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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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ABSTRACT

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-1β and IL-6 at high levels similar to mammalian species, duck PBMC expression levels were minimal or unchanged. Similarly, duck IFN-β expression was nearly unaffected, whereas chicken expression was highly upregulated. Chicken IFN-γ was expressed to higher levels than duck IFN-γ, while IFN-α 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 expressions 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.

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

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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 secreting 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 millilitres 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% CO_2 with a cell density of approximately 5×10^7 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 $10^{7.6}$ $TCID_{50}$/mL as determined in Madin-Darby Canine Kidney (MDCK) cells.

Table 1
Sequence of the oligonucleotide primers used in quantitative RT-PCR.

RNA target	Primer sequences		Size of PCR product (bp)	Target accession number
	Forward	Reverse		
Chicken				
GAPDH	5′-CCTCTCTGGCAAAGTCCAAG-3′	5′-CATCTGCCCATTTGATGTTG-3′	200	V00407
IL-1β	5′-GCTCTACATGTCGTGTGTGATGAG-3′	5′-TGTCGATGTCCCGCATGA-3′	80	NM204524
IL-2	5′-CGGGATCCATGATGTGCAAAGTACTG-3′	5′- CGGTCGACTTATTTTTGCAGATATCT-3′	80	AY510091
IL-6	5′-ATGTGCAAGAAGTTCACCGTG-3′	5′-TTCCAGGTAGGTCTGAAAGGCGAA-3′	171	EU170468
Interferon α	5′-ATGCCACCTTCTCTCACGAC-3′	5‘- AGGCGCTGTAATCGTTGTCT-3′	387	EU367971
Interferon γ	5′-GCTGACGGTGGACCTATTATT-3′	5′- TGGATTCTCAAGTCGCTCATCG-3′	248	DQ906156
MHC class I	5′-AAGAAGGGGAAGGGCTACAA-3′	5′-AAGCAGTGCAGGCAAAGAAT-3′	222	NM001031338
MHC class II	5′-CTCGAGGTCATGATCAGCAA-3′	5′-TGTAAACGTCTCCCCTTTGG-3′	312	DQ008588
TLR-7	5′-TGTGATGTGGAAGCCTTTGA-3′	5′-ATTATCTTTGGGCCCCAGTC-3′	218	DQ780342
Duck and chicken				
Interferon β	5′-CCTCAACCAGATCCAGCATT-3′	5′- GGATGAGGCTGTGAGAGGAG-3′	259	AY831397
Duck				
GAPDH	5′-ATGTTCGTGATGGGTGTGAA-3′	5′-CTGTCTTCGTGTGTGGCTGT-3′	176	AY436595
IL-1β	5′-TCGACATCAACCAGAAGTGC-3′	5′-GAGCTTGTAGCCCTTGATGC-3′	185	DQ393268
IL-2	5′-GCCAAGAGCTGACCAACTTC-3′	5′-ATCGCCCACACTAAGAGCAT-3′	137	AF294323
IL-6	5′-TTCGACGAGGAGAAATGCTT-3′	5′-CCTTATCGTCGTTGCCAGAT-3′	150	AB191038
Interferon α	5′-TCCTCCAACACCTCTTCGAC-3′	5′-GGGCTGTAGGTGTGGTTCTG-3′	232	EF053034
Interferon γ	5′-GCTGATGGCAATCCTGTTTT-3′	5′- GGATTTTCAAGCCAGTCAGC-3′	247	AJ012254
MHC class I	5′-GAAGGAAGAGACTTCATTGCCTTGG-3′	5′-CTCTCCTCTCCAGTACGTCCTTCC-3′	196	AB115246
MHC class II	5′-CCACCTTTACCAGCTTCGAG-3′	5′-CCGTTCTTCATCCAGGTGAT-3′	229	AY905539
TLR-7	5′-CCTTTCCCAGAGAGCATTCA-3′	5′-TCAAGAAATATCAAGATAATCACATCA-3′	154	AY940195

PCR primers and accession numbers of gene targets.