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H5N1 infection causes rapid mortality and high cytokine levels in chickens compared to ducks



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ABSTRACT

Infection with H5N1 influenza virus is often fatal to poultry with death occurring in hours rather than days. However, whilst chickens may be acutely susceptible, ducks appear to be asymptomatic to H5N1. The mechanisms of disease pathogenesis are not well understood and the variation between different species requires investigation to help explain these species differences. Here we investigated the expression of several key proinflammatory cytokines of chickens and ducks following infection with 2 highly pathogenic H5N1 (A/Muscovy duck/Vietnam/453/2004 (Vt453) and A/Duck/Indramayu/BBVW/109/2006 (Ind109)) and a low-pathogenic H5N3 influenza virus (A/Duck/Victoria/1462/2008 (Vc1462)). H5N1 viruses caused fatal infections in chickens as well as high viral loads and increased production of proinflammatory molecules when compared to ducks. Cytokines, including Interleukin 6 (IL6) and the acute phase protein Serum Amyloid A (SAA), were rapidly induced at 24 h post infection with H5N1. In contrast, low induction of these cytokines appeared in ducks and only at later times during the infection period. These observations support that hypercytokinemia may contribute to pathogenesis in chickens, whilst the lower cytokine response in ducks may be a factor in their apparent resistance to disease and decreased mortality.

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1. Introduction

Host–pathogen interactions are critical to the outcome of disease. The initial cellular recognition of pathogens by the pathogen recognition receptors (PRR) triggers downstream immune-related genes and rapidly sets up an overall immune response against the invading pathogen (Wang et al., 2007). Whilst the resultant milieu of cytokines are paramount in the host protection from viruses such as highly pathogenic avian influenza (HPAI), the upregulated cytokines also lead to inflammation at the sites of infection (Sladkova and Kostolansky, 2006; Kobasa et al., 2007). The level of cytokine impact on the disease state is unclear. Infections such as dengue fever (Leong et al., 2007), malaria (Clark et al., 2008) and severe acute respiratory syndrome (SARS) (Cameron et al., 2008) promote an inflammatory cytokine response during early infection which have been suggested to be responsible for cellular and organ

damage. H5N1 infections in humans also leads to an increased inflammatory response and appears to contribute to the severity of disease and associated mortality (Tran et al., 2004; Chan et al., 2005; de Jong et al., 2006). The H5N1-associated response includes the regulation of proinflammatory cytokines, antiviral cytokines and interferons (IFNs) (Sladkova and Kostolansky, 2006) which inhibit viral replication (Szretter et al., 2007; Karpala et al., 2008). However, the induction of some of the associated cytokines, like IFN, TNF α , IL1 β and IL6, are thought to be at least partially responsible for influenza-induced pathology (Cheung et al., 2002).

Interestingly, the rapid disease progression observed in influenza infected chickens is not observed in ducks (Alexander et al., 1986). Whilst ducks often maintain a healthy state they enable the continuing spread of influenza by acting as a viral reservoir and then mingling with chicken flocks, and so ducks are critical to the control of the influenza cycle (Webster et al., 1992). Research efforts show that humans and other animals, as well as chickens, suffer from high viral loads, extensive tissue tropism, and elevated immune responses following H5N1 infections (Cheung et al., 2002; Suzuki et al., 2009) whereas ducks often remain in good health, all the while continuing to shed virus (Alexander et al., 1986; Jeong et al., 2009). Recent comparative analyses indicates ducks and chickens respond differently to low pathogenic avian influenza

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(LPAI) infection (Mundt et al., 2009; Cornelissen et al., 2012). The lungs of the chicken, in contrast to ducks, endure high viral loads as well as high levels of IL6, type 1 IFNs, and PRR such as TLR3, TLR7 and Mda5 (Cornelissen et al., 2012). Nevertheless, studies with mammals and avians show that viral loads and immune responses associated with HP-influenza infections are quite different when compared with LP-influenza infections (Tumpey et al., 2005).

Since the clinical manifestations of influenza-infected chickens and ducks appear to be different, and further, the immune responses to LP-influenza, in contrast to HP-influenza, are distinctly divergent, we investigated the impact of HP-influenza H5N1 infection in chickens and ducks. In this study 2 H5N1 strains, derived from ducks, were used to infect chickens and ducks. The cytokine and acute phase immune responses were analyzed by observing the levels of IFN γ , SAA and the interleukins, IL1 β , IL6 and IL18. In addition, the levels of Toll-like receptor 7 (TLR7) were examined as H5N1 viral RNA interacts with TLR7 (Stewart et al., 2012) which further activate innate immune responses (Geeraedts et al., 2008). Vast differences were evident following HP-influenza infections in chickens and ducks. Furthermore, the findings were specific to HP-influenza since LP-influenza H5N3 virus (Vc1462) did not induce similar responses in the chicken. It is hoped that a better understanding of these host–pathogen interactions might be helpful in deriving new immune modulation strategies aimed at reducing HP-influenza induced illness in chickens and humans.

2. Results

2.1. H5N1 influenza has increased severity and mortality in chickens compared to ducks

Two groups of 12 chickens and 15 ducks were inoculated with either Vt453 or Ind109 H5N1 viruses and time to mortality indicated that Vt453-infected chickens succumb to infection most rapidly at 18–24 hours post infection (h.p.i.) (Fig. 1) (3 had to be euthanized due to severe symptoms). Ind109-infected chickens died (or were euthanized) between 24 and 28 h.p.i. The clinical signs associated with Vt453- or Ind109-infected chickens included weakness, depression, ataxia, swelling of the head, labored breathing, fever and seizures. In contrast, Vt453- and Ind109-infected ducks showed none of these clinical signs initially, but between 48 and 72 h.p.i., 3 Vt453-infected ducks showed some depression and weeping eyes. At 72–96 h.p.i. severe disease was observed in 8 of the 15 Vt453-infected ducks, including neurological signs, ataxia and in 4 cases death (or ducks requiring euthanasia). Ind109-infected ducks showed no signs of infection.

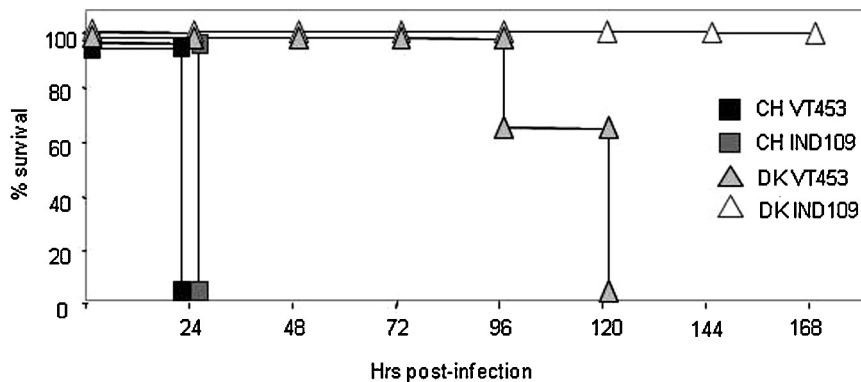


Fig. 1. Survival of chickens following H5N1 infection is decreased in comparison to ducks. Following inoculation with highly pathogenic H5N1 influenza (Vt453 or Ind109) the % survival of chickens (squares) or ducks (triangles) was measured. Chickens succumbed to both viral infections (or had to be euthanized) within 24 h whilst ducks infected with Ind109 appeared asymptomatic. Ducks infected with Vt453 succumbed to infection (or had to be euthanized) at 72–120 h.p.i.

2.2. Chickens appear to have rapid and systemic H5N1 viral infection compared to ducks

Tissues were analyzed for virus and the highest viral titers were identified in all H5N1-infected chickens. At 24 h.p.i., viral titers were between 6 and 7 log₁₀ tissue culture infectious doses (TCID₅₀) in the lung, spleen and heart whilst the brain contained between 5 and 6 log₁₀ TCID₅₀ (Fig. 2). In contrast, ducks had low levels of virus over a 168 h time-course (Fig. 2). Furthermore, ducks differentially responded to the different H5N1 strains. In Vt453-infected ducks, viral titers were 4, 2 and 7 log₁₀ TCID₅₀ in the lung, spleen and heart, respectively, at 48 and 72 h.p.i. whilst the Ind109-infected ducks, generally had lower viral titers (Fig. 2). H5N1-infected ducks had between 1 and 2 log₁₀ TCID₅₀ of virus in the brain at 72 h.p.i. (Fig. 2).

2.3. H5N1 Vt453 and Ind109 infection increases cytokine and acute phase expression in chickens

Rapid increases in mRNA levels of the proinflammatory cytokines IFN γ , IL1 β and IL6 were detected 24 h.p.i., in the spleen, brain, lung and heart of Vt453-infected chickens (Fig. 3). IL6 had the largest mRNA increases (heart 100-fold, spleen 80-fold and lung 70-fold) compared to uninfected controls (Fig. 3). The Ind109-infected chickens also had increased proinflammatory cytokine levels (IFN γ , IL1 β and IL6) at 24 h.p.i. although IL6 increases were far less in Ind109-infected chickens compared to Vt453-infected chickens (Fig. 3). Interestingly, the IL6 levels of Vt453-infected chickens were highly elevated compared to the IL6 levels of Vt453-infected duck (Fig. 3). The acute phase protein SAA increased following H5N1 infection in a profile similar to IL6. In the Vt453-infected chickens SAA increased in the heart 80-fold, spleen 40-fold and lung 40-fold – whilst SAA increases in the Ind109-infected chickens were heart 40-fold, brain 10-fold and lung 20-fold (Fig. 3). TLR7 levels were lower in H5N1-infected chickens when compared to the H5N1 infected duck in all tissues at 24 h.p.i.

H5N1 Vt453 and Ind109 infection influences duck inflammatory cytokine levels less than chickens but increases in TLR7 occurs in ducks, in contrast to chickens, both the Vt453- and Ind109-infected ducks had little to no change in IFN γ , IL1 β , IL6 and IL18 levels when compared to uninfected controls at 24 h.p.i. There was also no apparent change in the levels of the acute phase gene SAA in duck organs at 24 h.p.i. (Fig. 3). However, duck TLR7 levels were increased in spleen, brain and heart tissue between 2- and 4-fold at 24 h.p.i. At 72 h.p.i. some proinflammatory cytokines increased in the duck spleen, brain and heart which corresponded to peak virus titers at 72 h.p.i. However, in the brain of Vt453-infected ducks IFN γ increased 30- to 40-fold and IL18 5- to 10-fold at 72 h.p.i. (Fig. 4).

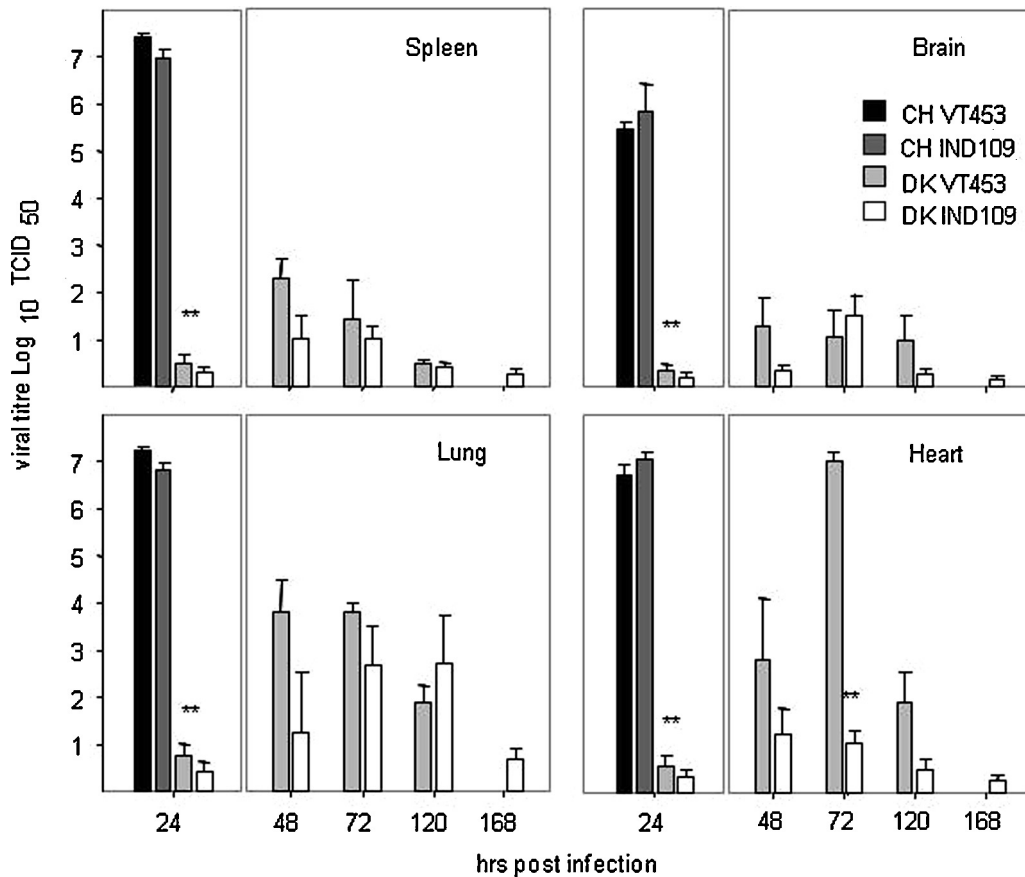


Fig. 2. High replication levels of H5N1 virus in chickens compared to ducks during the acute infection period. Viral titers were measured in a TCID₅₀ assay (Vero cells) in chicken and duck tissues. Mean virus titers derived from individual lungs, hearts, spleens and brains are shown. Virus titers were confirmed in embryonated chicken eggs (not shown). The mean virus titers were derived from 3 birds (log₁₀ TCID₅₀).

There were some proinflammatory increases in heart – IFN γ (2- to 5-fold), IL1 β (5- to 15-fold) and IL6 (15- to 20-fold) – and spleen IL1 β (10-fold) compared to uninfected ducks. The cytokine levels in H5N1-infected ducks were overall lower than H5N1-infected chickens at 24 h.p.i.

2.4. Elevated IL6 in sera of chickens and ducks infected with H5N1

Elevated IL6 mRNA levels prompted an analysis of IL6 in the sera of infected birds. Serum IL6 levels of Vt453-infected chickens were elevated between 8- and 10-fold compared to uninfected chickens. In contrast, serum IL6 levels in Vt453-infected ducks were not increased at 24 h.p.i., and only increased 2-fold at the observed peak of infection (72 h.p.i), compared to uninfected control ducks (Fig. 5A). To confirm IL6 assay specificity a neutralizing anti-IL6 antibody was included and reduced IL6 signals in a dose dependent manner. Chicken IL6 levels in sera were considerably higher than those observed in the duck following H5N1 infections (Fig. 5B).

2.5. IL6 and H5N1 antigens appear to co-localize in chickens

Increased IL6 levels following H5N1 influenza infection in chickens raised the question of whether IL6 co-localizes with H5N1 virus. H5N1 (Vt453) antigen was identified (Fig. 6B and E) in a range of chicken tissues at 24 h.p.i. H5N1 antigen was prevalent in endothelium of all tissues and in occasional necrotic foci of various tissues, including spleen, brain, lung and liver. In lung tissue (Fig. 6B), H5N1 antigen appeared throughout the parenchyma and around the epithelial cells of blood vessels. Serial sections of lung tissue were additionally stained with anti-IL6 antibody (Fig. 6C)

and apparent co-localization of IL6 with H5N1 antigen was identified. Liver hepatocytes (Fig. 6D) and sinusoidal cells (presumably endothelial cells) appeared necrotic and were positive for H5N1 antigen, mainly around the lesions observed (Fig. 6E). Serial sections of the liver tissue, led to IL6 being additionally identified in the foci of necrotic lesions and confirmed that the elevated IL6 expression was co-localizing with H5N1 infected tissue.

2.6. H5N3 Vc1462 influenza infection induces relatively low cytokine expression in chickens, compared to H5N1 infection

Chickens were infected with the LPAI H5N3 Vc1462 virus to determine if other H5 subtype influenza viruses increase proinflammatory cytokine levels similar to that observed following HPAI H5N1 infection. At the peak of Vc1462 infection (72 h.p.i.) IFN γ , IL1 β , and IL6 were increased 5- to 8-fold in the spleen and increases of IL1 β and IL6 levels were identified in the lung when compared to the uninfected chickens (Fig. 7A). When these cytokine levels were compared to those observed following H5N1-infection, IFN γ , IL1 β , IL6 and IL18 (lung) and IL1 β , IL6 and IL18 (spleen) were found to be 5- to 15-fold higher than H5N3-infected chickens (Fig. 7B). The levels of the acute phase gene, SAA were 25-fold higher in the lung and 13-fold higher in the spleen during H5N1 infection compared to the levels observed during H5N3 infection (Fig. 7B).

3. Discussion

Avian H5N1 influenza infection results in a high rate of mortality and a dysregulated inflammatory cytokine response (Kobasa et al.,

2007; de Jong et al., 2006). However, different species are likely to respond differently to a similar strain of virus. Additionally, various H5N1 influenza strains may have different clinical impacts in a specific animal (Perkins and Swayne, 2003). Here we show initially that H5N1 (Vt453) infection is fatal to chickens within 24 h whereas duck fatality occurs in around 120 h. Additionally, whilst the H5N1 (Ind109) is fatal to chickens, ducks overcame the same infection. This indicates that a HP influenza virus in one avian species is not always HP in other avian species and supports previous findings that ducks have a lower fatality incidence following H5N1 infection (Alexander et al., 1986; Bingham et al., 2009). It is unclear how this varying pathogenicity occurs, but differing host–pathogen immune responses during H5N1 influenza infection likely plays a role in disease.

Chickens generally had higher viral titers and higher inflammatory responses than ducks, either of which might explain morbidity and mortality. As expected, the lung tissue of H5N1-infected chickens had the highest viral titers (in excess of $7 \log_{10}$ TCID₅₀) and had correspondingly high levels of proinflammatory cytokines. Nevertheless, virus titers in the chicken spleen, heart and brain tissue were almost as high as those found in the lung. This intense viral replication and viremia may be promoted by excessive inflammation, which impacts on the permeability of blood vessels

allowing greater viral spread. Swayne et al. (2007) have suggested altered vascular permeability to explain edema and hemorrhage, as contributing to systemic viral replication and subsequent multiple organ failure then death. Furthermore, SARS, Hepatitis and Mareks research identified that altered permeability of blood vessels contributed to pathogenesis (Swayne et al., 1988; Liao et al., 2004) although it's less clear if increased permeability was caused by inflammation or viral proteins.

We showed that chickens infected with either H5N1 (Vt453 or Ind109) strains, had an early and increased production of proinflammatory molecules in comparison to ducks which initially appeared not to respond to these viruses. The key upregulated proinflammatory elements following H5N1 infection in the chicken included IL1 β and IL6. These increases appeared to be systemic since elevated levels were found in the lung, heart, spleen and brain. Both IL1 β and IL6 regulate epithelial and vascular endothelial cells and have been implicated in influenza pathogenesis in humans and primates (Skoner et al., 1999). IL6 may be released by influenza-damaged macrophages and epithelial cells (Hierholzer et al., 1998). SARS virus induced disease severity has similarly been linked with IL6 in the lung environment (Cheung et al., 2005). IHC showed that IL6 co-localized with H5N1-antigen and was highly associated with necrotic lesions in several tissues including

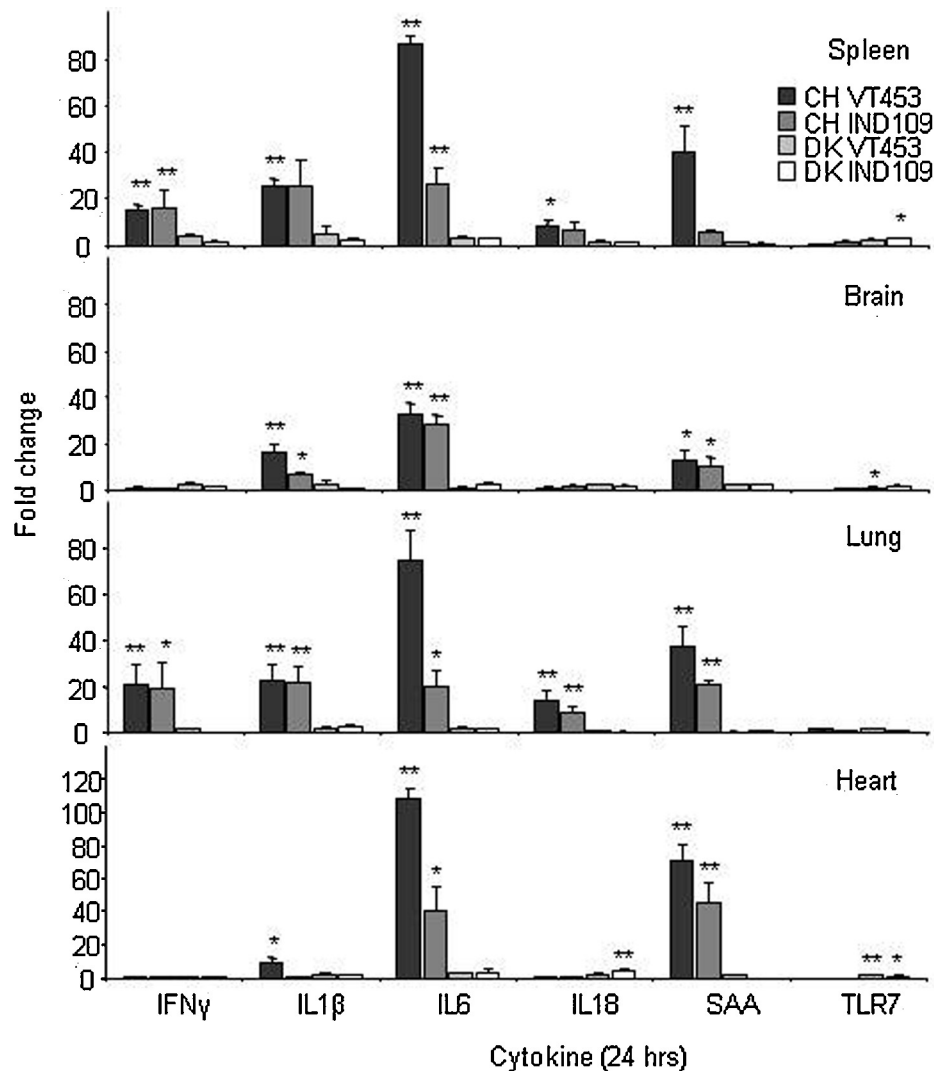


Fig. 3. Cytokines are increased in chickens compared to ducks following H5N1 influenza infection. Cytokine mRNA levels of chickens and ducks 24 h after H5N1 infection were measured in various tissues by qPCR. Data represents the mean fold expression of either duck or chicken mRNA relative to each uninfected tissue type after normalizing data to the housekeeping gene GAPDH (* $p < 0.05$, ** $p < 0.01$).

liver, providing further support that influenza-triggered IL6 may contribute to disease severity.

Ducks, on the other hand, produced only a moderate cytokine response late in the infection period. The peak cytokine levels of duck correlated with peak virus levels at 120 h.p.i. Vt453-infected ducks had the most pronounced changes, despite that these levels were much lower than in chicken. IFN γ and IL18 were upregulated in the brain of Vt453-infected ducks and may explain the neurological symptoms observed in some ducks. The increased IL6 levels in the heart of ducks could also have influenced the severity of infection although only a 2-fold increase in IL6 was observed in sera of infected ducks contrasting the 10-fold increase seen in chickens. IL6 levels are important in the regulation of the additional inflammatory pathways including the acute phase responses (Jensen and Whitehead, 1998).

The acute phase response is an arm of protection targeted at viruses and has been explored as a marker for virus (Nakayama et al., 1993). SAA elevation is triggered by IL6, but further, may increase the overall inflammation in a loop system (Ramadori et al., 1988). In H5N1-infected chickens SAA was elevated between

20- and 70-fold and the levels correlated with IL6 levels supporting that IL6 may influence SAA activation as others have shown (Ramadori et al., 1988). Excessive levels of SAA contribute to SARS disease (Huang et al., 2005) and may similarly contribute to H5N1-related disease observed in this study. In agreement with reduced H5N1-severity in ducks, the elevation of SAA was not observed.

The differential responses of chicken and ducks to H5N1 might further be explained by species-specific arrangements of PRR such as the RIG-I absence in chickens (Karpala et al., 2011). RIG-I overexpression in vitro benefits the chicken response to influenza and its natural absence may explain poor in vivo immunity to H5N1 (Barber et al., 2010). Clearly, the immune response is multifaceted and RIG-I alone may not protect an organism from influenza, especially since many organisms succumb to HPAI virus in the presence of RIG-I. What's more, both chickens and ducks overcame the LP H5N3 with only small increases in IFN γ , IL1 β , IL6 and SAA compared to the HP influenza viruses. Nevertheless future studies that investigate RIG-I-overexpression would be interesting for determining its protective functionality in vivo in HP-influenza-infected chickens.

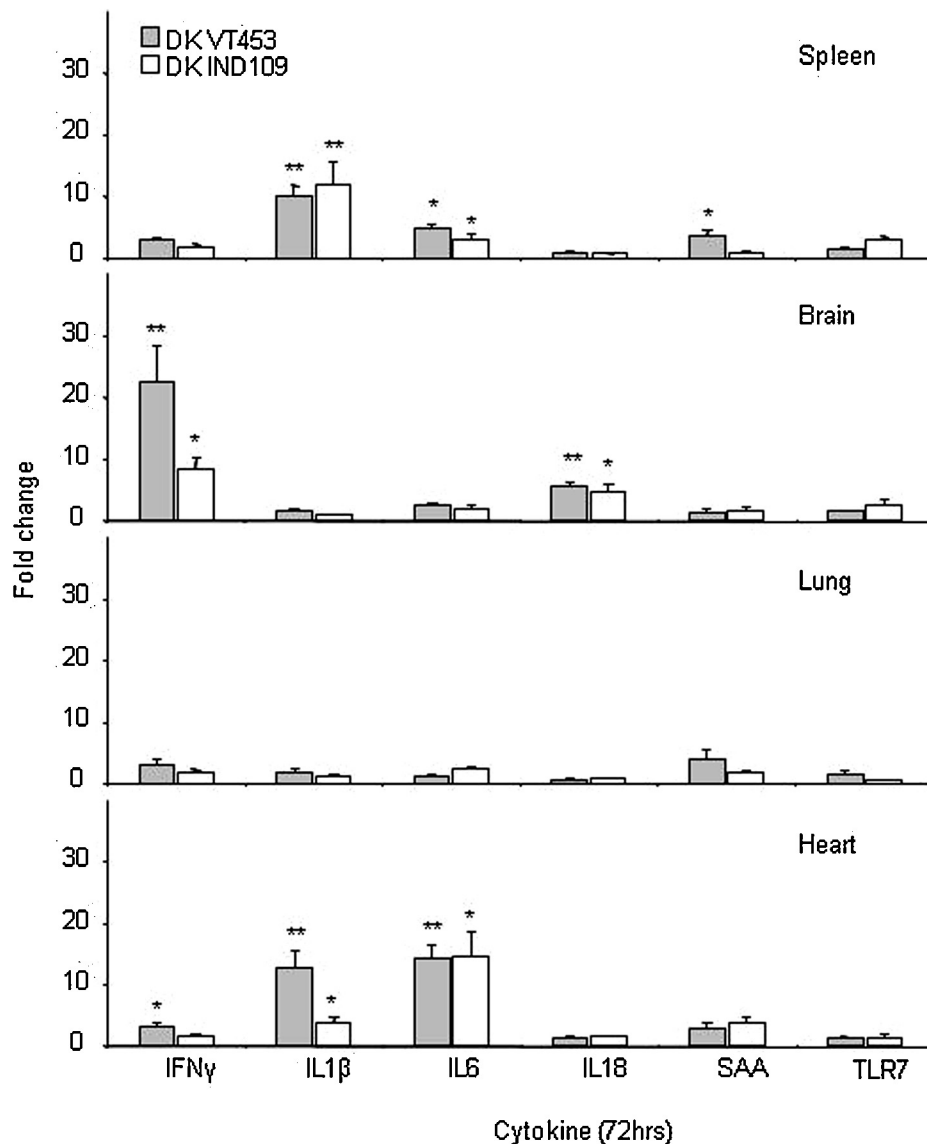


Fig. 4. An increase in some cytokine levels occurs in duck heart and brain following H5N1 infection after 72 h. Cytokine mRNA levels of ducks 72 h after H5N1 infection were measured in various tissues by qPCR. Data represents the mean fold expression of duck mRNA relative to each uninfected tissue type after normalizing data to the housekeeping gene GAPDH (* $p < 0.05$, ** $p < 0.01$).

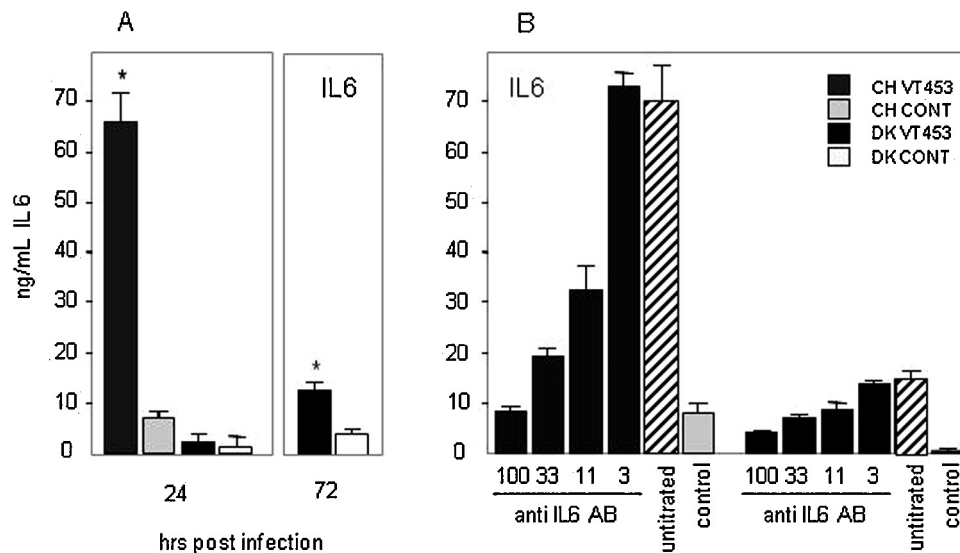


Fig. 5. Serum levels of IL6 are increased in chickens when compared to ducks following H5N1 influenza infection. (A) IL6 protein levels were measured in the sera of chickens and ducks using an IL6 dependant cell assay. The IL6 levels in Vt453-infected and uninfected chickens and ducks were examined at the relative peak of infection (24 h.p.i. for chickens and 72 h.p.i. for ducks). (A) Chickens had 8- to 10-fold increases and ducks had a 2-fold increase in IL6 levels. Anti-IL6 antibodies were used to confirm IL6 specificity. (B) Increased levels of anti-IL6 antibodies were required to neutralize infected chicken sera confirming the higher levels of IL6 and IL6 specificity in these tests (* $p < 0.05$).

Other factors that may explain differential duck and chicken responses to influenza include viral modification of the immune system such as by influenza NS1 which blocks key cellular protective mechanisms (Rajput et al., 2012). In particular, NS1 interacts with the RIG-like helicases further suggesting the importance of RIG-I for protecting influenza-infected organisms (Rückle et al., 2012). Recently, a species-specific interaction of NS1 and the ubiquitinase trim25, were identified which might explain an organisms differential response to influenza since the NS1-trim25 impacts the RIG-like helicases (Rajsbaum et al., 2012). Further research is required to delineate chicken and duck ubiquitinases as these are likely to have multiple cellular targets (Belgnaoui et al., 2012). Another interesting observation that may explain duck protection from influenza may be rapid cell death of influenza infected cells, which limits virus spread, before the virus can become well established in the race for survival (Kuchipudi et al., 2012).

In summary, chickens are overwhelmed by intense viral replication and an associated cytokine influx following H5N1 influenza infection. Conversely, ducks survive and their cytokine response is relatively low and parallels chickens infected with LP H5N3. With this in mind, protection strategies for chickens could target the virus or the host response. Research with H5N1-infected mice treated with the antiviral zanamivir showed that survival did not improve. However, when H5N1 infected mice were treated with a combination of an antiviral and cyclooxygenase-2 (COX-2) inhibitors, inflammatory markers decreased by almost half and survival was improved significantly (Zheng et al., 2008). Similarly, Imai et al. (2009), suggested that the administration of ETR-P1, an anti-inflammatory, could protect 10-day-old chickens from H5N1 influenza infection. With this in mind, future work, directed at modulating the levels of proinflammatory responses, such as IL6, following H5N1 influenza infection may be beneficial for developing potential immunotherapies.

4. Materials and methods

4.1. Virus strains

Two HPAIV H5N1 isolates and a LPAIV H5N3 originally isolated from ducks were used in infection experiments. The A/Muscovy duck/Vietnam/453/2004 (Vt453) virus is a clade 1

strain received from Vietnam as a Madin-Darby Canine Kidney (MDCK) cell culture supernatant. The Indonesian strain, A/Duck/Indramayu/BBVW/109/2006 (Ind109) of clade 2.1.3 origin was from West Java and received as infected allantoic fluid. Both were passed twice in chicken eggs to obtain the working stock virus. The working inoculum consisted of a 1:100 dilution of infected allantoic fluid. Each chicken and duck was inoculated through an oral-nasal-ocular route with a total volume of 0.2 and 0.5 mL, respectively. On back-titration the doses for Vt453 was approximately $10^{6.1}$ and $10^{7.2}$ median egg infectious doses (EID₅₀) for chickens and ducks, respectively, for Ind109 the doses were $10^{6.3}$ and $10^{6.0}$ EID₅₀, respectively, for chickens and ducks. The LPAIV used was of the H5N3 subtype, A/duck/Victoria/1462/2008 (Vc1462). Chickens were similarly infected with $10^{6.0}$ EID₅₀.

4.2. Virus titration

Heart, brain, lung and spleen tissues were homogenized and 10% (w/v) homogenates in phosphate-buffered saline were prepared. These were titrated in Vero cells which were found to be slightly less sensitive than eggs, but more sensitive than MDCK cells for this H5N1 strain. Flat-bottomed 96-well micro-titer plates were seeded with a Vero cell suspension (10^6 cells per plate). Ten-fold dilutions of the samples were prepared and 0.1 mL each dilution was added, with four replicates, to sequential wells of the plates. An uninfected cell control was present on each plate. The plates were incubated at 37 °C in a humidified CO₂ incubator and examined for the presence of cytopathic effect after 5 days. The lowest possible limit of viral detection, equivalent to a single infected well with optimal cell growth in all wells, was $10^{0.75}$ 50% tissue culture infectious doses (TCID₅₀) per 0.1 mL.

4.3. Chickens and ducks

Five-week-old Pekin ducks were purchased from Luv-a-Duck (Nhill, Victoria, Australia). Four to six-week-old broiler chickens were purchased from a high-biosecurity commercial supplier. Prior to challenge both chickens and ducks were swabbed by the oral and cloacal routes and bled for serum and tested to confirm the absence of prior infection. Each of the groups of ducks ($n = 15$) and chickens

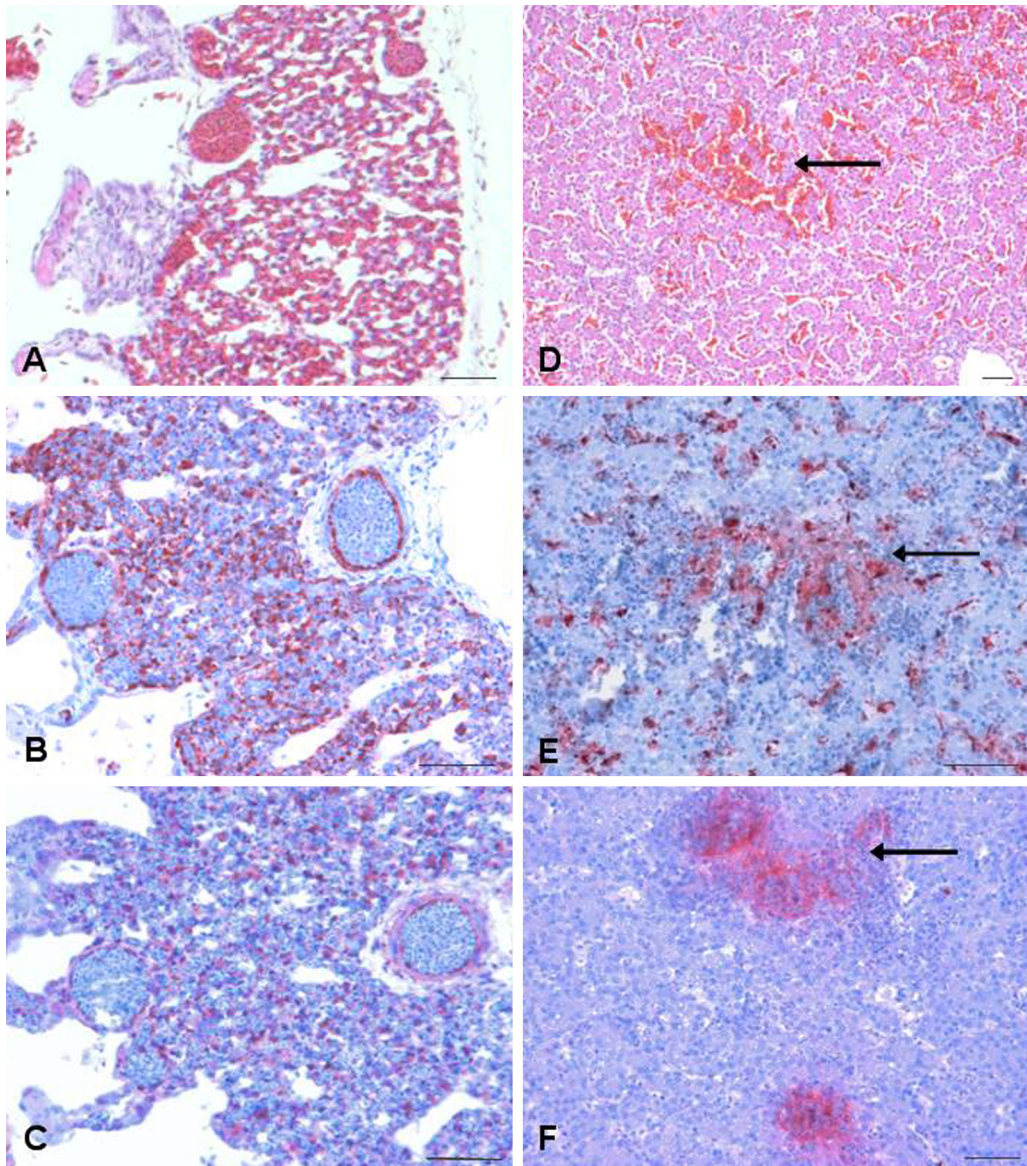


Fig. 6. H5N1 and IL6 appear co-localized in chicken lung and liver tissues as determined by immunohistochemistry. Left hand panel shows H5N1 infected chicken (94) lung tissue, right hand panel shows H5N1 infected chicken (76) liver tissue. (A) Lung tissue with blood vessels and lesions in parenchyma (hematoxylin and eosin). (B) Lung tissue showing H5N1 viral antigen staining in blood vessel endothelium and wall and within the lung parenchyma. (C) Lung tissue with IHC staining for IL6 within blood vessel walls and throughout the parenchyma. (D) Liver tissue with arrow identifying necrotic lesion (hematoxylin and eosin). (E) Liver tissue showing same lesion with IHC for H5N1 antigen staining throughout sinusoidal endothelial cells and necrotic hepatocytes. (F) Liver tissue showing same lesion with IHC for IL6 staining throughout necrotic cells (arrow). All scale bars = 50 μ m.

($n = 12$) were housed in separate rooms at microbiological security level 3.

4.4. Ethics statement

All animal work was conducted with the approval of the Commonwealth Science and Industrial Research Organisation (CSIRO), Australian Animal Health Laboratory (AAHL) and Animal Ethics Committee (AEC). Experiments were conducted under AEC applications 1211 and 1255 – study of pathogenesis of H5N1 influenza in ducks and chickens.

4.5. RNA isolation and reverse transcription

RNA was harvested using Tri-reagent (Sigma–Aldrich, St. Louis, MO). Extracted RNA was subjected to DNase treatment using a

DNase 1 (Sigma–Aldrich). The DNase-treated RNA was then reverse transcribed to complementary DNA (cDNA) using a reverse transcription kit (Promega, Madison, WI).

4.6. Quantitative RT-PCR (Q-RT-PCR)

The relative quantitation of gene expression following treatment was carried out on an ABI Prism 7700 sequence detection system and used the comparative threshold cycle (Ct) method to derive fold change gene expression, according to the manufacturers instructions (Applied Biosystems, Foster City, CA). Primers and probes were designed using Primer express software and where possible were designed across intron:exon boundaries. Probes were labeled with the reporter dye carboxyfluorescein (FAM) and the quencher tetramethyl-6-carboxyrhodamine (TAMRA). Briefly, triplicate or more samples were each measured in 25 μ L reactions. PCR

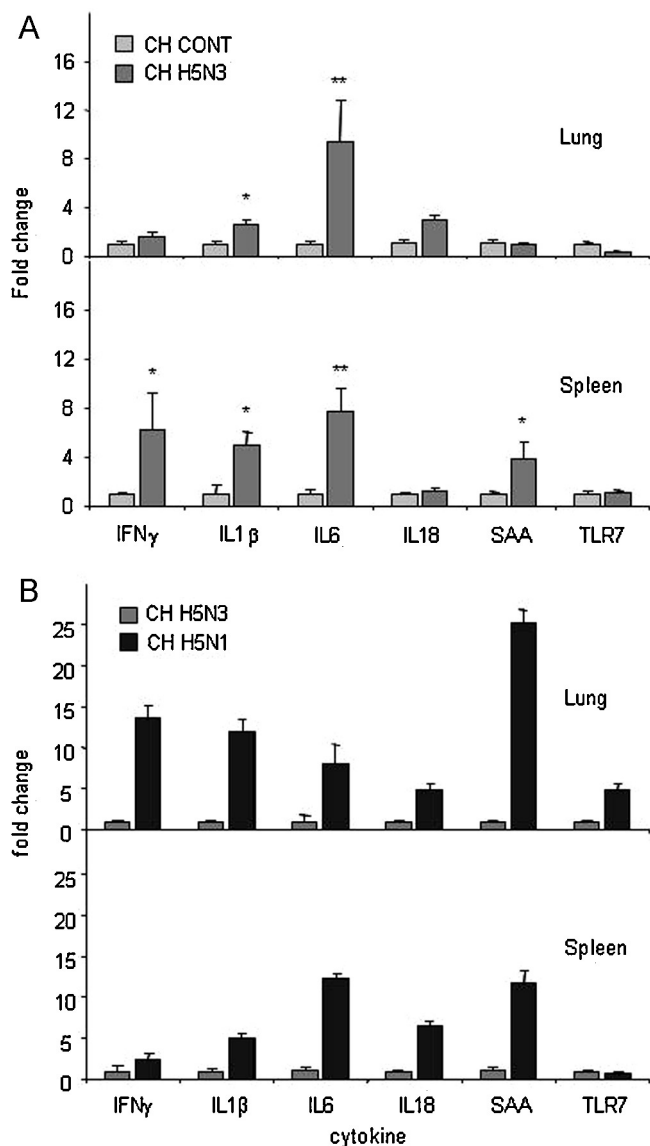


Fig. 7. H5N3 influenza infection induces large changes in cytokine levels in chicken lung and spleen tissues when compared with H5N1 infection. (A) Chickens were infected with H5N3 (Vc1426) influenza virus and at 72 h.p.i. various tissues were analyzed for mRNA levels by Q-RTPCR then compared with uninfected controls. (B) Lung and spleen tissues from H5N1- (Vt453) or H5N3-infected chickens were analyzed for mRNA levels by qPCR and compared for fold change. Data were normalized to the house keeping gene GAPDH ($n=4$, $*p < 0.05$).

cycling was performed as follows: 95 °C for 15 s, 61 °C for 30 s and 68 °C for 30 s. Threshold values were set at a standard value (0.2) which corresponded to the midway point of the amplification plots. Relative gene expression was calculated using the mean values obtained with the arithmetic formula $\Delta\Delta Ct$ (Applied Biosystems) as follows. Target gene Ct values were normalized to the endogenous control glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), a house-keeping gene, to derive the ΔCt . This was compared with an untreated calibrator to derive the $\Delta\Delta Ct$ and relative gene quantitation, or fold expression relative to the untreated control (calibrator), was derived using $2^{-\Delta\Delta Ct}$. Primer and probe sets were checked for efficiency by comparing the gradients of target and house keeping scores from 5 ten-fold dilutions of a reference and primer sequences can be seen in Table 1.

4.7. Detection of IL6 activity in chicken and duck sera

IL6 activity in chicken and duck plasma was measured by the ability to stimulate the proliferation of 7TD1 murine hybridoma cells in a 3H-thymidine incorporation assay. Briefly, 7TD1 cells were cultured in DMEM supplemented with 5% FBS, glutamine, 0.55 mM arginine, 0.24 mM asparagine, penicillin (100 U)/streptomycin (100 $\mu\text{g}/\text{mL}$), and recombinant ChIL6 (100 ng/mL) until the cells reached the logarithmic phase of growth. The cells were washed three times with DMEM to remove the residual ChIL6 in the medium and cultured further for 3 days in the medium without ChIL6. Test samples were serially diluted in 96-well Nunc tissue culture plates, then starved 7TD1 cells were added to each well, and plates were incubated at 37 °C in a humidified CO₂ incubator for 48 h. For the last 5 h of incubation, 0.5 Ci of 3H-thymidine (Amersham Biosciences, Buckinghamshire, UK) was added to each well, and cells were harvested onto glass fiber filter mats using a cell harvester (Tomtec, Hamden, CT). Filters were placed in plastic pouches with 5 mL scintillant, and radioactivity was measured on a Micro-Beta TriLux 1450 β counter (EG&G Wallace, Turku, Finland). The presence of the IL6 in chicken and duck sera was further confirmed by inhibition of its activity in the 7TD1 bioassay by the addition of rabbit anti-ChIL6 anti-sera [61]. Preimmunization sera were used as a negative control serum.

4.8. Immunohistochemistry

Tissue from chickens and ducks were placed into 4% formaldehyde in neutral buffered saline. After no more than 24 h of formalin fixation, tissues were processed into paraffin wax by routine histological methods. Sections of tissues were cut and stained using an immunoperoxidase test. Sections were quenched with 10% hydrogen peroxide for 10 min and digested with 5–7 mg/mL proteinase K for 6 min. They were then incubated for 1 h with either a rabbit serum directed against a recombinant-expressed purified H5N1 influenza virus nucleoprotein (produced by AAHL) or with a rabbit polyclonal antibody directed against IL6 (produced by AAHL). A secondary horseradish peroxidase-conjugated antibody (DAKO Envision) was then used for 45 min. Sections were finally stained with a commercial aminoethylcarbazol substrate chromogen (AEC) kit from DAKO Envision for 5–6 min, and counterstained with Mayer's hematoxylin.

4.9. Statistical analyses

To determine the significant differences between experimental groups, ANOVA or the Mann-Whitney *U* test were performed using the fold change scores. *p*-values were set at 0.05 ($*p \leq 0.05$) or 0.01 ($*p \leq 0.01$) unless indicated otherwise. Error bars represent the standard error of the mean (SE).

Conflict of interest

The authors declare no conflict of interest.

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