Modulation of the immune responses in chickens by low-pathogenicity avian influenza virus H9N2

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Most low-pathogenicity avian influenza (LPAI) viruses cause no or mild disease in avian species. Little is known about the mechanisms of host defence and the immune responses of avian influenza-infected birds. This study showed that chicken macrophages are susceptible to infection with LPAI H9N2 and H6N2 viruses and that infection led to apoptosis. In H9N2 virus-infected chicken macrophages, Toll-like receptor 7 responded to infection and mediated the cytokine responses. Whilst pro-inflammatory cytokines were largely upregulated, the interferon (IFN) response was fairly weak and IFN-inducible genes were differentially regulated. Among the regulated genes, major histocompatibility complex (MHC) antigens II were downregulated, which also occurred in the lungs of H9N2-infected chickens. Additionally, interleukin (IL)-4, IL-4 receptor and CD74 (MHC class II invariable chain) were also downregulated, all of which are pivotal in the activation of CD4+ helper T cells and humoral immunity. Remarkably, in H9N2 virus-infected chickens, the antibody response was severely suppressed. This was in contrast to the robust antibody response in chickens infected with H6N2 virus, in which expression of MHC class II antigens was upregulated. These data suggest that neutralizing antibodies and humoral immunity may not be developed efficiently in H9N2-infected chickens. These findings raise questions about how some LPAI viruses differentially regulate avian immune responses and whether they have similar effects on mammalian immune function.

INTRODUCTION

Antigen-presenting cells are critical in processing and presenting pathogen antigens and activating adaptive immunity under major histocompatibility complex (MHC) restriction in infectious immunity. Virus infections generally upregulate MHC antigens and co-stimulatory molecules on antigen-presenting cells and induce pro-inflammatory cytokines and chemokine expression, which enhances dendritic cell and macrophage activation of T lymphocytes. Macrophages play important roles in innate immunity, such as phagocytosis, containment of pathogens and the secretion of cytokines and chemokines important in the inflammatory responses that contain infection and augment immune regulation (Taylor et al., 2005; Twigg, 2004). In influenza virus-infected hosts, mononuclear cells become infected after the primary target cells, respiratory ciliated epithelial cells (Fujisawa et al., 1987; Kaufmann et al., 2001). How macrophages function after influenza virus infection is critical in the host’s immune response to the virus and, thus, to viral pathogenesis.

H9N2 viruses are among the most commonly occurring in domestic poultry populations, with several outbreaks reported in Asia and North America since 1990. H9N2 subtype viruses are classified as low-pathogenicity viruses both by molecular characterization and by pathotyping. This subtype is unique among low-pathogenicity avian influenza (LPAI) viruses in that they infect a wide variety of species including chickens, quail, turkeys, ducks, geese, pigs and humans (Alexander, 2000; Peiris et al., 2001; Tang et al., 1998). Because H9N2 viruses infect a number of species, they may be significant donors of genetic material to emerging human pathogens (Guo et al., 1999; Lee et al., 2000; Matrosovich et al., 2001; Peiris et al., 1999). There is no evidence to suggest that H9N2 subtype viruses can be transmitted from human to human (Uyeki et al., 2002). However, they could emerge as human pathogens through reassortment in intermediate hosts, such as pigs (Peiris et al., 2001) and in avian species, or through direct adaptation in the human host (Guan et al., 1999, 2000). The ability of H9N2 subtype viruses to spread widely in domestic poultry populations (Alexander, 2000; Chen et al., 1994; Naem et al., 1999), to cross species barriers and
potentially to impact on emerging viruses (Guo et al., 1999; Peiris et al., 1999) warrants further studies on the pathogenesis of H9N2 viruses in humans as well as in avian species.

Extensive studies on influenza virus-affected gene-expression profiling have been performed (Baskin et al., 2004; Degen et al., 2006; Diaz-Mitoma et al., 2004; Geiss et al., 2001, 2002; Huang et al., 2001; Kash et al., 2004, 2006; Kobasa et al., 2007; Marshall et al., 2005; Wei et al., 2006). It was indicated that the drastic increase in interleukin (IL)-6 in the lungs may be a key factor for pulmonary pathology. The data from A/PR/8/34 (H1N1) virus-infected lung epithelial cells also showed that non-structural gene 1 (NS1) is critical for suppression of selected genes involved in interferon (IFN) and IFN-inducible gene expression (Geiss et al., 2002). In contrast to the extensive work performed in mammalian model systems, little is known about the global gene-expression profiles associated with LPAI viruses, which mostly restrict their host ranges to avian species. How avian hosts survive LPAI virus infections remains unclear. In this study, we examined the susceptibility of the chicken HTC macrophage cell line to LPAI virus strains. Our interest was in examining the consequences of LPAI virus infections on the immune responses in macrophages and in chickens. Our results indicated that chicken macrophages were highly susceptible to infection with A/phi/CA/2373/98 (H9N2) virus resulting in apoptosis. In macrophages and lung tissues infected with A/phi/CA/2373/98 (H9N2), class II MHC antigens were downregulated. Whilst many pro-inflammatory cytokines, chemokines or their receptors were upregulated, some were selectively reduced, including IL-4 and the IL-4 receptor (IL-4R), both crucial in the activation of CD4+ helper T (Th) cells and humoral immunity. When chickens were challenged with A/phi/CA/2373/98 (H9N2), we found that the antibody responses in infected chickens were largely suppressed. Taken together, these findings suggest that some LPAI viruses may specifically modulate host adaptive immune responses negatively in avian species.

**METHODS**

**Cell lines and reagents.** The HTC monocytic/macrophage cell line was obtained from Dr N. C. Rath (USDA, USA; Rath et al., 2003). These cells were derived from peripheral blood monocytic/macrophage cells and have been spontaneously transformed. The HTC cells were grown under conditions described previously (Rath et al., 2003). Madin–Darby canine kidney (MDCK) cells were purchased from the ATCC and grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Chicken blood was from Colorado Serum Co. Fluorescein isothiocyanate (FITC)-conjugated anti-influenza A nucleoprotein (NP) monoclonal antibody was purchased from ViroStat. The In situ Cell Death Detection Kit used for terminal dUTP nick-end labelling (TUNEL) assays was applied following the manufacturer’s instructions (Roche Diagnostics).

**Animals and viruses.** A/phi/CA/2373/98 (H9N2) was isolated from a domestically raised pheasant and A/ch/CA/1772/02 (H6N2) was isolated from a chicken during an LPAI outbreak in California in 2002–2003 (Woolcock et al., 2003). The eight genomic segments of both viral strains have been completely sequenced and analysed phylogenetically (J. Li, unpublished data). The viruses were grown in 10-day-old embryonated eggs from specific-pathogen-free hens (Charles River SPAFAS) and allantoic fluid was harvested 48 h after inoculation. Virus in the allantoic fluid was titrated using a standard haemagglutination test (Hirst, 1942) and infectious viral titres were determined in 10-day-old embryonated eggs (EID$_{50}$) and in MDCK cells (TCID$_{50}$).

**Virus infection in macrophages and chickens.** HTC cells (5×10^6) were plated on 10 cm tissue culture plates 16 h before infection. In one-cycle infections, cells were infected with group A/phi/CA/2373/98 (H9N2) or A/ch/CA/1772/02 (H6N2) virus in allantoic fluid at an m.o.i. of 1 and incubated at 37 °C. Chickens from commercial egg production flocks were used in all challenge experiments. Both 20–30-month-old and 2–3-week-old hens were challenged with 10^7 EID$_{50}$ virus delivered intranasally. In each challenge experiment, the birds were housed in groups in separate HEPA-filtered, negative-pressure isolators.

For microarray studies, 2–3-week-old birds were maintained in groups of ten; one group was an uninfected control group and the other group was inoculated with either H9N2 or H6N2 virus as described above. Two birds from each group were sacrificed on days 1, 3, 6, 9 and 12 and their lungs were taken for total RNA preparation. Both oropharyngeal and cloacal swabs were taken before challenge and on days 1, 3, 6, 9 and 12 post-challenge (p.c.). Swabs were placed immediately in virus transport medium on ice and subsequently used for virus isolation or viral RNA detection.

Additional challenge studies were carried out to determine the full range of antibody responses in H9N2- and H6N2-infected birds. Briefly, chickens were held in groups of 12. One group was maintained as an uninfected control group and two groups were challenged with either H9N2 or H6N2 virus. All chickens were bled and swabs were collected prior to challenge. After virus inoculation, the chickens were bled on days 3, 7, 10, 14, 17, 20 and 24, and both cloacal and oropharyngeal swabs were collected on days 2, 3, 4, 5, 6, 7, 11, 14 and 21. Chicken sera were prepared for haemagglutination inhibition (HI) antibody detection and swabs were handled as described above for examination of virus shedding. Two chickens from each group were euthanized, necropsied and examined for grossly evident lesions on days 3, 7 and 10. The challenge study was terminated on day 24 post-inoculation, with six chickens remaining in each group throughout the period of the challenge experiment.

**Immunofluorescence staining and TUNEL assay.** HTC cells were grown on four-chamber slides, fixed and permeabilized with 2% paraformaldehyde and 0.5% Triton X-100, prior to incubation with FITC-labelled anti-NP antibody diluted 1:20 in PBS at 4 °C for 30 min. The cells were washed three times for 10 min each with PBS, after which they were air-dried and rewetted with glycerol and a cover slip applied. Cells were viewed under a Nikon Eclipse E400 fluorescence microscope. TUNEL staining was performed following the instructions of the In situ Cell Death Detection kit. To quantify apoptosis, infected and non-infected cells were trypsinized at certain time points p.i. (p.i.) using trypsin/EDTA. After two washes with PBS, the cells were stained with 7-amino-actinomycin D (7-AAD; Sigma-Aldrich) on ice for 30 min. The cells were washed three times with PBS containing 0.5% BSA, followed by flow-cytometric analysis on a FACScan cell sorter (Benton Dickinson).

**RNA preparation from cell cultures and chicken lungs.** An RNeasy RNA extraction kit (Qiagen) was used to prepare total RNA from HTC cell cultures and the lungs of chickens. Control and infected macrophages (5×10^6 total) were trypsinized and washed
twice with PBS before being resuspended in 350 μl RLT buffer (Qiagen). Flash-frozen lung samples (40 mg each) were placed into 600 μl RLT buffer before being homogenized with a MagNA Lyser instrument (Roche Diagnostics). The quantity and integrity of the resultant RNA were examined using an Agilent Nano LabChip (Agilent Technologies) and the RNA preparations were used for probe labelling or RT-PCR.

cRNA probe amplification and labelling, and microarray analysis. Reverse transcription, second-strand cDNA synthesis, and cRNA probe generation and biotin labelling were accomplished following standard Affymetrix protocols. cRNA probe hybridization to Chicken GeneChips (Affymetrix) and scanning were performed at the Genome Center, University of California, USA, following the Affymetrix Expression Analysis Technical Manual. Fluorescence intensity values (*.cel files generated from Microarray suite 5.0) from scanned GeneChips were subsequently subjected to analyses utilizing a DNA-Chip Analyser (dChip; http://www.dchips.org) (Schadt et al., 2001) database (forward and reverse: GSM158130). The intensity value of each sample was normalized. Briefly, by default, an array with median overall intensity was chosen as the baseline array against which other arrays were normalized at probe intensity level by dChip and the gene expression was calculated in a PM (perfect matching) model (Zhong et al., 2003). Changes in the level of mRNA of any gene were selected only when the following two criteria were met: (i) the alteration in expression was statistically significant (F value for pair Student’s t-test of ≤0.05); and (ii) the change was at least 50% (equivalent to a 1.5-fold change where the value for no change is 0) above or below the baseline expression level. The baseline was calculated as the expression level of the 0 h or day 0 for a particular probe set. Genes with significant transcriptional changes were analysed by the NetAffx Analysis Center (http://www.affymetrix.com/software/index.affx).

Real-time RT-PCR. The presence of mRNAs for MHC classes I and II, β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in control and infected chicken macrophages was analysed using a two-step real-time RT-PCR. Total RNA (1 μg) was used for the reverse transcriptase reaction with a GeneAmp RNA PCR kit (Perkin Elmer) as described previously (Xing & Schat, 2000). Real-time PCR was carried out with 2.5 μl cDNA in a total volume of 25 μl using iQ SYBR Green Supermix (Bio-Rad) following the provided instructions. Relative expression values were normalized using either chicken β-actin or GAPDH. The fold change in relative gene-expression levels was calculated using the formula: 2ΔΔCt (of gene−ΔCt of β-actin or GAPDH). Melting curves were analysed to determine the specificity. Reactions were conducted in duplicate for each sample and the mean value was calculated.

The sequences for the primers used for real-time RT-PCR were chosen using the web-based software Primer3 (http://frodo.wi.mit.edu/primer3/input.htm; v.0.4.0) as follows (forward and reverse primers, respectively): MHC class II B-LA, 5'-CTCGAGGTCATGA-TGCAGCAA-3' and 5'-TTGAAAGGCC-GTACGATGAC-3'; MHC class II B-LB, 5'-GAGGTTTCCTGACAGC-3' and 5'-GGTAAAGGCC-GTACGATGAC-3'; MHC class I B1F2, 5'-GGCTGAGGAGGACTCG-TGAGG-3' and 5'-AATCCAACACCAACACGAT-3'; IL-4, 5'-GAGGTGTTTCCTGGTGCA-3' and 5'-TGGTGAAGAGGACTCG-TAGG-3'; IL-1β, 5'-GGCTCAACACTTGCGCCTGAC-3' and 5'-CC-CATTACTCTCTCTGAGC-3'; TLR7, 5'-TGACTGAGGAGGACTCG-TGAGG-3' and 5'-TTATCCTTCTGGGCCCCAGT-3'; β-actin, 5'-CATGAACTACATTGCTA-3' and 5'-GATTTCATGCTCCTCTGCTT-3'; and GAPDH, 5'-CCCTCTGGCGAAGTCC-AAG-3' and 5'-CATCTGGGCTTTGGTGTT-3'.

The real-time RT-PCR with the TaqMan protocol for the identification of influenza virus was performed based on a published protocol (Spackman et al., 2002). The primers used for detection of influenza A virus matrix (M) gene were 5'-AGATGATCTTTCTAACC-GAGTTCG-3' (forward), 5'-TGACAAAACTCTCTCTGCT-3' (reverse) and 5'-FAM-TCAGGCCCCCTCAAAAGCGCA-TAMRA-3' (probe).

Flow-cytometric staining of macrophages. Whole blood was taken from chickens inoculated with H6N2 or H9N2 virus on days 3 and 5 p.i. as described above. Peripheral blood mononuclear cells (PBMCs) were prepared with Ficoll (Mediatech) after low-speed centrifugation. A total of 106 cells was incubated with R-phycocerythrin-conjugated anti-chicken macrophage/monoocyte, FITC-conjugated anti-chicken MHC class I or FITC-conjugated anti-chicken MHC class II antigen (Southern Biotech) on ice for 30 min. After three washes with PBS containing 0.5% BSA, the cells were subject to flow-cytometric analysis using a FACScan cell sorter.

Virus shedding and HI assay. Chickens were inoculated and samples were collected as described above. The virus load in the swab samples was determined by real-time RT-PCR. Antibody responses in uninected and infected chickens were tested by HI assay based on Hirst’s principle (Hirst, 1942). The serum was diluted 10-fold with saline before making an additional 2-fold dilution with PBS. Virus with haemagglutination titres of 1:8 to 1:16 was then added to each diluted serum sample and mixed for approximately 15 min. An equal volume (50 μl) of 0.5% chicken red blood cells was added to the virus/serum mixture and incubated for 30–60 min before reading the results.

RESULTS

Susceptibility of chicken HTC macrophages to infection by H9N2 and H6N2 viruses

To understand the role macrophages may play in the pathogenesis of LPAI virus infections, chicken HTC macrophages were cultured and infected with LPAI viruses. The cells were susceptible to both the H9N2 and H6N2 virus (Fig. 1e and f). Twelve hours after infection, the morphology of H9N2 virus-infected HTC macrophages altered. More dendrites were produced and the cells flattened and became more strongly adherent to the culture plates. After 24 h, these same cultures deteriorated (Fig. 1c). A large number of H9N2 virus-infected macrophages became apoptotic, as demonstrated by a TUNEL assay (Fig. 1i), which was positive as early as 12 h p.i. Comparatively fewer obvious morphological changes and less apoptosis were observed in cells infected with H9N2 virus (Fig. 1b and h). Quantitatively, at 18 h p.i., about 40 and 20% of the cells infected with H9N2 and H6N2 viruses, respectively, underwent apoptosis. The number of apoptotic cells rose to 80 and 45%, respectively, at 36 h p.i. (Fig. 1f).

MHC antigen expression in H9N2 virus-infected macrophages

Affymetrix microarray chips were used to analyse the global expression patterns of genes in A/ph/CA/2373/98 (H9N2)-infected HTC macrophages. The expression of
multiple MHC antigen genes was affected in the infected cultures (Fig. 2a). MHC antigen genes, located in different spots with distinct probe-set IDs in the chips, were downregulated to various degrees in infected macrophages compared with uninfected cultures. Whilst expression of MHC class I antigens (B-FIV) were not changed (1.1–1.3-fold), expression of MHC class II antigens (B-LA and B-LB) detected at various loci was suppressed from 6 h p.i. MHC class II antigens were downregulated between 1.5- and 5.3-fold by 12 h p.i. (Fig. 2a and Table 1). It was notable that CD74, the MHC class II invariant peptide, was also downregulated by 1.2–2.0-fold.

In real-time RT-PCR analysis, whilst the expression of the MHC class I antigen showed slight increases at the earlier stage, the expression of MHC class II antigens was downregulated by 12 h p.i. (Fig. 3a). Functionally, MHC class II antigens are responsible for presentation of antigenic peptides to activate CD4+ Th lymphocytes. The decrease in MHC class II antigens on the macrophages, together with the lowered CD74, could negatively impact on the activation of CD4+ Th cells, which may lead to a suppression of both Th1 and Th2 responses.

In addition to MHC class I and class II antigens, chickens have unique molecules, important for non-classical antigen presentation and lymphocyte activation. Among them are the MHC restrictive fragment protein-Y (Rfp-Y) and CD1. As shown in Fig. 2(a) and Table 1, both MHC Rfp-Y and CD1 antigens were downregulated between 1.2- and 4.8-fold.
Differential regulation of cytokines in H9N2 virus-infected avian macrophages

Pro-inflammatory cytokines IL-1β and IL-8, and chemokines K203, ah221 (CCL7), ah294, K60 (CXCL1), ligand 3 (CCL3), ligand 14 (CXCL14) and ligand 20 (CCL20) were all upregulated 1.3–26.7-fold. However, cytokines IL-4, IL-6, IL-16 and IL-17, as well as IL-4R α-chain and IL-21R, were downregulated by between 1.2- and 10.8-fold (Fig. 2b and Table 1). The regulation of IL-1β and IL-4 expression was reproducible in the real-time RT-PCR analysis (Fig. 3b). Both IL-4 and IL-4R are critical to the Th2 response, and are required for activation and differentiation of Th2 CD4⁺ T cells. When considered together with the downregulation of MHC class II antigens, the activation of specific B lymphocytes to produce neutralizing antibodies in the Th2 response might be affected in A/ph/CA/2373/98 (H9N2) virus-infected chickens.

Regulation of IFN-related genes

Interferons were weakly induced in infected macrophages. As shown in Table 1, IFN-β expression was only increased up to 1.6-fold at 12 h p.i., whilst IFN-α and -γ expression
Table 1. Summary of pathogen-regulated gene expression in HTC macrophages infected with A/ph/CA/2373/98 (H9N2) virus

Changes (fold) in expression levels are shown relative to those of uninfected cells. Genes that were induced or reduced by more than 1.5-fold relative to those of uninfected cells were considered to be significantly regulated and are shown in bold.

<table>
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<th>Gene</th>
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<tbody>
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<tr>
<td>MHC class I glycoprotein (B-FIV)</td>
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<td>MHC class II β1 domain (B-LBI)</td>
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<td>Similar to C-type lectin/MHC Rfp-Y class I α-chain</td>
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<td>MHC class II antigen z (B-LA)</td>
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<tr>
<td>CD74 antigen (MHC class II invariant polypeptide)</td>
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<td>Cathepsin S</td>
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<tr>
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did not change in H9N2-infected macrophages. Weak induction of IFNs probably contributed to differential regulation of IFN-inducible genes. 2′-5′-Oligoadenylate synthetase (OAS)-like gene was upregulated 6 h p.i. by 3.3-fold, but the Mx protein gene was unchanged or marginally downregulated (Table 1). Among the strongly downregulated genes was IFN-induced protein with RNA helicase domain 1 (IFIH1), also known as melanoma differentiation associated protein 5 (MDA-5), which is considered crucial in IFN activation and host defence (Kato et al., 2006; Kawai et al., 2005). Additionally, IFN regulatory factor (IRF)-8 was downregulated between 2.1- and 4.1-fold but IRF-1 was slightly upregulated (Table 1). Among the strongly upregulated genes was the IFN-inducible 58 kDa protein with tetratricopeptide repeats 5 (IFIT-5; upregulated 20.1-fold) at the early stage of infection (6 h p.i.). Interestingly, IFN-inducible protein IP-30, a lysosomal thiol reductase also involved in MHC class II antigen processing and functional in antigen-presenting cells (Arunachalam et al., 2000; Maric et al., 2001), was suppressed up to 2.4-fold.

Regulated expression of genes involved in immunity in the lungs of LPAI virus-infected chickens

Gene-expression profiling of the lungs of chickens infected with A/ph/CA/2373/98 (H9N2) was evaluated. As shown in Table 2, multiple MHC class I and II antigens were downregulated to variable degrees in the lungs of infected chickens. MHC class I antigens were reduced by between 1.3- and 30.3-fold, whilst MHC class II antigens (B-LBI and B-LBVI) were downregulated by between 1.3- and 30.3-fold. CD74 and cathepsin S were also downregulated by

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between 1.6- and 1.7-fold on day 6 p.i. Among the genes that were reduced were the Ig light chain precursor and the polymeric immunoglobulin receptor (Table 2). Functionally these two genes may recognize specific pathogen antigens on the surface of immune cells, such as B lymphocytes, and help in the endocytic process.

**Table 2.** Summary of pathogen-regulated gene expression in the lungs of 2–3-week-old chickens infected with A/PH/CA/2373/98 (H9N2) or A/ch/CA/1772/02 (H6N2) virus

Changes (fold) in expression levels are shown relative to those from the lungs of uninfected chickens. Genes that were induced or reduced by more than 1.5-fold relative to those from the lungs of uninfected chickens were considered to be significantly regulated and are shown in bold.

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<td>Polymeric Ig receptor</td>
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<td>2'-5'-oligoadenylate synthetase-like</td>
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<tr>
<td>MHC B-G antigen</td>
<td>−1.5</td>
<td>−1.1</td>
</tr>
<tr>
<td>Mx protein</td>
<td>−2.7</td>
<td>−1.6</td>
</tr>
<tr>
<td>2'-5'-oligoadenylate synthetase-like</td>
<td>−1.9</td>
<td>−1.8</td>
</tr>
<tr>
<td>IRF-2</td>
<td>+1.5</td>
<td>+1.5</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>+1.7</td>
<td>+1.8</td>
</tr>
</tbody>
</table>
ultimately resulting in antigenic presentation by MHC class II proteins and the activation of helper T cells. Interestingly, no IFN induction was observed in infected lungs, which is probably explained in part by the observation that the Mx protein and 2′,5′-OAS IFN-inducible genes were both downregulated in the lungs. IFIT-5 was marginally transiently induced on day 1, but downregulated up to 2-fold on days 3 and 6 (Table 2). The expression levels of most interleukins and chemokines remained unchanged in the lungs of virus-infected chickens (data not shown), suggesting that pro-inflammatory responses might all be inhibited.

Chickens were also infected with A/ch/CA/1772/02 (H6N2) virus. As shown in Table 2, both the Mx protein (1.6–2.7-fold) and 2′,5′-OAS (1.8–1.9-fold) genes were downregulated. MHC class I antigens were downregulated by 2.2–6.3-fold between days 1 and 3 p.i., all of which were consistent with the results from A/ph/CA/2373/98 (H9N2)-infected chickens. However, in contrast to the findings with A/ph/CA/2373/98 (H9N2), the expression of MHC class II genes was either unchanged or upregulated (1.5–4.7-fold) in the lungs of H6N2-infected chickens.

Comparative analysis of H9N2 and H6N2 infection in chicken macrophages also indicated differences in the regulation of MHC class II antigens and IL-4 expression as revealed by the results of real-time RT-PCR (Fig. 4a). At 8 and 24 h p.i., the MHC class II antigens B-LA and B-LB were upregulated in H6N2-infected cells, whilst they were downregulated in H9N2-infected cells. A similar pattern was shown for IL-4 expression, but IL-1β expression was upregulated in both H6N2- and H9N2-infected macrophages (Fig. 4a).

### Regulation of Toll-like receptors (TLRs)

Regulation of TLR expression was also observed, and at 18 h p.i. TLR-2 and TLR-7 were downregulated up to 3.2- and 3.7-fold, respectively (Table 1). Several chicken TLRs including TLR-1, TLR-4 and TLR-15, which are highly expressed in uninfected HTC macrophages, remained unchanged or changed only marginally after infection (data not shown). Interestingly, when the expression of TLR-7 was further analysed at the earlier stages of infection using real-time RT-PCR, we found that TLR-7 responded to infection and expression was actually upregulated up to 7.8-fold at 8 h p.i. (Fig. 4b). It appeared that expression of TLR-7 was only suppressed during the later stage of infection, which echoes the downregulation of expression of multiple cytokines during the later stages of infection.

### Expression of MHC antigens on macrophages in infected chickens

PBMCs were prepared from whole blood taken from H6N2-infected, H9N2-infected or uninfected chickens on...
day 3 p.i. and stained with labelled anti-macrophage/monocyte and anti-MHC antigen antibodies (data not shown). MHC class I antigens were downregulated on the surface of monocytes from both H6N2- and H9N2-infected chickens. However, MHC class II antigens were upregulated in H6N2-infected birds, but downregulated in H9N2-infected birds.

Suppressed HI antibody responses in chickens challenged with H9N2 virus

We next examined how the host adaptive immunity responded in infected chickens. On day 7 p.c., HI antibodies were detectable in 100% of H6N2-infected chickens but in only 20% of H9N2 virus-infected birds became seropositive (Fig. 5a). Fifty per cent of chickens infected with H9N2 virus became seropositive on day 14 p.c. A comparison of the mean HI antibody titres between H6N2 and H9N2 virus-infected chickens is shown in Fig. 5(b). A vigorous HI antibody response was observed in H6N2-infected chickens starting at day 7, reaching its peak at day 10 and declining thereafter, whilst the antibody response in H9N2-infected chickens remained low throughout. The differences in the seroconversion and HI antibody titres between H9N2- and H6N2-infected chickens were significant (Student's t-test, P<0.01). The HI antibody titres are also shown in Table 3 for three pairs of chickens infected with either H6N2 or H9N2, which showed comparable virus shedding detected in oropharyngeal swabs using real-time RT-PCR but distinct antibody responses. All challenged birds survived infection, and necropsy of the birds showed that both H6N2- and H9N2-infected hens had no or mild grossly detectable lesions in the respiratory tract and lungs.

DISCUSSION

The primary target cells for influenza A virus infection and replication are ciliated respiratory epithelial cells. In avian species, intestinal epithelia are also targets of infection. In the later stages of infection, mononuclear cells become involved (Fujisawa et al., 1987; Kaufmann et al., 2001). Unlike epithelial cells, infected macrophages/monocytes are the main source of cytokine and chemokine production in response to influenza virus infection (Kaufmann et al., 2001). In this study, we found that the pro-inflammatory cytokines IL-1β, IL-8 and IL-18 and the chemokines CCL3, CCL7, CCL20, CCL294, K203, K60 (CXCL1) and CXCL14 were all upregulated, suggesting that chicken macrophages may play a key role in responding to LPAI virus infections. Among the genes downregulated in A/ph/CA/2373/98 (H9N2)-infected macrophages were IL-4, IL-4R, IL-17 and IL-21R. Both IL-4 and IL-4R are pivotal in the activation of Th2 responses and neutralizing-antibody production. IL-21R is related to IL-2Rβ in that it transduces the signals of IL-21, a member of the IL-2 cytokine family (Asao et al., 2001) and is important in the differentiation of T, B and NK cells (Strengell et al., 2002, 2003). Therefore, H9N2 virus-regulated changes in IL-4, IL-4R and IL-21R expression in macrophages may have an overall negative impact on the development of adaptive immunity in chickens.

Among the key findings of the current analysis was that the MHC antigens, and the MHC class II antigens in particular, were extensively downregulated by A/ph/CA/2373/98 (H9N2). In fact, MHC class II α-chain (B-LA), β-chain (B-LB), α-chain 1B locus, β1 domain (B-LBI) and β1 domain (B-LBVI) were all downregulated at the various time points examined following infection, making it one of the most consistent effects of this LPAI virus in infected chicken macrophages. The MHC class I antigens B-FIV, ID B-F2 minor, YFV (MHC class I heavy chain) and Rfp-Y α-chain were also downregulated, some, at least transiently, at certain time points p.i. In addition, CD74 and cathepsin S also had reduced expression. CD74 functions mainly as an MHC class II chaperone, promoting MHC class II antigen exit from the endoplasmic reticulum and entry into endocytic compartments. It also contributes to peptide
isms (Alexander, 2000; Guan, 2000; Marshall et al., 2005). Previous studies have found that LPAI viruses induced apoptosis to variable degrees in human and mouse macrophages (Brydon, 2005; Mok et al., 2007). Chicken macrophages are susceptible to infection with the LPAI H9N2 and H6N2 subtype viruses used in these studies. In our study, infection in the absence of trypsin led to aggressive apoptosis in A/ph/CA/2373/98 (H9N2) virus-infected macrophages. This is in contrast to reports in which productive infection in mammalian macrophages with H1N1 and H3N2 subtype viruses did not result in apoptosis (Seo et al., 2004; Tyner et al., 2005). It has been postulated that macrophages have evolved mechanisms to allow them to remain viable during all types of infection. In keeping with this strategy, it has been reported that the upregulation of chemokine ligand CCL5 and the induced downstream pathways for survival signals by respiratory syncytial virus and human influenza virus (H1N1) infection are critical in preventing apoptosis (Tyner et al., 2005). In the chicken microarray chips used in this study, CCL5 was absent. In order to determine whether there was

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**Table 3.** HI antibody titration and M gene copy numbers of 3–4-week-old chickens infected with A/ch/CA/1772/02 (H6N2) or A/ph/CA/2373/98 (H9N2) virus

The antibody titres from three chickens of each group with comparable virus shedding in oropharyngeal swabs sampled on day 3 after virus challenge were compared. Real-time RT-PCR for the influenza A virus M gene was used to quantify the M gene copy number on day 3 p.c.

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>H6N2</th>
<th>H9N2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>&lt;1:2</td>
<td>&lt;1:2</td>
</tr>
<tr>
<td>3</td>
<td>&lt;1:2</td>
<td>&lt;1:2</td>
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<tr>
<td>24</td>
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</tr>
</tbody>
</table>
a CCL5 homologue in chickens, we compared the sequences of a number of chicken chemokines and found that chemokine ah294 may be the chicken counterpart of CCL5. A sequence comparison showed that ah294 had the highest sequence identity with duck (77%), pig (68%), and mouse and human (64%) CCL5 (data not shown). In this study, ah294 was upregulated to 26.7-fold in A/ph/CA/2373/98 (H9N2)-infected macrophages (Table 1), although this clearly did not prevent the cells from becoming apoptotic. More evidence is needed to determine whether ah294 is indeed the counterpart of CCL5 in mammals and whether it plays a role in apoptosis in chicken macrophages.

It has been suggested that T lymphocytes or CD8+ T cells from H9N2-infected chickens may be sufficient to prevent chickens from disease caused by H5N1 infection through cross-reactive cell-mediated immunity in the absence of cross-reactive neutralizing or HI antibodies (Seo & Webster, 2001). Specific neutralizing or HI antibodies are still considered protective and can clear the virus in birds and humans. Frankly, we know little about how birds recover from LPAI infections. In this study, we observed mixed responses among IFN and IFN-inducible genes and β-defensins in response to infection in chickens. Whilst 2’5’-OAS was upregulated, the Mx protein gene was marginally downregulated in A/ph/CA/2373/98 (H9N2)-infected macrophages. However, they were both downregulated in infected chicken lungs. IFIT-5 was highly induced in macrophages, but this induction appeared only on day 1 and started to decline thereafter in chicken lungs. Differential regulation of IFN-inducible genes is probably due to weak IFN induction in H9N2-infected macrophages and the lung tissues (Table 1). Interestingly, IFIH1 (MDA-5) was highly downregulated up to 6.9-fold and is considered to be critical, like RIG-1, as an RNA virus sensor in IFN-β induction (Kato et al., 2006; Kawai et al., 2005). Differential regulation of IFN-inducible genes was also observed in other microarray studies, including one using macaques (Baas et al., 2006). In the lungs of A/Texas/36/91 (H1N1)-infected macaques on day 2 p.i. in this study, OAS1, OAS3, MX1 and IFIT2 were upregulated, whilst MX2, GBP-1 and GBP-2 were downregulated. Our data clearly showed that TLR-7 is upregulated as the sensor responsive to avian influenza virus in chicken macrophages at the earlier stages of infection, but is suppressed at later times. We consider that down-regulation of TLR-7 may be one of the mechanisms causing the downregulation of IFNs and IFN-inducible genes including MHC antigens. Suppression of IFNs and IFN-inducible genes in influenza virus-infected cells and in the host is unique among virus infections.

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REFERENCES


