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Avian influenza rapidly induces antiviral genes in duck lung and intestine

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Abstract

Ducks are the natural reservoir of influenza A and survive infection by most strains. To characterize the duck immune response to influenza, we sought to identify innate immune genes expressed early in an infection. We used suppressive subtractive hybridization (SSH) to construct 3 libraries enriched in differentially expressed genes from lung RNA of a duck infected with highly pathogenic avian influenza virus A/Vietnam/1203/04 (H5N1), or lung and intestine RNA of a duck infected with low pathogenic avian influenza A/mallard/BC/500/05 (H5N2) compared to a mock-infected duck. Sequencing of 1687 clones identified a transcription profile enriched in genes involved in antiviral defense and other cellular processes. Major histocompatibility complex class I (*MHC I*), interferon induced protein with tricopeptide repeats 5 (*IFIT5*), and 2'–5' oligoadenylate synthetase-like gene (*OASL*) were increased more than 1000-fold in relative transcript abundance in duck lung at 1 dpi with highly pathogenic VN1203. These genes were induced much less in lung or intestine following infection with low pathogenic BC500. The expression of these genes following infection suggests that ducks initiate an immediate and robust response to a potentially lethal influenza strain, and a minimal response to a low pathogenic strain.

1. INTRODUCTION

Ducks are the natural reservoir of influenza A, and all subtypes of the virus are perpetuated in the duck host (Hinshaw et al., 1980; Webster et al., 1992). Since ducks typically do not show signs of disease when infected with avian influenza, classification of viruses as low or highly pathogenic is based on their virulence in domestic galliformes (Suarez and Schultz-Cherry, 2000). Low pathogenic avian influenza usually causes minor signs of disease such as reduced egg production, ruffled feathers and occasionally mild respiratory distress in chickens. In contrast, highly pathogenic avian influenza infections lead to rapid onset of severe, contagious disease that can result in mortality rates approaching 100% within 48 hours of an outbreak (Pantin-Jackwood and Swayne, 2009). Since 1996, strains of H5N1 influenza have arisen that are highly pathogenic to chickens and humans, and some can be pathogenic to ducks while others cause no harm (Hulse-Post et al., 2005; Kim et al., 2009; Sturm-Ramirez et al., 2004; Sturm-Ramirez et al., 2005). This emphasizes that influenza

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virulence is multifactorial and depends on the viral strain and interference in the host immune response (Tscherne and Garcia-Sastre, 2011).

Due to the acute nature of influenza infection, the innate immune system is most critical in determining severity and outcome. We postulate that limited pathogenesis of avian influenza in ducks reflects a successful antiviral innate immune response. In mammals, influenza is detected by endosomal toll-like receptors 7/8 (TLR7/8) in plasmacytoid dendritic cells leading to the production of IFN- α (Diebold et al., 2004; Lund et al., 2004). A cytoplasmic influenza sensor, retinoic acid-inducible gene I (RIG-I), is present in most other cell types (Pichlmair et al., 2006), and signalling leads to the production of IFN- β (Kato et al., 2006; Loo et al., 2008). Detection by pattern recognition receptors leads, in turn, to the upregulation of IFN-stimulated genes (ISGs), including RIG-I itself, and others that directly combat the infection. Recently, investigation of the human ISGs showed many directly inhibit viral replication (Schoggins et al., 2011). The effectors responsible for the successful antiviral response to influenza in ducks are unknown.

White Pekin ducks are an important agricultural species and a potential host of influenza viruses. As such, there are growing genetic resources for White Pekin ducks with the forthcoming genome sequence, BAC (Yuan et al., 2006) and cosmid libraries (Moon and Magor, 2004), and expressed sequence tags (Xia et al., 2007). We have been characterizing immune genes in the response to influenza in this host. We showed previously that *RIG-I* was highly upregulated in lung tissues of White Pekin ducks infected with highly pathogenic avian influenza, and only slightly upregulated in intestine tissues of ducks infected with low pathogenic virus (Barber et al., 2010). We predict that detection of influenza leads to expression of ISGs in infected tissues, but these are largely uncharacterized in the duck host. Furthermore, we predict that immune responses differ depending on strain of virus. H5N1 strains replicate in the respiratory tract of ducks (Sturm-Ramirez et al., 2004), while low pathogenic strains replicate in the intestinal tract (Webster et al., 1978). Thus, with the dual goals of identifying duck genes that contribute to the antiviral response, and adding more expressed sequence tags to the genomic resources available for ducks, we undertook a differential subtraction approach to identify and sequence genes expressed in tissues of influenza-infected ducks.

Here we perform suppressive subtractive hybridization (SSH) to construct libraries enriched in genes showing increased transcript abundance in duck tissues following infection with highly pathogenic A/Vietnam/1203/04 (H5N1) or low pathogenic A/mallard/BC/500/05 (H5N2) avian influenza relative to mock-infected duck tissues. In both infected lung and intestine tissues, the upregulation of *RIG-I* was higher on 1 dpi than 3 dpi (Barber et al., 2010). Therefore, to identify genes downstream of RIG-I involved in the innate immune response, we generated subtracted cDNA libraries using RNA from tissues obtained at 1dpi. These libraries contain many immune genes including genes involved in antigen processing and presentation, transcription factors, and IFN stimulated genes (ISGs) downstream of RIG-I. Key ISGs showed greater than 1000-fold increase in relative transcript abundance in duck lung following VN1203 infection, and less upregulation by low pathogenic infection. The timing of this response was remarkably rapid as these genes were upregulated at one day post-infection.

2. MATERIALS AND METHODS

2.1 Viruses, infections and RNA extraction

A/Vietnam/1203/04 (H5N1) (VN1203) was made by reverse genetics (Salomon et al., 2006) and later characterized as less pathogenic in ducks than the original virus (Marjuki et al., 2010). A/mallard/BC/500/05 (H5N2) (BC500) was isolated during surveillance of wild

ducks in Canada (J. Pasick). Infections were described previously (Barber et al., 2010). Briefly, six week-old White Pekin ducks were infected with 10^6 of 50% egg infectious doses of BC500 or VN1203. Ducks were mock-infected with PBS-only, infected with BC500 or VN1203 by dripping in nares, eyes and trachea. Ducks were confirmed infected by recovering live virus by egg inoculation of material from tracheal (VN1203) or cloacal swabs (BC500). Swabs were stored in freezing medium (Sturm-Ramirez et al., 2004) and infectivity of virus in positive swab samples was titrated by determining the EID₅₀ (Reed and Muench, 1938) (Table 1). Tracheal swabs were negative for ducks infected with BC500, and cloacal swabs were negative for ducks infected with VN1203. Cloacal swabs were negative at 1 dpi with BC500, and positive at 2 and 3 dpi. Lung and intestine samples were harvested and total RNA extracted using TRIzol (Invitrogen), DNase treated, cleaned up with RNeasy columns (Qiagen) then stored at -80°C . PolyA⁺ RNA was isolated using the oligo(dT)₂₅ Dynabead mRNA Purification kit (Invitrogen).

2.2 Construction of suppressive subtractive hybridization (SSH) cDNA libraries

An RNA sample from each infected tissue having high expression of *RIG-I* (Barber et al., 2010) was selected for subtractive hybridization with a matched tissue from a mock-infected animal. Total (or polyA⁺) lung RNA from a mock, BC500 and VN1203-infected duck was used to synthesize and amplify cDNA using the SuperSMART cDNA Synthesis kit (Clontech). cDNA from BC500-infected and mock-infected intestine was made directly from mRNA without prior amplification. SSH subtraction between cDNA samples was done with the PCR-Select cDNA Subtraction kit following the manufacturer's protocol (Clontech). For the generation of forward subtracted libraries (Table 2), cDNA from the mock-infected tissue was used as a driver, and cDNA from the infected tissue was used as the tester to identify genes upregulated by influenza infection. Plasmid DNA was isolated by alkaline lysis or the GeneJET Plasmid Miniprep kit (Fermentas), and sequenced using BigDye Terminator version 3.1 (Applied Biosystems) and vector-specific primers M13F: GTAAAACGACGGCCAG and M13R: CAGCAAACAGCTATGAC. Sequences were edited in ContigExpress, VectorNTI Advance 11 (Invitrogen). The identity of insert was determined by nucleotide BLAST (blastn; www.ncbi.nlm.gov/blast) and a mass BLASTx program to search for potential matches in the TrEMBL protein database.

2.3 Deoxygenin-11-dUTP (DIG) labelling probes and dot blotting

Nylon membranes (Roche) were spotted with clones for each unique insert in the 3 SSH libraries. Plasmid DNA was denatured with 0.6 N NaOH at room temperature, spotted onto nylon membranes and fixed by UV crosslinking. Duplicate membranes were hybridized overnight (~16 h) at 42°C with forward and reverse SSH PCR amplification products as probes. Following an *RsaI* restriction digest to remove adaptors, probes were purified with the QIAquick PCR Purification kit (Qiagen), and labelled with deoxygenin-11-dUTP (DIG) using the DIG High Prime DNA Labeling and Detection (Roche) as per kit instructions. Greater spot intensity of the forward probe hybridization compared to the reverse, confirmed upregulation of that transcript in RNA from influenza-infected tissue.

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

RNA was DNase treated and cDNA synthesized with Oligo(dT)₂₀ primer (50 μM) using the SuperScript III reverse transcriptase (Invitrogen). qRT-PCR amplifications were carried out using FastStart TaqMan® Probe Master Mix (Roche) and gene-specific primer-probe sets from Integrated DNA Technologies. Primers and probes (Table 3) were designed using Primer Express version 3.0 software (Applied Biosystems) and validated for linear amplification and amplification efficiency matched to the *GAPDH* control. Influenza matrix (M) gene was readily amplified from VN1203-infected lung, with 12-fold more M gene present at 1 dpi than 3 dpi. M gene was at the limit of detection using 10 times as much

template in BC500 infected intestine (Fleming et al., 2011). All qRT-PCR assays were performed in a 7500 Fast Real-Time PCR System machine (Applied Biosystems). Each duck cDNA sample was 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 ($\Delta\Delta CT$) and the analysis was performed with the 7500 Fast System software version 1.4 (Applied Biosystems).

3. RESULTS

3. Sequencing clones from SSH libraries to identify immune genes

3.1.1 Immune transcripts enriched in lung cDNA at 1 dpi with VN1203—To identify genes contributing to the innate response to VN1203 H5N1 influenza, which replicates in the lung, we sequenced to saturation, dot-blotted and hybridized representative clones from the forward-subtracted VN1203 lung cDNA library. We identified several immune genes that appeared enriched in lung tissues of ducks infected with VN1203 (Table 4). The most abundant transcript, which accounted for over a third of the clones, was MHC class I. While some were too short to identify, most were transcripts from the predominantly expressed MHC class I locus *UAA*. Other upregulated genes included *HSP90*, *HSP70* and *LY6E* and *IFITM1*. Three additional innate immune genes were among the sequenced clones, oligoadenylate synthetase-like (*OASL*), interferon regulatory factor-1 (*IRF-1*) and an interferon-induced protein with tricopeptide repeats (*IFIT*) that resembles chicken *IFIT5*.

To identify transcripts downregulated by VN1203 infection we sequenced 100 clones of the reverse subtracted library. Most sequences were α -globin and β -globin. Seven transcripts were unique to the reverse subtraction, but only one had an immune function, *YBOX1*. *YBOX1* is a repressor that binds the Y box of MHC genes (Lloberas et al., 1995), and its downregulation is consistent with the upregulation of MHC class I observed. We showed *YBOX1* is indeed less abundant in lung tissues from influenza-infected ducks by RT-PCR (data not shown). Thus, sequencing clones from the reverse subtraction yielded mainly housekeeping genes.

3.1.2 Immune transcripts enriched in lung cDNA at 1 dpi with BC500—To identify genes upregulated in lung tissue following initial viral challenge with BC500, which does not replicate in the lung, we sequenced to saturation, dot-blotted and hybridized representative clones from the forward subtracted BC500 lung cDNA library. Only a few genes appear upregulated in lung tissues of BC500 infected ducks (Table 5). These include selenoprotein P1 (*SEPP1*), a putative ubiquitin-conjugating enzyme E2 D3 protein (*UBE2D3*), and GTP binding protein 4 (*GBP4*). The majority of clones showed no difference in hybridization between the forward and reverse-subtracted probes, likely because expression differences are too subtle for this technique. Other immune genes were among the sequenced transcripts of the forward subtraction library, nuclear factor related to kappa binding protein (*NFR κ B*), *IFIT5*, *CXCR4*, *STAT1* and *CD44*.

3.1.3 Immune transcripts enriched in intestine cDNA of BC500-infected ducks—To identify genes upregulated following infection with BC500 in intestine, the site of replication, we sequenced to saturation and dot-blotted representative clones from the forward-subtracted BC500 intestine cDNA library. We identified several immune gene transcripts that were enriched in the intestine of ducks infected with BC500 (Table 6). These include *MHC I UAA*, $\beta 2m$, MHC II β -chain, *ISG12-2*, and *IFIT5*. The two most abundant transcripts sequenced from this tissue were intestinal 15kDa protein (*ILLBP*) and *MHC I* with over 80 clones sequenced for each.

3.1.4 Transcripts upregulated in duck tissues following influenza infection—

To determine whether the same transcripts were enriched following influenza infection with different viruses or in different tissues, we compared the transcripts sequenced from the forward subtraction libraries using a VENN diagram (Figure 1). Only 7 transcripts were identified in all 3 SSH libraries, lung from VN1203-infected ducks, and lung and intestine from BC500-infected ducks. Of these, only one is an immune gene, *IFIT5*. More immune genes were shared between lung of ducks infected with VN1203 and intestine of ducks infected with BC500, perhaps because these are the primary sites of replication for these viruses. Common transcripts include *MHC I UAA*, invariant chain (*CD74*), and proteasome subunit α -type 6 (*PSMA6*).

3.2 MHC class I is upregulated in ducks infected with VN1203

To confirm the relative transcript abundance for MHC class I in tissues from influenza infected ducks, we performed qRT-PCR using RNA from lung tissue of 3 mock-infected ducks, 3 infected with BC500, and 3 infected with VN1203 (Figure 2). We observe 1695-fold more *MHC I UAA* transcript in lung RNA from VN1203-infected relative to mock-infected ducks at 1 dpi, and 340-fold more *MHC I UAA* transcripts in BC500 lung tissue, with induction ranging from 7 to >800-fold, depending on the animal. At 3dpi, MHC was upregulated 3-fold by VN1203 in comparison to mock-infected animals. *MHC I UAA* expression was increased in intestine tissue from ducks infected with BC500 by 13-fold above the mock-infected animals. However, by 3 dpi *MHC I* transcripts were less abundant in the BC500-infected intestine than in the mock-infected samples. Some variation between animals may also be due to sequence polymorphisms preventing the binding of primers to some allelic MHC variants in these genetically different animals. To minimize this, we designed primers in the more conserved transmembrane region and cytoplasmic tail.

3.3 Interferon stimulated genes are rapidly upregulated in ducks infected with VN1203

To determine the extent of upregulation of ISGs identified in our subtractive hybridizations, *ISG12-2*, *IFIT5*, *OASL* and *IFITM1*, we examined their relative expression in duck lung by qRT-PCR (Figure 3). At 1dpi, we observed 1392-fold more *ISG12-2* transcript in lung tissues from ducks infected with VN1203, and 36-fold more *ISG12-2* transcript in ducks challenged with BC500 (Figure 3A). By 3dpi, *ISG12-2* transcript was only 35-fold more abundant for VN1203, and not upregulated by BC500. *ISG12-2* expression was low in one VN1203-infected duck lung, which corresponded to the sample used for subtraction. *IFIT5* transcripts were 1254-fold more abundant in duck lung at 1 dpi with VN1203, and 81-fold more abundant in the lung tissue from BC500-infected than mock-infected ducks (Figure 3B). By 3dpi, *IFIT5* induction was 83-fold for VN1203 or 9-fold for BC500, respectively. *OASL* was 1410-fold more abundant in VN1203 infected lung, and elevated 95-fold in BC500-infected duck lung compared to mock-infected lung (Figure 3C). By 3dpi, the induction of *OASL* was significantly lower at 72-fold and 6-fold for VN1203 and BC500 respectively. The relative amount of *IFITM1* transcript was 67-fold greater in lung tissue of VN1203-infected, and 8-fold more abundant in BC500 infected, compared to mock-infected ducks at 1 dpi (Figure 3D). By 3dpi, the relative expression of *IFITM1* was 6-fold greater in VN1203 or 2-fold greater in BC500 than in mock-infected duck lung. At 1 dpi, individual ducks showed different expression levels for each ISG. At 3dpi, one duck (318) had the highest expression of all 4 ISG genes, and its tracheal influenza titre was 100-fold lower (Table 1).

We examined the relative transcript abundance of two ISGs identified by SSH in duck intestine at 1 and 3 dpi (Figure 4). *ISG12-2* transcripts were 4-fold more abundant in intestine 1 dpi with BC500 virus, compared to mock-infected ducks (Figure 4A). A 3dpi, *ISG12-2* was 6-fold more abundant in BC500 infected intestine than mock-infected ducks.

IFIT5 was 10-fold more abundant in BC500 infected duck intestine compared to mock-infected ducks at 1dpi, and *IFIT5* transcript abundance was still increased (8-fold) in duck intestine at 3dpi with BC500, relative to mock-infected ducks.

4. DISCUSSION

Here we use suppressive subtractive hybridization to identify genes showing increased transcript abundance in duck lung or intestine tissues following infection with a low or highly pathogenic avian influenza virus. The transcriptional profile of MHC class I and ISGs in lung tissue of influenza-infected Pekin ducks suggests a remarkably rapid induction of the interferon response with >1000-fold upregulation of key genes at 1 dpi with VN1203. In comparison, the low pathogenic strain BC500 induced much lower expression of these same antiviral genes in lung or intestine tissue, despite higher replication of this virus in ducks. Titres of cloacal swabs from BC500 infected ducks were 10^5 – 10^7 EID₅₀ compared to 10^4 EID₅₀ from tracheal swabs for VN1203 infected ducks. One interpretation of our data is that VN1203 does not antagonize the interferon response in ducks, while BC500 can. A delayed and modest upregulation of ISGs is seen following PR8 influenza infection of mice, due to antagonism of the interferon response by influenza NS1 protein, allowing the virus a period of stealth replication (Molledo et al., 2009). Similar kinetics, with a delayed interferon response, are seen in the ferret depending on influenza strain and NS1 (Meunier and von Messling, 2011). Viral subversion of IFN by a low pathogenic avian H7N1 isolate was seen in duck embryonic fibroblasts, while an NS1 truncation mutant was a strong IFN inducer (Soubies et al., 2010). Alternatively, the differences in response to VN1203 and BC500 could be due to inherent immunological capability of the tissues of the sites of replication, with VN1203 replicating in lung, while BC500 replicates in the intestinal tissues. Finally, since transcripts for M gene could be readily detected in VN1203 infected lung, while they were at the limit of detection in BC500 infected intestine tissue, viral replication may be more widespread in VN1203-infected lung.

Several genes encoding proteins involved in antigen processing and presentation, including *MHC I*, *β2M*, *MHC II B chain*, *HSP70*, and proteasome genes (*PSMA6* and *PSMD13*), were induced in the duck lung and intestine. Real time PCR confirmed upregulation of MHC class I *UAA* by 1700-fold in VN1203 infected lung, and by 13-fold in BC500 infected intestine tissues. MHC class I upregulation is most likely due to upregulation of Type 1 IFN upon viral infection. Chicken and duck MHC class I genes have an interferon response element in their promoters (Moon et al., 2005; Zoller et al., 1992). Alternatively, MHC class I may be regulated by NF-κB, independent of the interferon response, as recently shown for dengue virus (Othman et al., 2012). The induction of MHC class I by 1918 influenza virus in macaques (Kobasa et al., 2007), despite concomitant subversion of the interferon response, is consistent with this possibility. Others have noted only slightly upregulated, or decreased, MHC class I expression following influenza infection in birds. Infection of chicken and duck PBMCs with A/duck/WA/633/97 (H11N9) increased MHC class I expression by 2–3 fold (Adams et al., 2009). Infection of chicken embryonic fibroblast (CEF) cells with Egret/Hong Kong/757.2/02 (H5N1) or CK/HK/97 (H5N1) decreased expression of *RFP-Y*, a non-classical MHC gene (Sarmiento et al., 2008). Surprisingly, MHC was not significantly upregulated in duck spleen at 2 dpi with highly pathogenic A/dk/VN88/2007 (H5N1) (Cagle et al., 2011), however, this strain upregulated RIG-I by only 13-fold. Infection of duck embryonic fibroblasts (DEF) with A/plateau pike/Qinghai/04/2007 (H5N1) led to the down regulation of MHC I (Liang et al., 2011). Although these latter studies differ markedly from our results, this may reflect the ability of different influenza strains to elicit or block interferon. Also, it is not clear whether a direct comparison can be made, since the tissues and primers used were different.

The consequences of upregulation of MHC class I in influenza-infected tissues to both the host and virus are unclear. Early in an infection, increased MHC I expression may be advantageous to the virus by interfering with NK cell killing. However, we should note that inhibition of NK cells by binding to self-MHC has not been established in birds. Increased MHC class I expression would generally result in presentation of more influenza epitopes to CTL and presumably more efficient killing, however, 1 dpi is too early for initiation of CTL responses. Instead this massive upregulation of MHC could give rise to an unfocused response due to recruitment and expansion of T cells of lower avidity. These T cells may compete for MHC, but interfere with killing. Ducks express a single dominant MHC class I gene (Mesa et al., 2004; Moon et al., 2005), thus would be expected to have a limited repertoire of appropriate T cells selected on either MHC allele. To understand the significance for host and virus, however, we must first know whether MHC is upregulated on infected cells, on all cells, or primarily on leukocytes that have relatively higher MHC expression. We can rule out that the increase in MHC transcripts in lung and intestinal tissues is simply due to the influx of leukocytes to the site of infection, because we still observe extensive leukocyte accumulation in duck lung tissue at 3 dpi with VN1203 (Fleming-Canepa et al., 2011), but MHC I gene expression is no longer elevated.

In addition to MHC class I, four ISGs (*ISG12-2*, *IFIT5*, *OASL* and *IFITM1*) with previously reported antiviral activity were identified by SSH and shown to be significantly upregulated by qRT-PCR in duck lung and intestine tissues. Although the functions of these ISGs in the context of an avian immune response to influenza are unknown, they each warrant further investigation. Duck *ISG12-2* was induced 1400-fold in VN1203 infected lung, induced 36-fold in lung and 4-fold in intestine tissues following BC500 infection. *ISG12-2* was similarly identified by SSH as induced in the chicken kidney following H5N1 infection (Zhang et al., 2008). Implicating *ISG12* in an antiviral role, neuronal expression of *ISG12b1* significantly delayed Sindbis virus-induced death in neonatal mice (Labrada et al., 2002). Human *ISG12* has a mitochondrial location (Rosebeck and Leaman, 2008), and it has been postulated that it may sensitize cells to IFN-induced apoptosis by destabilizing the mitochondrial membrane (Cheriyath et al., 2011). It is tempting to speculate that *ISG12* is involved in apoptosis of H5N1 influenza infected duck cells, which has been suggested as a mechanism of viral resistance (Kuchipudi et al., 2009).

An *IFIT* homologue was enriched in all three SSH libraries, and transcripts were upregulated 1200-fold by VN1203. The duck homologue resembles chicken *IFIT5*, the sole representative of the *IFIT* family in birds, which is upregulated 30-fold by H7N2 influenza infection of avian macrophages (Keeler et al., 2007), and 20-fold by H9N2 infection of the chicken macrophage cell line, HTC (Xing et al., 2008). *IFIT* proteins are involved in restriction of several viruses by binding to RNA (Daffis et al., 2011). Recently, mammalian *IFIT1* and *IFIT5* were shown to detect and bind to 5' triphosphate-RNA, and potentially sequester this RNA in a multiprotein complex with other *IFIT* proteins in the infected cell (Pichlmair et al., 2011).

Duck *OASL* was induced 1400-fold by infection with VN1203 and 95-fold by BC500. Duck *OASL* resembles chicken *OASL*, which 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 the human *OASL* lacks oligoadenylate synthetase activity, the UbL domains are necessary for antiviral function (Marques et al., 2008). Human *OASL* inhibits replication of RNA viruses including picornaviruses and encephalomyocarditis virus (Marques et al., 2008) and hepatitis C virus (Ishibashi et al., 2010). Human *OASL* is upregulated by influenza or Sendai viral infection, dependent on IRF3 (Melchjorsen et al., 2009). *OASL* was upregulated by influenza infection in chicken lung tissues, and implicated in survival of chickens to lethal influenza strains (Uchida et al., 2012).

An *IFITM* transcript was enriched in both the VN1203 and BC500-infected duck lung. The duck *IFITM* homologue was most similar to *IFITM1* of the 3 predicted *IFITM* genes. A chicken *IFITM* blocks entry of MLV-GFP pseudovirus particles with influenza H5 entry proteins (Huang et al., 2011). Human *IFITM1*, 2 and 3 proteins mediate cellular resistance to early replication of influenza (Brass et al., 2009). Further analysis of human *IFITM3* shows that it accounts for 50–80% of the antiviral activity of Type I and Type II interferon to block an influenza infection *in vitro* (Brass et al., 2009). Human *IFITM3* resides in late endosomes and blocks cytosolic entry of influenza genomes (Feeley et al., 2011).

Some known genes (such as *Mx*, *PKR* and the Type I interferons) that we anticipated to be upregulated by influenza were not recovered in this study. *ISG12-2*, identified in the BC500 subtraction, was not seen among the SSH clones sequenced from the VN1203 library. This can be explained by the fact that the animal used for library construction showed low *ISG12* expression, despite high expression of other ISGs. Clearly, SSH does not provide a comprehensive list of upregulated transcripts. Coupled with hybridization to confirm upregulated transcripts, it is a relatively insensitive method, which only detects genes that demonstrate large differences in abundance.

Studies investigating influenza infection in lungs of chickens and ducks suggest that the host response varies greatly depending on the viral strain. Infection of chickens with low pathogenic strain A/ph/CA/2373/98 (H9N2) did not upregulate MHC class I, *IFIT5*, *Mx* or *OASL* in the infected lung at 1 dpi (Xing et al., 2008), and A/ch/CA/1772/02 (H6N2) resulted in slight down regulation of MHC class I, *Mx* and *OASL* in lung tissues (Xing et al., 2008). In contrast, infection of chickens with the highly pathogenic strain A/chicken/Yamaguchi/7/2004 (H5N1) upregulated *OASL* in chicken lung tissues (Uchida et al., 2012). The Yamaguchi 7 strain induced high levels of bioactive interferon in lung, spleen and plasma, while a low pathogenic strain A/vac-1/Hokkaido/04 induced much less (Moulin et al., 2011). Highly pathogenic strains A/Cygnus cygnus/Germany/R65/2006 (H5N1) and A/seal/Mass/1/80 (H7N1) also elicited high levels of interferon in chickens, however these strains were resistant to antiviral effects of IFN (Penski et al., 2011). Therefore a picture is emerging where highly pathogenic avian influenza strains elicit high levels of Type I IFN in chickens, while low pathogenic strains do not. Our data on induction of ISGs suggests the same is true in ducks, where Type I IFN is strongly induced by VN1203, and to a lesser extent by BC500 infection.

Comparison of ducks and chickens infected with the same highly pathogenic influenza strain reveals a very different outcome. A/Vietnam/1203/04 (VN1203) causes 100% mortality in chickens in 1 to 3 days, and 50% mortality in ducks (Hulse-Post et al., 2005). Infection of chickens with A/Vietnam1203/2004 (H5N1) virus resulted in a 20 and 40-fold induction of IFN- α and IFN- β transcripts in lung tissue at 36 hpi, while the same strain with a deletion of the polybasic HA cleavage site did not elicit IFNs (Karpala et al., 2011). The recombinant rgVN1203 we used is less pathogenic in ducks due to 3 changes in PB1-F2 (Marjuki et al., 2010) and results in only 20% mortality in ducks. Thus the induction of ISGs by VN1203 is part of a successful immune response in ducks, which results in reduction of virus by 3 dpi, as estimated by M gene amplification. The induction of IFNs in chickens suggests that ISGs would also be induced, however, the outcome of infection is very different. Most likely, VN1203 can subvert the antiviral effect of Type I IFNs in chickens. This highlights the need to compare the expression and function of induced antiviral effectors and the proinflammatory cytokine profile, between chickens and ducks to investigate the molecular basis of different pathogenicity in these two species.

Here, our SSH libraries contribute new expressed sequences and provide a crude picture of the antiviral response mounted by the natural host after challenge with high and low

pathogenic viruses. We saw minimal overlap between gene sets obtained from the three SSH libraries, suggesting that the transcriptional response is specific for the virus and the infected tissue. Innate immune transcripts were enriched in SSH libraries and upregulation was confirmed by qRT-PCR for MHC class I and 4 ISGs. The expression profile is remarkable both for its rapid timing and the magnitude of gene induction. We observed large increases in transcript abundance of ISGs in ducks at 1 dpi with VN1203, and a modest response against low pathogenic BC500 in the natural host. This may also reflect duck immune responses to these influenza strains in nature.

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Highlights

- Suppressive subtractive hybridization libraries made for influenza-infected tissues.
- Immune genes were enriched by infection with high and low pathogenic strains.
- MHC class I was highly upregulated in A/Vietnam1203/04 infected lung.
- Interferon stimulated genes were upregulated 1000-fold by highly pathogenic influenza.
- Innate responses were initiated immediately at one day post-infection.

Fig. 1.

IFIT5 was enriched in lung and intestine SSH libraries from influenza-infected ducks. Venn diagram of sequences identified in forward-subtracted SSH cDNA libraries prepared from lung RNA from a duck infected with VN1203, and lung and intestine RNA of a duck infected with BC500.

Fig. 2.

MHC class I *UAA* is upregulated in lung and intestine tissues from influenza-infected ducks. Lung RNA was extracted from ducks 1 dpi and 3 dpi with BC500 or VN1203 and analyzed in comparison to mock-infected ducks by qRT-PCR. Fold expression of *UAA* mRNA in duck lung tissues at 1 dpi and 3 dpi (A) and in duck intestine tissue at 1 dpi or 3 dpi (B) is shown relative to a mock-infected animal from each day. Dots represent individual ducks (n=3) and the mean is indicated. qRT-PCR was performed twice and data from one replicate plotted.

Fig. 3.

Interferon-stimulated genes are vastly upregulated in duck lung at 1 dpi with VN1203 virus. Lung RNA extracted 1 dpi and 3 dpi with BC500 or VN1203, was analyzed for expression of ISGs in comparison to mock-infected ducks by qRT-PCR. Fold expression of mRNA transcripts for (A) *ISG12-2*, (B) *IFIT5*, (C) *OASL* and (D) *IFITM1* is shown relative to a mock infected duck from each day. Dots represent individual ducks (n=3) and the mean is indicated. qRT-PCR was performed twice and data from one replicate plotted.

Fig. 4.

Interferon-stimulated genes are upregulated in intestine tissue of BC500 infected ducks relative to mock-infected animals. Intestine RNA extracted 1 and 3 dpi with BC500 was analyzed for expression of ISGs relative to mock-infected ducks by qRT-PCR. Fold expression of mRNA transcripts for A) *ISG12-2* and B) *IFIT5* are shown relative to a mock-infected RNA sample. Bars represent individual ducks (n=3) and the mean is indicated. qRT-PCR was performed twice and data from one replicate plotted.

Table 1

Titre (EID₅₀/mL) of swabs taken from ducks infected with VN1203 and BC500.

Duck	virus	Tracheal swab 3dpi	Cloacal swab 2 dpi	Cloacal swab 3dpi
316	VN1203	5.58×10 ⁴	ND	–
317	VN1203	1×10 ⁴	ND	–
318	VN1203	5.58×10 ²	ND	–
68	BC500	–	2.49×10 ⁵	+
69	BC500	–	1×10 ⁶	5.18×10 ⁴
70	BC500	–	2.71×10 ²	3.16×10 ⁷

* duck 68 3dpi swab positive but titre not determined. Swabs for BC500 at 1dpi were negative.

Table 2
Suppression subtracted hybridization cDNA libraries and EST sequences deposited

Tester sample	Driver sample	RNA	library	#clones	unique	accession numbers
Lung VNI203	mock-infected lung	poly A+	LIBEST_027385	621	125	JK311321-939, 312443-4
Lung BC500	mock-infected lung	total	LIBEST_027384	504	171	JK311940-2442, 312923
Intestine BC500	mock-infected intestine	poly A+	LIBEST_027386	562	161	JK310758-311320

Table 3

Primer and probe sequences for qRT-PCR.

Gene	Primer and Probe Sequence (5'→3')
<i>MHC I UAA</i>	Primer 1: CCAACCTGATCCCCATTGTG Primer 2: TTGTAGCCCTTCCCTTCTTC Probe:/56-FAM/CTGTCATCGCTGCCCTGGCTGG/3IABkFQ/
<i>IFT5</i>	Primer 1: GGTGTCAGTGTAAAGGCTTTTCTCA Primer 2: TCCTGCGATATGCTGCTATATTTAT Probe:/56FAM/CTCCAGTGCCTTGTCACCTTCCCTTTC/3IABkFQ/
<i>OASL</i>	Primer 1: GACCAACACACACTGCAGCACTA Primer 2: CCGTAGCCGCAGAAGCA Probe:/56-FAM/ACGTCCAGCCCAAGAGCACCATCTAC/3IABkFQ/
<i>IFTM1</i>	Primer 1: CACCGCCAAGTACCTGAACA Primer 2: CGATCAGGGCGATGATGAG Probe:/56-FAM/CACGGCCCTGCTGCTCAACATCT/3IABkFQ/
<i>ISG12-2</i>	Primer 1: AAAATGGCTGACCGAAACGT Primer 2: TGTGTGAAGCAAGCGAACCT Probe:/56-FAM/ACAACGCTG/ZEN/GCTTCGGCTCTTCCT/3IABkFQ/
<i>GAPDH</i>	Primer 1: AAATTGTCAGCAATGCCTCTTG Primer 2: TGGCATGGACAGTGGTCATAA Probe (single quenched):/56-FAM/ACCACCAACTGCCTGGCGCC/3IABkFQ/ Probe (double quenched):/56-FAM/ACCACCAAC/ZEN/TGCCTGGCGCC/3IABkFQ/

Table 4

Immune genes enriched in duck lung 1 dpi with VN1203

Insert Identity	Number of Clones
Major histocompatibility complex class I (<i>MHC-I</i>)	257
Heat shock protein 90 (<i>HSP90</i>)	27
Heat shock protein 70 (<i>HSP70</i>)	18
Fibrinogen-like 1 (<i>FGL1</i>)	11
Selenoprotein P, plasma, 1 (<i>SEPP1</i>)	5
Complement regulatory protein CD59	5
2'-5' oligoadenylate synthase-like (<i>OASL</i>)	4
MHC-II invariant chain (CD74)	2
Lymphocyte antigen 6 complex locus E (LY6E)	2
Interferon regulatory factor 1 (IRF-1)	2
Interferon induced protein with tripeptide repeats 5 (IFIT5)	2
IFITM1	1

Table 5

Immune gene transcripts enriched in duck lung 1 dpi with BC500.

Insert Identity	Number of Clones
Fibrinogen-like 1 (<i>FGL1</i>)	20
Nuclear factor related to kappa binding protein (<i>NFRKB</i>)	12
Vascular endothelial cadherin 5 (<i>CDH5</i>)	8
Selenoprotein P, plasma, 1 (<i>SEPP1</i>)	8
Interferon induced protein with tricopeptide repeats 5 (<i>IFIT5</i>)	6
GTPase activating Rap/RanGAP domain-like 1 (<i>GARNLI</i>)	3
Lysozyme (<i>LYZ</i>)	2
Complement regulatory protein CD59	1
Ubiquitin conjugating enzyme E2 D3-like (<i>UBE2D3</i>)	1
Signal transducer and activator of transcription 1 (<i>STAT1</i>)	1
CD44 (<i>CD44</i>)	1
Chemokine receptor 4 (<i>CXCR4</i>)	1

Table 6

Immune gene transcripts enriched in intestine 1 dpi with BC500.

Insert Identity	Number of Clones
Intestinal 15kDa protein (<i>ILLBP</i>)	83
Major histocompatibility complex I (<i>MHC-I</i>)	80
Interferon-stimulated gene 12-2 (<i>ISG12-2</i>)	24
Beta ₂ -microglobulin (<i>β2m</i>)	10
Major histocompatibility complex II (MHC-II) <i>β-chain</i>	4
Interferon-induced protein with tricopeptide repeats 5 (<i>IFIT5</i>)	3
ISG12-1 protein (<i>IFI27L2</i>)	1