IFN-β–KO mice show reduced survival and enhanced influenza viral titers in the lung (35). Thus, IFN-β appears to be protective during an influenza infection and cannot be compensated for by IFN-α. Chicken embryonic fibroblasts infected with influenza produce IFN and inhibit an IFN-sensitive GFP-tagged vesicular stomatitis virus (27); however, this response is 80% IFN-α (36). Infection of primary chicken embryo fibroblasts with H5N1 strains induced low Mx1 and IFN-α gene expression, whereas IFN-β was not significantly induced (37). Similar to influenza infection, Newcastle Disease Virus, also detected by RIG-I, causes substantially more pathology in chickens than ducks. In accord with our findings, others have found that Newcastle Disease Virus infection does not induce IFN-β promoter activity in chicken embryonic fibroblasts (38), also consistent with lack of RIG-I.

Here we provide evidence for the antiviral function of RIG-I in ducks, and its apparent absence in chickens. RIG-I independent pathways, such as TLR7, also contribute to influenza detection and IFN production in chickens (39) and ducks (40). However, chickens lack a key regulator of the antiviral response, so it is not surprising that they suffer remarkable pathology from HPAI infection compared with ducks. Chickens lacking RIG-I would be without a first line of defense at the lung epithelial cell layer during influenza infection, and antiviral genes downstream of RIG-I may not be expressed (11). Considering that RIG-I may also inhibit influenza replication directly and independent of IFN (10), loss of this undefined pathway may also contribute to unchecked virus
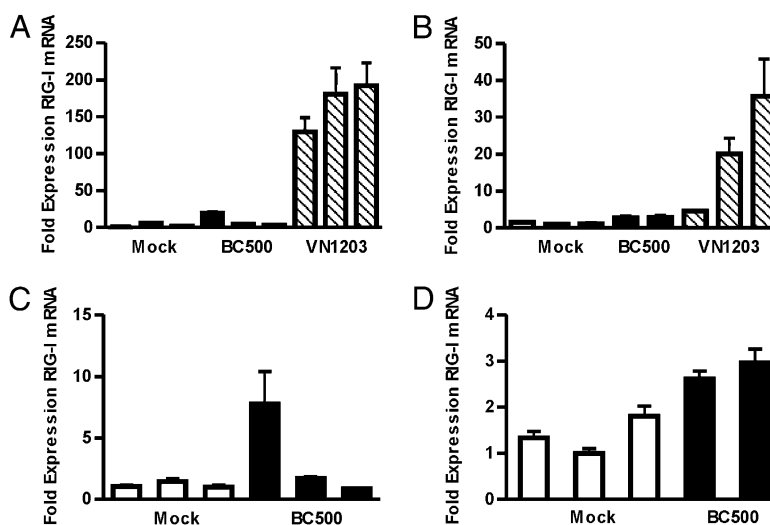
Fig. 4. *RIG-I* is dramatically up-regulated in duck lung infected with VN1203 but not BC500. Lung and intestinal RNA was extracted d 1 or d 3 PI and analyzed by qRT-PCR for *RIG-I* expression compared with a mock-infected animal. Fold expression of RIG-I mRNA in duck lung tissue infected with BC500 or VN1203 at d 1 PI (*A*) or d 3 PI (*B*). Fold expression of RIG-I mRNA in duck intestine infected with BC500 at d 1 PI (*C*) or d 3 PI (*D*). Error bars show RQ$_{Min/Max}$ at a 95% confidence level and represent SE (*n* = 4).

replication in chickens. RIG-I loss-of-function mutants in humans (41, 42) may also be predicted to cause increased viral pathogenesis. The presence of a functional RIG-I in ducks eliciting an early antiviral response may contribute to survival of what may otherwise be a lethal influenza infection.

Materials and Methods

Identification and Cloning of Duck RIG-I. A PCR fragment of RIG-I was obtained from duck (*A. platyrhynchos*) splenic cDNA using primers 5′-GAT CCC AGC AAT GAG AAT CCT AAA CT-3′ and 5′-CAA TGT CAA TGC CTT CAT CAG C-3′ based on a conserved region of the human RIG-I sequence. The complete cDNA sequence was obtained via 5′ and 3′ RACE using the SMART RACE cDNA Amplification Kit (Clontech). The complete coding region of duck RIG-I was amplified using primers in the 5′UTR 5′-CGG CCG GCA GAG CCC AGC C-3′ and 3′ UTR 5′-GTG TAG GAG AGT AAT AGA TGC ACT A-3′ using Phusion High-Fidelity DNA Polymerase (New England Biolabs), cloned into pCR 2.1-TOPO (Invitrogen) and completely sequenced.

Plasmids. pcDNA-RIG was obtained by cloning duck RIG-I into the mammalian expression vector pcDNA 3.1 hygro+ (Invitrogen). Duck RIG-I was digested out of pCR 2.1-TOPO (Invitrogen) using SpeI and NotI. RIG-I was then inserted between NheI and NotI sites of pcDNA 3.1 hygro+ (Invitrogen). The chicken IFN-β promoter luciferase reporter (pGL3-chIFNβ) was constructed from White Leghorn chicken genomic DNA using primers with incorporated BglII and MluI sites that amplified −158 to +14 of the chicken IFN-2 promoter, as previously done (24, 38). The promoter fragment was then inserted between BglII and MluI sites of the pGL3-basic luciferase reporter vector (Promega).

In Vitro Transcription and RNAs. The 21-mer RNA, 5′-pppGGGGCUGACCCU-GAAGUUCCC-3′ (23) was transcribed from annealed DNA oligonucleotides 5′-TAA TAC GAC TCA CTA TAG GG-3′ and 5′-GGG AAC TTC AGG GTC AGC CCC TAT AGT GAG TCG TAT TA-3′ containing a T7 promoter site, using the T7 Megashortscript kit (Ambion). In vitro transcription was carried out overnight, followed by DNaseI digestion and precipitation. Calf intestinal alkaline phosphatase (CIAP) treatment was carried out for 3 h using 30 μg of in vitro transcribed RNA, 30 U of CIAP (Invitrogen), 1× CIAP buffer, and 200 U RNase Out (Invitrogen). CIAP-treated 5′ppp RNA was purified by phenol-chloroform extraction and precipitation. Poly (I:C) (25 mg/mL) was obtained from InVivogen.

Cell Culture, Infections, and Transfections. UMNSAH/DF-1, a spontaneously immortalized chicken embryonic fibroblast cell line derived from East Lansing strain eggs (43), was maintained in DMEM plus 10% FBS. Cells (1.25×10^5) were seeded overnight in 24-well plates. Cells were cotransfected with 150 ng of pcDNA-RIG or empty pcDNA, 150 ng of pGL3-chIFNβ, and 10 ng of the constitutive renilla luciferase reporter phRG-TK (Promega). Thirty hours after plasmid transfection, cells were challenged with 21-mer 5′ppp RNA (800 ng), CIAP-treated RNA (800 ng), or poly (I:C) (25 μg/mL). Plasmids and RNA ligands were transfected using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) 15 h after challenge. For infection of transfected cells, DF-1 cells were maintained in DMEM plus 10% FBS and 1.25×10^5 cells were seeded overnight in 24-well plates. Cells were transfected with 1 μg of pcDNA-RIG or empty pcDNA. Twenty-four hours after transfection, cells were infected at a multiplicity of infection (MOI) of 1 with H5N2 A/mallard/BC/500/05 or H5N1 A/Vietnam/1203/04 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) in view of the trypsin sensitivity of DF-1 cells (44). 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 influenza virus titer was determined by plaque assay on MDCK cells.

Viruses and Duck Infections. The H5N1 A/Vietnam/1203/04 HPAI was generated by reverse genetics (45) and H5N2 A/mallard/BC/500/05 LPAI was isolated by screening of environmental samples. The viruses were propagated in 10-d-old embryonated chicken eggs and handled at St. Jude Children's Research Hospital, with VN1203 handled in biosafety level 3+ facilities approved by the United States Department of Agriculture and Centers for Disease Control and Prevention. Outbred White Pekin ducks (*A. platyrhynchos*) were purchased from Ideal Poultry or Metzer Farms, and all animal experiments were approved by the Animal Care and Use Committee of St. Jude Research Hospital and performed in compliance with relevant institutional policies, National Institutes of Health regulations, and the Animal Welfare Act. A total of 10^6 of 50% egg infectious dose of BC500 and VN1203 were used to inoculate 6-week-old mallards via the natural route, in

nares, eyes, and trachea. Mock infection was PBS only. Ducks were killed and tissues collected at d 1 and d 3 PI (*n* = 3, except for BC500 d 3 samples, *n* = 2). Tracheal and cloacal swabs were collected to monitor viral shedding.

Southern Hybridization. Genomic DNA was extracted from blood of White Pekin ducks (*A. platyrhynchos*), White Leghorn chickens (*G. gallus*), and pigeon (*Columba livia*). Genomic DNA (10 μg) was digested to completion, separated on 0.8% agarose, and blotted to Nytran Supercharge (Schleicher & Schuell). DNA was immobilized by UV cross-linking and baking for 3 h at 80 °C. A multiple-exon 307-bp RIG-I probe in the helicase domain was amplified using the primers 5′-ACA GGT ATG ACC CTC CCA AGC CAG-3′ and 5′-CAT CCC ATT TCT GGA TCT TTT CAA CAG-3′ from a defined duck RIG-I clone. A 171-bp probe predicted to contain a single exon was amplified from the same template using 5′-GTA TGA CCC TCC CAA GCC AGA AGG -3′ and 5′-CTC TGA CTT GGA TCA TTT TGG TCA CG-3′. A 150-bp duck MDA5 probe was amplified with the following primers: 5′-GAG CAA AGG GAA GTC ATT GAT AAA TTC C-3′ and 5′-GGC CTG CAA CAT AGC AAT TTC ATT-3′ from a duck MDA5 clone. All probes were radiolabeled with ^{32}P α-dCTP by random priming (PrimeIt Random Primer Labeling Kit; Stratagene). Blots were hybridized overnight at 42 °C in 50% formamide, 5× Denhardt solution, 4× SSPE, 5% dextran sulfate, 1% SDS, and 100 μg/mL salmon sperm DNA. Washes were carried out at low stringency in 1× sodium chloride/sodium phosphate/EDTA and 0.1% SDS at 52 °C. Film was exposed for 3 d.

Real-Time qRT-PCR. RNA was extracted using TRIzol (Invitrogen), followed by purification from the final aqueous phase using the RNeasy Mini Kit (Qiagen). First strand cDNA synthesis was performed using the SuperScript III kit (Invitrogen) using OligodT (Invitrogen) and random primers (Invitrogen). To quantify *RIG-I* gene expression from duck tissues, 50 ng of cDNA was amplified in a 10-μL reaction using the Applied Biosystems 7500 real-time PCR system (Applied Biosystems). Duck primers and fluorogenic TaqMan FAM/TAMRA (6-carboxyfluorescein/6-carboxytetramethylrhodamine)–labeled hybridization probe mixes were obtained from Applied Biosystems and used with FastStart Universal Probe Master (Rox; Roche Applied Science). Duck *GAPDH* was used as an endogenous control. Primer and probe sequences were as follows: duck *RIG-I* primers 5′-GTG TAT GGA GGA AAA CCC TAT TTC TTA ACT-3′ and 5′-GGA GGG TCA TAC CTG TTG TTT GAT-3′, and probe 5′-TTC CGC GCC CCA TCA A-3′. Duck GAPDH primers were 5′-GCC TCT TGC ACC ACC AAC T-3′ and 5′-GGC ATG GAC AGT GGT CAT AAG AC-3′ and probe 5′-CAC AAT GCC AAA GTT G-3′. Changes in gene expression PI were expressed as a ratio of the level observed in a mock-infected animal. RT-PCR was performed for *RIG-I* and *GAPDH* in a single-plex format, with the following cycling conditions: 95 °C for 10 min for activation, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Quadruplicate cycle threshold C_T values were analyzed with SDS software (Applied Biosystems) using the comparative C_T ($\Delta\Delta C_T$) method.

To quantify gene expression from transfected DF-1 cells, 20 ng of cDNA was amplified in a 10-μL reaction using the Applied Biosystems 7500 real-time PCR system (Applied Biosystems). Chicken 28s RNA (endogenous control) and IFN-β probes and primer (46) were obtained from Applied Biosystems and used with TaqMan Fast Universal PCR Master Mix (Applied Biosystems). For Influenza A matrix gene expression, primers were designed according to CDC recommendations. InfA forward primer was 5′-GAC CRA TCC TGT CAC CTC TGA C-3′; InfA reverse was 5′-AGG GCA TTY TGG ACA AAK CGT CTA-3′; and InfA probe 5′-TGC AGT CCT CGC TCA CTG GGC ACG-3′ (Centers for Disease Control and Prevention). The primers and probes for chicken Mx1 (accession no. NM_204609) and PKR (accession no. NM_204487.1) were designed with the Roche online Universal Probe Library (UPL) system Assay Design Center. The primers for Mx1 were 5′-GTT TCG GAC ATG GGG AGT AA-3′ and 5′-GCA TAC GAT TTC TTC AAC TTT GG -3′ (UPL probe 80). The primers for PKR were 5′-TGC TTG ACT GGA AAG GCT ACT-3′ and 5′-TCA GTC AAG AAT AAA CCA TGT GTG-3′ (UPL probe 29). Cycling conditions were 95 °C for 20 s for activation, followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. Changes in gene expression were expressed as a ratio of the level observed with cells transfected with RIG-I or vector only.

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