Immune-related gene expression in response to H11N9 low pathogenic avian influenza virus infection in chicken and Pekin duck peripheral blood mononuclear cells

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\textbf{A B S T R A C T}

The duck and chicken are important hosts of avian influenza virus (AIV) with distinctive responses to infection. Frequently, AIV infections in ducks are asymptomatic and long-lasting in contrast to the clinically apparent and transient infections observed in chickens. These differences may be due in part to the host response to AIV infection. Using real-time quantitative PCR, we examined the expression of immune-related genes in response to low pathogenic AIV H11N9 infection in peripheral blood mononuclear cells (PBMC) isolated from the blood of chickens and Pekin ducks. While chicken PBMC expressed IL-1\textbeta and IL-6 at high levels similar to mammalian species, duck PBMC expression levels were minimal or unchanged. Similarly, duck IFN-\textbeta expression was nearly unaffected, whereas chicken expression was highly upregulated. Chicken IFN-\textgamma was expressed to higher levels than duck IFN-\textgamma, while IFN-\textalpha was expressed similarly by both species. IL-2 was elevated early in infection in duck PBMC, but returned to baseline levels by the end of the experiment; in contrast, IL-2 was weakly induced in chicken PBMC at late time points. TLR-7 and MHC class I molecule expression were conserved between species, whereas duck MHC class II expression was downregulated and chicken expression was unchanged. These results show distinct PBMC expression patterns of pro-inflammatory cytokines and IFNs between species. The differences in pro-inflammatory cytokine and IFN expression reflect the asymptomatic and lasting infection observed in ducks and the tendency towards clinical signs and rapid clearance seen in chickens. These results highlight important differences in the host response to AIV of two species thought to be critical in the genesis and maintenance of epidemic strains of AIV.

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1. Introduction

Avian influenza viruses (AIV) belong to the Orthomyxoviridae, are of the Type A genus, and have negative-sense, single-stranded, segmented genomes. Avian influenza viruses are encapsulated by envelopes containing the surface proteins hemagglutinin (HA) and neuraminidase (NA) and are classified by 16 identified HA and 9 NA subtypes (Fouchier et al., 2005), all of which occur in their reservoir hosts, free-flying waterfowl and shorebirds (Spackman, 2008; Webster et al., 1992).

The roles of birds as reservoir hosts and hosts in which viruses with pandemic potential can be amplified and transmitted to humans have become a focus of interest with the emergence and perpetuation of H5N1 highly pathogenic avian influenza virus (HPAIV). Despite this, few studies have been done to elucidate basic viral pathogenesis and host response questions in avian species resulting in incomplete and confusing data on AIV in avian hosts. For example, chickens and ducks generally respond to AIV infections differently and there are many instances demonstrating that infection with a specific AIV isolate may cause lesions and even death in a chicken host, while infection of a duck with the same virus would be asymptomatic, rarely resulting in death (Homme and Easterday, 1970; Kida et al., 1980; Narayan et al., 1969; Otsuki et al., 1982; Slemons and Easterday, 1972; Slemons et al., 1990). AIV shedding in chickens is transient with a rapid clearance by the host (Kwon et al., 2008; Lee et al., 2004; Otsuki et al., 1982; Smith et al., 1980); in contrast, prolonged, intermittent shedding is observed in infected ducks (Higgins et al., 1987). While chickens can mount a strong humoral immune response to AIV infection (Suarez and Schultz-Cherry, 2000), it has been reported that ducks do not (Kida et al., 1980; Philpott et al., 1989). Further, though AIV replication has been reported in both the respiratory system and the intestinal
tract for both species (Wood et al., 1995). AIV are usually limited in distribution to the intestinal tract in the duck (Scholtissek, 1995), while replication in the upper respiratory tract of chickens with some migration to the intestinal tract is more common (Lee et al., 2007; Swayne, 1997). To add to the complexity, influenza viruses adapted to efficient growth in ducks, do not always grow in chickens and vice versa and thus, very few studies have compared viral pathogenesis of or host responses to the same AIV.

AIV replicates primarily in the epithelial cells of the lung and intestines, however, spread to resident macrophages and recruited monocytes are also a feature of infection (Herold et al., 2006). Macrophages, among other cells that make up PBMC, possess a battery of receptors responsible for the recognition of conserved molecular patterns expressed by pathogens and the subsequent induction of a signaling cascade that culminates in the activation of an immune response. One such receptor, toll-like receptor (TLR) 7 is found in the endosomal compartment and recognizes single-stranded viral RNA released during the uncoating of internalized virus (Barton, 2007). The recognition of viral RNA results in the secretion of pro-inflammatory cytokines such as IL-1β and IL-6 as well as anti-viral cytokines such as the interferons (IFNs) (MacDonald et al., 2008). Expression of IFNs and pro-inflammatory cytokines influences both viral clearance and clinical disease presentation. MHC class I and II antigen presentation, though selectively utilized on the basis of pathogen uptake, both serve to activate cellular members of the adaptive immune response such as B cells and T cells (CD4+ and CD8+) (Gromme and Neefjes, 2002; Williams et al., 2002). Endogenously processed antigen is presented by MHC class I molecules and activates CD8+ cytotoxic lymphocytes (CTL), while antigen processed exogenously is presented by MHC class II molecules activating antibody secretory B cells and helper CD4+ T cells (Germain, 1994; Lennon-Dumenil et al., 2002). The differential expression of the MHC molecules and the expression of cytokines such as IL-2 and IL-6 may provide clues as to whether a cell-mediated (Th1) or antibody (Th2) response are being initiated by the cells responding to influenza virus infection.

The in vitro infection of chicken and duck PBMC with AIV serves as a starting point for observing host responses. By comparing the expression of cytokines involved in pathogen responses including the pro-inflammatory, anti-viral, and cell-mediated and adaptive responses in PBMC, we can better understand how immune responses and thus, pathogenesis might differ between two highly relevant agricultural species. Here we present the results of quantitative real-time RT-PCR analysis of several cytokines, the TLR-7, and the MHC class I and II molecules expressed in response to infection with the same low pathogenic avian influenza virus (LPAIV) H11N9 in chicken and duck PBMC.

2. Materials and methods

Birds: Two 1-year-old Pekin ducks serologically negative for AIV antibodies were obtained from a commercial breeder (Metzer Farms, Gonzales, CA). Two Hyline W-36 egg-laying hens serologically negative for AIV antibodies were obtained from the University of California, Davis avian research facility (Hopkins Avian Research Facility, UC Davis). Blood was collected from all birds by wing venipuncture into heparinized RPMI medium (Invitrogen Corp., Carlsbad, CA). Ten milliliters of blood was collected from each bird and subsequently pooled by species.

2.1. PBMC cell culture

Peripheral blood mononuclear cells (PBMC) were purified by ficoll gradient (Lymphocyte Separation Media, Mediatech, Inc., Herndon, VA) separation. The PBMC were grown overnight in RPMI supplemented with 10% fetal bovine serum, 5% chicken serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C, 5% CO2 with a cell density of approximately $5 \times 10^6$ cells/60 mm tissue culture dish. After overnight growth, non-adherent cells were removed by washing the monolayers with sterile PBS to enrich the cultures for adherent macrophages, monocytes, and dendritic cells.

2.2. Virus and cell culture infection

The AIV strain used in this study was A/duck/WA/663/97 (H11N9), a duck-adapted virus that has been well-characterized in our laboratory (Li et al., 2008). The virus stocks were propagated in SPF chicken eggs (Charles River, CA) using standard methods (Woolcock, 2008) to a titer of $10^{7.6}$ TCID50/mL as determined in Madin-Darby canine kidney (MDCK) cells.

<table>
<thead>
<tr>
<th>RNA target</th>
<th>Primer sequences</th>
<th>Size of PCR product (bp)</th>
<th>Target accession number</th>
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<td>Reverse</td>
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<td>5'-GGATGAGGCTGTTGGCAGGAC-3'</td>
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Table 1: Sequence of the oligonucleotide primers used in quantitative RT-PCR.
Cultures of PBMC were made in triplicate for both birds. One PBMC culture was trypsinized and the cells counted. This representative count was used to calculate the volume of virus stock necessary to infect the other three PBMC cultures at an m.o.i. of 4 for duck. Chicken PBMC were infected at an m.o.i. of 0.5 because an m.o.i. of 4 resulted in the death of all cells in the culture by 8 hpi and the failure to recover RNA (data not shown). To infect cells, growth media was replaced with infection media containing RPMI and supplemented as before, but without serum, and with the addition of viral allantoic fluid (VAF). Negative control PBMC cultures were set up identically but without the addition of VAF. Culture plates were gently rocked every 15 min for 1 h after which the media was replaced with RPMI supplemented with 0.2% fetal calf serum (Invitrogen Corp., Carlsbad, CA) and 1 μg/mL TPCK trypsin (Sigma–Aldrich, St. Louis, MO). Cultures were incubated and RNA extracted from the cell monolayer at 8, 24, and 36 h post-infection (hpi).

2.3. RNA and cDNA preparation

Total RNA was isolated from infected and control PBMC at each time point using QIAshredder columns and the RNeasy mini RNA Purification kit following manufacturer’s instructions (Qiagen Inc., Valencia, CA). RNA in each sample was quantified using Ultrospec 2000 mass spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Three hundred nanograms of RNA from each culture was treated with DNase to remove genomic DNA and then used to produce cDNA using a Quantitect® Reverse Transcription kit (Qiagen Inc., Valencia, CA).

2.4. Quantitative real-time RT-PCR

qRT-PCR was performed using primers designed by Primer3 software [http://frodo.wi.mit.edu/] based on published target sequences. Primers were developed for IL-1β, IL-2, IL-6, interferon alpha (IFN-α), interferon beta (IFN-β), interferon gamma (IFN-γ), TLR-7, and MHC class I and class II molecules based on published sequences and the predicted product sizes are shown in Table 1. Primers pairs were selected based on specificity as determined by dissociation curves. qRT-PCR was performed using 7500 Real-Time PCR System (Applied Biosystems) and Smartcycler (Cepheid) thermocyclers. PCR conditions were the same for each targeted gene and are as follows: 1 min at 50 °C, 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s and 58 °C for 32 s. Cycling was terminated after 45 cycles with 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Dissociation curves of the products were generated.
by increasing the temperature of samples incrementally from 55 to 100 °C as the final step of the real-time PCR. Amplified products were run on a gel and extracted using a PCR and Gel Purification kit (Qiagen Inc., Valencia, CA). For the purpose of assay validation, purified products were cloned into pCR-TOPO2.1 subcloning kit (Qiagen Inc., Valencia, CA) and sequenced to verify proper target amplification using M13 forward and reverse primers as provided by Davis Sequencing (Davis, CA).

2.5. Calculations and statistics

Expression fold change was determined by the \( \frac{C_t}{94} \) method using glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as the endogenous reference gene to normalize the level of target gene expression. Logarithmic transformation was performed on fold change values before being analyzed by the Student’s T-test. Error bars represent standard error.

3. Results

3.1. Differential expression of pro-inflammatory, anti-viral, and Th1-associated cytokines in duck and chicken PBMC

The expression of pro-inflammatory cytokines IL-1β and IL-6 were statistically different \((p < 0.01)\) at each time point for duck and chicken PBMC infected with H11N9. In chicken, both IL-1β and IL-6 messages were induced by 8 hpi (9- and 1006-fold respectively) and peaked at 24 hpi (16- and 1322-fold). While also upregulated, IL-6 expression in duck cells was induced at a far lower level (peak expression was 12-fold at 36 hpi). In contrast, IL-1β expression was suppressed for the duration of the experiment 1.4- to 2-fold in duck cells (see Fig. 1A). Taken together, these results indicate weak pro-inflammatory signaling in duck PBMC, which is different from the robust expression of pro-inflammatory cytokines observed in chicken PBMC.

We compared type I and II IFN expression in chicken and duck cells infected with H11N9 and observed a similar species-dependent response. The pattern of IFN-α responses in chicken and duck were similar with peak expression occurring at 8 hpi followed by a decrease to baseline expression between 24 and 36 hpi. The differences in expression levels for IFN-α were not statistically different \((p > 0.05)\) between duck and chicken. The comparative expression of IFN-β represents the most striking finding in this study: chicken IFN-β is highly expressed and peaks at 238-fold expression while the duck response is never more than 1.6-fold elevated (24 hpi). IFN-γ is induced, although insignificantly different \((p = .32)\) to 406-fold for duck and 757-fold for chicken cells by 8 hpi, but a decline in expression in duck is observed by 24 hpi (39-fold induction), whereas chicken IFN-γ continues to rise and peaks at 1698-fold expression at 24 hpi (see Fig. 1B).

The expression of the Th1-involved cytokine, IL-2, was significantly higher in duck cells compared to chicken cells at 8 hpi, while at the other time points IL-2 was not significantly different between the species. Despite the lack of statistically significant differences at the 24 and 36 hpi points, the trend of IL-2 expression is clearly different: duck PBMC express elevated levels (4-fold induction) of IL-2 early during the infection, followed by a gradual decrease to baseline expression by 36 hpi. In contrast, chicken PBMC expression of IL-2 was gradually induced from baseline levels at 8 hpi to a 3-fold induction by 36 hpi.

3.2. Conserved downregulation of TLR-7 expression in chicken and duck PBMC

We compared the expression of TLR-7 in response to infection with H11N9 in chicken and duck PBMC. The results showed that both duck and chicken expressed TLR-7 only transiently at the early stage of infection followed by a decline (see Fig. 2). Peak expression occurred at 8 hpi for both species PBMC, and never surpassed baseline expression levels. At 24 and 36 hpi, the infection process appeared to result in approximately the same level of downregulation of TLR-7, between 1.5- and 2-fold decrease in expression in duck and chicken cells (see Fig. 2).

3.3. MHC class I and II molecule expression preference by species

MHC class I molecule expression was upregulated in duck PBMC throughout the duration of the experiment. By 8 hpi, MHC class I was upregulated 2-fold whereas MHC class II molecules were downregulated by 1.5-fold. The trend of MHC class II expression continued through the experimental period, reaching its lowest level at 36 hpi with a 3-fold downregulation. MHC class I, on the
other hand, was upregulated to 5-fold by 24 hpi, followed by a decrease to a 3-fold induction at 36 hpi. In chicken and duck PBMC, MHC class I molecules were expressed at nearly identical levels, whereas MHC class II molecules were maintained at baseline levels with no significant changes. The difference in MHC class I and class II expression between duck and chicken PBMC was not significant for the 8 and 24 hpi time points ($p = 0.19$, $p = 0.13$), however, by 36 hpi, the MHC class II expression levels were significantly different ($p = .01$) with duck suppression by 3-fold and chicken upregulation at 1.4-fold induction (see Fig. 3).

4. Discussion

In mammals, signs of influenza stem from the host’s inflammatory response to infection (Durbin et al., 2000). It has been shown that infected cells and the immune cells that respond to infection express early cytokines in response to the presence of the pathogen. The cytokines, IL-1β, IL-6, TNF-α, and IFN-α, are expressed by primary and continuous macrophage cultures from mice, swine, and humans immediately after influenza virus infection (Peschke et al., 1993; Van Reeth, 2000). The results from our study indicate that, with the exception of IFN-α, the expression of the pro-inflammatory cytokines IL-1β and IL-6 in chicken and duck PBMC are distinct. The increased expression of IL-1β and IL-6 in chicken and the skew towards a Th1 response are similar to the responses of mammals. In contrast, the downregulation of IL-1β and IL-6 and the skew towards a weak Th1 response in duck PBMC is very different both from the responses of chickens and mammals infected with influenza A viruses. In addition, it has been demonstrated in mice, swine, and humans that disease outcome is correlated with the in vitro expression of IL-1β, IL-6, TNF-α, and IFN-α (Hayden et al., 1998; Kaiser et al., 2001; Skoner et al., 1999; Van Reeth, 2000). Though our results were derived in vitro, they are consistent with what is observed in experiments carried out in vivo, i.e., an absence of signs of disease in ducks correlates with low pro-inflammatory cytokine levels and the presence of lesions in chickens correlated with high pro-inflammatory cytokine levels.

Differential expression of the type I IFNs in chicken and duck PBMC evidenced by the weak induction of IFN-β in duck PBMC and the approximately 100-fold induction in chicken PBMC may suggest differing susceptibilities of the two species to the suppressive effect of the NS1 gene on IFN expression. Reduction in IFN-β expression after infection with influenza virus was likely due to NS1A expression, a virally encoded immunomodulator. The interaction between NS1A and the cellular protein cleavage and polyadenylation specificity factor, which is required for the processing of the 3′-end of mRNA, results in suppression of IFN-β expression in human airway epithelial cells (Noah et al., 2003). In comparing the expression levels of both type I IFNs, chicken PBMC express higher levels of both IFN-α and IFN-β than duck PBMC. A weak induction of IFN has been correlated in chickens with higher virus titers and a longer shedding period, whereas strong IFN expression results in lower virus titers and a shorter shedding period (Cauhen et al., 2007). Based on these findings in chickens, we conclude that the lower overall expression of IFN-γ by duck PBMC in response to AIV infection reflect what happens at the organismal level, longer shedding and weaker viral clearance observed in duck, and more rapid clearance and thus a relatively shorter shedding period in chicken.

Ducks and chickens are among the most important hosts in the maintenance and transmission cycles of influenza A viruses. Some of the reasons that the duck and chicken agricultural systems have been so critical in the emergence of epidemic AIV are that AIV can replicate at low levels in duck for at least 32 days and be shed intermittently (Higgins et al., 1987) and infected ducks are frequently asymptomatic (Webby and Webster, 2001; Webster et al., 1978) and thus may contact many new individuals while shedding virus. However, it may also be that different species may exert variable immunological pressures on AIV and their combined presence results in greater changes in the virus than would happen if a single species were infected. It has been suggested that since ducks and chickens are short-lived, they do not put immunological pressure on pathogens such as AIV and therefore there is little antigenic drift (Austin and Webster, 1986; Kida et al., 1987). Despite this argument, our findings would suggest that antigenic drift probably does occur in chickens, as others have reported (Garcia et al., 1997; Suarez et al., 1999) and it is driven by a robust host response to infection. In contrast, our findings of MHC II downregulation would suggest that there is a much less robust antibody response in ducks and that antigenic drift may not occur in ducks as has been suggested by others (Kida et al., 1987). However, ducks do immunologically respond to AIV infection and their responses may exert a different type of selective pressure whose impact is yet to be understood. The real-life complexities of understanding the emergence of novel disease agents in agricultural systems surely requires more study, including more attention to host immune responses.

References


