Characterisation of the chicken and duck response to H5N1 avian influenza infection

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Abstract

Avian influenza viruses are increasingly widespread in poultry and show varied disease severity depending on their hemagglutinin and neuraminidase structure. Whilst many influenza viruses, such as the H5N3 subtype, are of low-pathogenicity, H5N1 influenza viruses result in rapid mortality that in poultry occurs in a matter of hours. The mechanisms of disease pathogenesis are still somewhat poorly understood. Ducks often appear asymptomatic, with few strains of H5N1 causing severe pathogenesis. The gradual progression of infection in ducks contrasts the rapid nature of infection in chickens and it is not clear how this difference in virulence comes about. One possibility is that the innate immune response in chickens and ducks varies during infection and this may be critical to the clinical outcome. With this in mind, we investigated the expression of several key proinflammatory cytokines following infection of chickens and ducks with highly-pathogenic H5N1 and low-pathogenic H5N3 influenza virus. Two H5N1 strains, A/Muscovy duck/Vietnam/453/2004 (Vt453) and A/Duck/Indramayu/BBVW/109/2006 (Ind109) were compared, as well as the low pathogenic H5N3 A/Duck/Victoria/1462/2008 (Vc1462) strain. Intriguingly, in the chicken, H5N1 viruses caused fatal infections, a high viral load and increased production of proinflammatory molecules. Inflammatory molecules such as IL6, IFNγ, the acute phase reactant SAA and also NO inducing gene iNOS, were raised by up to 80 fold at 24 hours post infection. Meanwhile, infection with the Vc1462 H5N3 influenza strain induced a comparably low cytokine response in chickens. In contrast, ducks displayed only small changes in these cytokines and this only occurred later in the infection period. Only the Vt453 H5N1 strain caused mortality in ducks and this was associated with increased levels of cytokines, such as IFNγ and iNOS, as well as increased virus replication in the lung and heart. These observations support the belief that hypercytokinemia may contribute to pathogenesis in chickens, whilst the lower cytokine response in ducks may explain their resistance to disease and decreased mortality. Given the increased levels of inflammatory molecules and the observation that IL6, a pleitropic inflammatory gene, is highly upregulated in the sera of H5N1 infected patients, it appeared likely that IL6 had an

impact on hypercytokinemia induced disease severity. Since IL6 signalling results in increased levels of downstream inflammatory molecules, potentially triggering hypercytokinemia, we aimed to suppress the levels of IL6 during H5N1 infection. To investigate whether a more moderated IL6 response may improve the severity of H5N1 infection in chickens, we aimed to inhibit the signalling of IL6 using Madindoline-A (Mad-A) and Galielallactone (Gal). Following H5N1 infection, chickens treated with Mad-A/Gal inhibitors had reduced levels of IL6 and IL6-stimulated genes, such as SAA and AGP as well as lower NO and iNOS levels. However, even though this decreased IL6 response was associated with reduced viral titres, chickens did not appear to have increased survival. Nevertheless, these studies provide insight into the role possible therapeutics could play to target inflammation and improve the immune response during H5N1 infection. Therefore, examining the key components of the inflammatory response during H5N1 induced hypercytokinemia in chickens and ducks may help us understand how to ameliorate the initial hyperinflammatory response and prolong survival so that an adaptive response which alleviates viral replication can commence.

Declaration

This is to certify that:

(i) this thesis comprises only my original work except where assistance has been specifically acknowledged in individual chapters of this thesis.

(ii) due acknowledgement has been made in the text to all other materials used.

(iii) the thesis is less than 100 000 words in length, exclusive of figures, tables, references and appendices.

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Abstracts and oral presentations

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List of Abbreviations

aa	amino acids
AAHL	Australian animal health laboratory
ABI	applied biosystems international
AEC	animal ethics committee
AGP	alpha-1-acid glycoprotein
AI	avian influenza
APC	antigen presenting cell
APP	acute phase protein
BCDF	b cell differentiation factor
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
BSF2	b cell stimulation factor
CDF	cytotoxic t cell differentiation factor
cDNA	DNA complementary to mRNA
Ch	chicken
CRP	c-reactive protein
Ct	threshold cycle
CPE	cytopathic effect
CSIRO	Commonwealth scientific and industrial research organisation
DC	dendritic cell
DEPC H_2O	milli-Q water with 0.1% diethylpyrocarbonate
DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease cleavage enzyme
ds	double stranded
Du	duck
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetracetic acid

ETR-P1	endothelin receptor derived antisense homology box peptide
FACS	fluorescence activated cell scanning
FAM	6-carboxyfluorescein
FCS	foetal calf serum
GAL	galiellalactone
gDNA	genomic DNA
HA	hemagglutinin
HPAI	highly pathogenic avian influenza
HPGF	hybridoma plasmacytoma growth factor
HSF	hepatocyte stimulating factor
Hu	human
IBDV	infectious bursa disease virus
IFN	interferon
IFNGR	interferon gamma receptor
IFNα	interferon alpha
IFNβ	interferon beta
IFNγ	interferon gamma
IGIF	interferon gamma inducing factor
IHC	immunohistochemistry
IL	interleukin
iNOS	inducible nitric oxide synthase
IPTG	isopropyl-β-D-thiogalactopyranoside
JAK	janus kinase
kDa	kilo Dalton
LB	luria broth
LPAI	low pathogenicity influenza virus
LPS	lipopolysaccharide
MAD-A	madindoline-A
MDa5	melanoma differentiation-associated gene 5
MDCK	madine darby canine kidney cell line
MDT	mean death time
MODS	multiple organ dysfunction syndrome

MW	broad range molecular weight marker
MWCO	molecular weight cut off
NA	neuraminidase
NALP	nucleotide-binding domain, leucine-rich repeat protein
NCBI	national centre for biotechnology information
NEP	nuclear export protein
NOS	nitric oxide synthase
NP	nucleoprotein
NS	non-structural
PA	acid polymerase
PAMP	pathogen associated molecular pattern
PB1/2	polymerase basic 1/2
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reactions
Poly (I:C)	polyinosinic-polycytidylic acid
PRR	pattern recognition receptor
PKR	protein kinase receptor
Q-RTPCR	quantitative real time polymerase chain reaction
RACE	rapid amplification of cDNA ends
RIG-I	retinoic acid-inducible gene-I
RNA	ribonucleic acid
RNI	reactive nitrogen intermediate
RNP	ribonucleoprotein
ROS	reactive oxygen species
RT-PCR	reverse transcribed PCR
SAA	serum amyloid A
SARS	severe acute respiratory syndrome
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	standard error
SPF	specific pathogen free
STAT	signal transducer and activator of transcription

TAE	tris acetic acid
TAMRA	tetramethylrhodamine
TCID50	50% tissue culture infectious dose
TLR	toll-like receptor
TNF	tumor necrosis factor

Chapter 1

Introduction

1.1 General introduction

The ability of a host to recognise and eliminate deleterious pathogens is essential for survival. These host-pathogen interactions are critical in the outcome of disease and have been the focus of much new research, highlighting the crucial role of the innate immune response and its antiviral receptors (Kato *et al.,* 2006; Wang *et al.,* 2007; Loo *et al.,* 2008; Ehrhardt *et al.,* 2009). Of particular concern is the management of new highly pathogenic veterinary viruses which are capable of jumping the species barrier. The emergence of zoonotic viruses, such as Severe Acute Respiratory Syndrome (SARS), Henipavirus, Ebolavirus and Highly Pathogenic Avian Influenza Virus (HPAIV) (Kobasa *et al.,* 2007) have resulted in severe infections and complicated syndromes which have made treatment and control of these diseases difficult. Additionally, the continued persistence of these viruses suggests a lack of understanding of host-pathogen interactions (Webster *et al.,* 1992; Guan *et al.,* 2004). The inability to prevent and control HPAIV has lead to some viral strains of H5N1 Influenza becoming endemic in many countries and this may allow for H5N1 viruses to reasort with other influenza strains which are more adapted to human infection (de Jong *et al.,* 2005; Gilbert *et al.,* 2008). Associated with a high rate of mortality, a dysregulated immune response (de Jong *et al.,* 2006b), and a large, changeable viral reservoir (Webster *et al.,* 1992), Avian Influenza (AI) poses a significant threat to human and animal populations. A better understanding of host-pathogen interactions will help in developing improved strategies for the prevention and control of viral infections. These developments are critically important considering the continuing challenge of viral diseases, such as HPAIV.

1.2 Avian influenza virus

Influenza is an infectious respiratory virus that has been shown capable of circulating in a range of hosts including humans (Subbarao *et al.,* 2000), pigs (Alexander *et al.,* 2000), cats (Burns, 2006), dogs (Harder *et al.,* 2009), ferrets (Herlocher *et al.,* 2001), and a wide variety of domesticated and wild birds (Perkins *et al.,* 2001; Isoda *et al.,* 2006; Keawcharoen *et al.,* 2008). The close association of migratory birds and domesticated poultry with humans provides the potential for interspecies transmission, as observed in the recent outbreaks of H5N1 influenza between 1997 and 2007 (Chen *et al.,* 2004). Influenza outbreaks have always been a global infectious disease threat. There have been 10 epidemics of influenza in the past 300 years (Osterholm, 2005). The devastating Spanish influenza virus infected around a third of the world's population during the pandemic of 1918, killing an estimated 40 - 100 million people worldwide (Osterholm, 2005; Loo *et al.,* 2007). More recent outbreaks include the spread of influenza viruses from Qinghai Lake, southwest China in 1997, which led to the prominence of H5N1 viruses throughout south east Asia (Chen *et al.,* 2006). Similarly, the 2009 H1N1 swine flu outbreak was thought to have originated from either infected pigs in Mexico or the United States of America and spread quickly across the globe, reaching pandemic levels (Cohen, 2009).

1.3 Influenza genome organization

Influenza viruses are enveloped, negative-sense, single-stranded RNA viruses, with an eight-segmented genome encoding ten proteins. Influenza, part of the Orthomyxoviridae family, is classified by the antigenicity of the nucleocapsid and matrix proteins (Lee *et al.,* 2009). A diagram of the structure of influenza virus is shown in Fig. 1.1. Protruding from the lipid envelope are two distinct glycoprotein's, the hemagglutinin (HA) and the neuraminidase (NA) (de Jong *et al.,* 2006a).

1.3.1 *HA (hemagglutinin) and NA (neuraminidase)*

HA attaches to cell surface sialic acid receptors, thereby facilitating entry of the virus into host cells. The NA gene is involved in the release of viral progeny from the infected host cell, by cleaving sugars that bind the mature viral particles (Suzuki, 2005). The HA and NA genes are extremely variable in sequence, with less than 30% of the amino acids being conserved among all the virus subtypes. A total of 16 different HA subtypes (H1-H16) and 9 different NA subtypes (N1-N9) have been identified (Fouchier *et al.,* 2005). Avian species, particularly aquatic birds, are the reservoir for influenza viruses as they harbour all 16 HA and 9 NA subtypes within their population (Webster *et al.,* 1992). However, only H1 to H3 and N1 to N2 viral subtypes are commonly found in humans with influenza virus infections. Although some internal proteins induce an antibody response, the HA and NA genes are the most important antigenic determinants for a protective immune response (Slepushkin *et al.,* 1995; Chen *et al.,* 1998).

1.3.2 *NP (nucleoprotein)*

The RNA genome of the influenza virus is encapsidated in the form of ribonucleoprotein (RNP) complexes. The nucleoprotein (NP), the major protein component of RNPs, binds along the entire length of each genomic RNA segment forming a double-helical RNP structure in mature viruses (Wang *et al.,* 2010). The NP has many essential roles, including intracellular trafficking of the viral genome, viral RNA replication, packaging and assembly. The NP gene has also been shown to control the induction of cytotoxic T cells targeting infected cells (Townsend *et al.,* 1984).

1.3.3 *M (matrix)*

The matrix (M) gene of the influenza virus encodes two matrix proteins (M1 and M2) from the same RNA segment through the use of different reading frames. In addition

to the HA and NA proteins, a small amount of the M2 protein is present on the surface of the viral envelope (de Jong *et al.,* 2006a). The M2 protein acts as an ion channel, playing a role in initiating the viral uncoating in the endosome (de Jong *et al.,* 2006a; Betakova, 2007). The matrix protein (M1), which lies inside the lipid envelope, is associated with both the RNP and the viral envelope, forming a coat inside the viral envelope. The M1 protein is thought to play a fundamental role in virus assembly and also facilitates budding of mature influenza viruses (Ruigrok *et al.,* 2000).

1.3.4 *PA, PB1 and PB2 (polymerase genes)*

The influenza viral polymerase is a heterotrimeric complex, consisting of PA, PB1 and PB2 subunits, harbouring several enzymatic activities for catalysing both viral RNA transcription and replication. PB1 is the nucleotide polymerase and likely the RNA endonuclease (Li *et al.,* 2001), although PB2 recognises the cap structures on host cell mRNAs (Blaas *et al.,* 1982). The role of the PA subunit is less well defined, however, mutations in the PA of the polymerase gene affect both genome replication and mRNA transcription (Fodor *et al.,* 2002).

1.3.5 *NS, NEP (nuclear export protein)*

The NS gene directs the synthesis of two mRNAs in infected cells. One is collinear with the viral RNA segment and encodes for NS1 protein and the other, NS2, is derived by alternative splicing from the NS1 mRNA. The NS1 protein is designated as a non-structural protein because it is synthesised in infected cells, but is not incorporated into virions (Krug *et al.,* 2003). The NS1 protein participates in both protein-protein and protein-RNA interactions and is one of the virally encoded interferon (IFN) agonists. The NS2 protein, also known as the nuclear export protein (NEP), was thought to be a non-structural protein, but is present in small amounts in the virions in association with the RNP through an interaction with the M1 protein (Townsend *et al.,* 1984). The NS2 functions to mediate the export of newly synthesized RNPs from the nucleus.

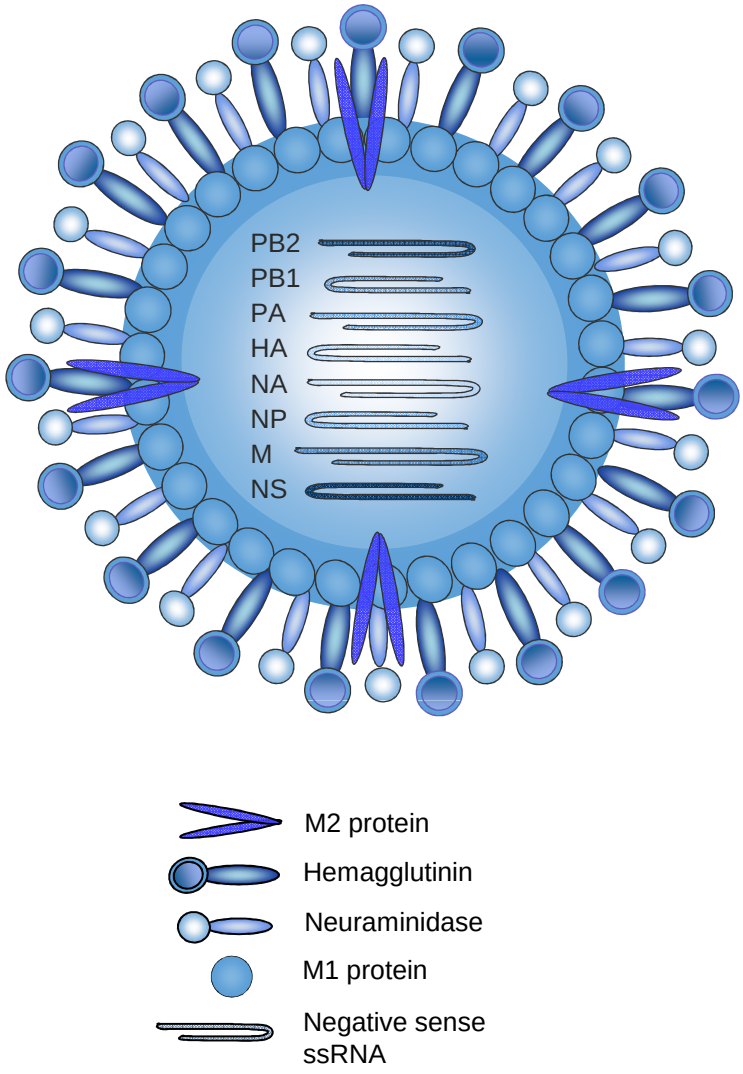

Figure 1.1. Influenza A virus. Schematic representation of influenza A virus outlining the segmented genome organization and structural components. Typically, influenza viruses are encapsulated, with the hemagglutinin (HA), neuraminidase (NA), matrix one and two (M1, M2) proteins making up the outside shell, with the eight segmented genome contained within.

1.4 Antigenic shift / drift

As a result of its segmented genome, shuffling of gene segments can occur if two different strains of influenza virus infect the same cell (Yuen *et al.,* 2005). This antigenic change may be due to immune selection pressure or other factors that are involved in virus adaptation to a new host. Antigenic variations result from an accumulation of molecular changes in the viral RNA that occurs primarily through point mutations but also through RNA recombination and RNA segment reassortment (Loo *et al.,* 2007). Antigenic drift has been well characterised in human H3 influenza viruses that are suggested to exhibit approximately 7.9 nucleotide and 3.4 amino acid substitutions per year in the HA1 gene (Bean *et al.,* 1992). Similarly, analysis of the mutation rate of the HA1 of H5 AI viruses from live bird markets indicated 7.8 substitutions per 1000 nucleotide sites per year (Suarez *et al.,* 2000). The ability of the influenza genome to rapidly evolve, in concert with a large natural reservoir, has made influenza viruses both difficult to control and in some cases more pathogenic. Studies of genetically modified influenza virus containing some of the genes from the 1918 virus suggest that its genetic reassortment may have been crucial to its pathogenicity (Takeda *et al.,* 2003; Tumpey *et al.,* 2005).

Influenza virus infectivity is influenced by the type of linkage to galactose on the host cell surface (Connor *et al.,* 1994). The HA of human influenza virus preferentially adheres to sialic acids attached to galactose by an alpha-2,6-linkage (α2,6), which is the predominant type of sialic acid receptor on the surface of human respiratory epithelial cells (Yuen *et al.,* 2005). The HA subtypes of avian influenza viruses, such as the H5N1 subtypes, is thought to preferentially attach to the alpha-2,3-linked (α2,3) sialic acid receptor present on the respiratory epithelium of many avian species. However, there are exceptions and studies by Yamada *et al*., (2006), indicate that single amino acid substitutions at the receptor binding site of the HA molecule allow H5N1 viruses to recognise the human sialic acid receptor (Yamada *et al.,* 2006). Typically, host range restriction of influenza virus also depends on the receptor distribution in the hosts. Humans have sialic acid α2,6 linked galactose receptors

throughout the respiratory tract from the nose to the lungs, but also have sialic acid α2,3 linked galactose receptors in and around the alveoli in the lungs (Nicholls *et al.,* 2007). Thus, H5N1 preferentially infects cells in the lower respiratory tract and induces heavy damage to the lungs but may have little involvement of the upper respiratory tract (Yuen *et al.,* 2005; Gu *et al.,* 2007). Furthermore, the epithelial cells of the trachea in swine produces both (α2,3) and (α2,6) linked sialic acids. This is believed to be the reason why swine can be infected with both avian and human influenza virus strains and serve as an intermediate host for the emergence of new viruses (Nicholls *et al.,* 2008; Liu *et al.,* 2009). Clearly, a better understanding of the genetic determinants that confer transmissibility of an influenza virus from avian species to humans will help in determining the pathogenicity of these viruses.

1.5 Avian influenza pathogenicity

Historically, the pathogenicity of AI viruses has been based on their lethality in the major domesticated poultry species, the chicken. All AI viruses are categorised as either low pathogenicity (LP) or high pathogenicity (HP) (Alexander, 2000). Within each category, pathobiological changes vary within the host species and virus strain (Swayne *et al.,* 2006; Alexander, 2007). Typically, highly pathogenetic avian influenza viruses (HPAIV) produce severe, systemic disease with high mortality in humans and chickens and a similar pathobiology in other galliform birds. However, HPAIV usually produce either no infection or mild disease in ducks or other migratory birds (Kishida *et al.,* 2005). The role of wild birds in the epidemiology of the Asian lineage H5N1 avian influenza virus and their contribution to the spread of the virus through Eurasia and Africa is unclear (Brown *et al.,* 2008). Although H5N1 viruses have been isolated from dead wild birds, usually within the flight range of infected poultry farms (Liu *et al.,* 2005; Isoda *et al.,* 2006; Lee *et al.,* 2008).

Aquatic avian species have been shown to be a reservoir for AI viruses (Webster *et al.,* 1992) and are generally asymptomatic to viral infection (Guan *et al.,* 2004). Nevertheless, new viruses have been shown to be pathogenic in ducks. These migratory birds may act as vectors for the virus, spreading new isolates into domestic

poultry and the thus causing the virus to become endemic in parts of Asia (Lipatov *et al.,* 2005a). An unprecedented epizootic outbreak was caused by H5N1 viruses in 8 countries in the winter of 2003 to 2004 (Alexander, 2003; Ellis *et al.,* 2004; Sims *et al.,* 2005). Associated with this outbreak in poultry was the spill-over of influenza viruses into other species. Fig 1.2. illustrates how H5N1 influenza viruses are maintained in aquatic avian species but have the ability to be transmitted to other species which are more susceptible to influenza virus infection. Purely avian influenza viruses of subtypes H5N1 and H7N7 have crossed the species barrier to directly cause fatal disease in humans in Hong Kong and Holland (Webster *et al.,* 1992; Subbarao *et al.,* 1998). It is thought that an intense cytokine dysregulation resulting from H5N1 infection is involved in the subsequent fatalities of chickens and humans (To *et al.,* 2001; de Jong *et al.,* 2006b; Us, 2008). The spread of H5N1 through Eurasia and its pleiotropic pathogenicity in avian populations is a cause for alarm (Palese, 2004; Lipatov *et al.,* 2005a).

1.6 H5N1 influenza in ducks and other migratory birds

In ducks, Asian H5N1 viruses have changed from producing inconsistent respiratory infections to some strains being HP (Swayne *et al.,* 2006). Most studies emphasize the resistance ducks have to disease development after experimental infection with H5N1 influenza virus. In fact there were few reported cases of AI that were pathogenic in wild aquatic birds (Becker, 1966), and none of the HPAIV strains had been shown to be pathogenic in ducks until 2002 to 2003 (Alexander *et al.,* 1986; Webster *et al.,* 2002; Perkins *et al.,* 2002). H5N1 virus isolates from 1997 did not replicate efficiently in ducks (Shortridge *et al.,* 1998) and induced only mild tissue lesions, if any (Perkins *et al.,* 2002). However, in the past few years, there have been more frequent reports of ducks and other migratory water fowl developing severe disease, such as neurologic signs and mortality after infection with HPAI viruses (Brojer *et al.,* 2009). Specifically, the H5N1 virus isolated in 2002 in Hong Kong, which caused the first wave of lethal influenza virus infection reported in wild aquatic birds, were antigenically distinct, neurotropic and pathogenic in ducks (Vascellari *et al.,* 2007). After infection, H5N1 virus has been isolated from a range of organs, and the

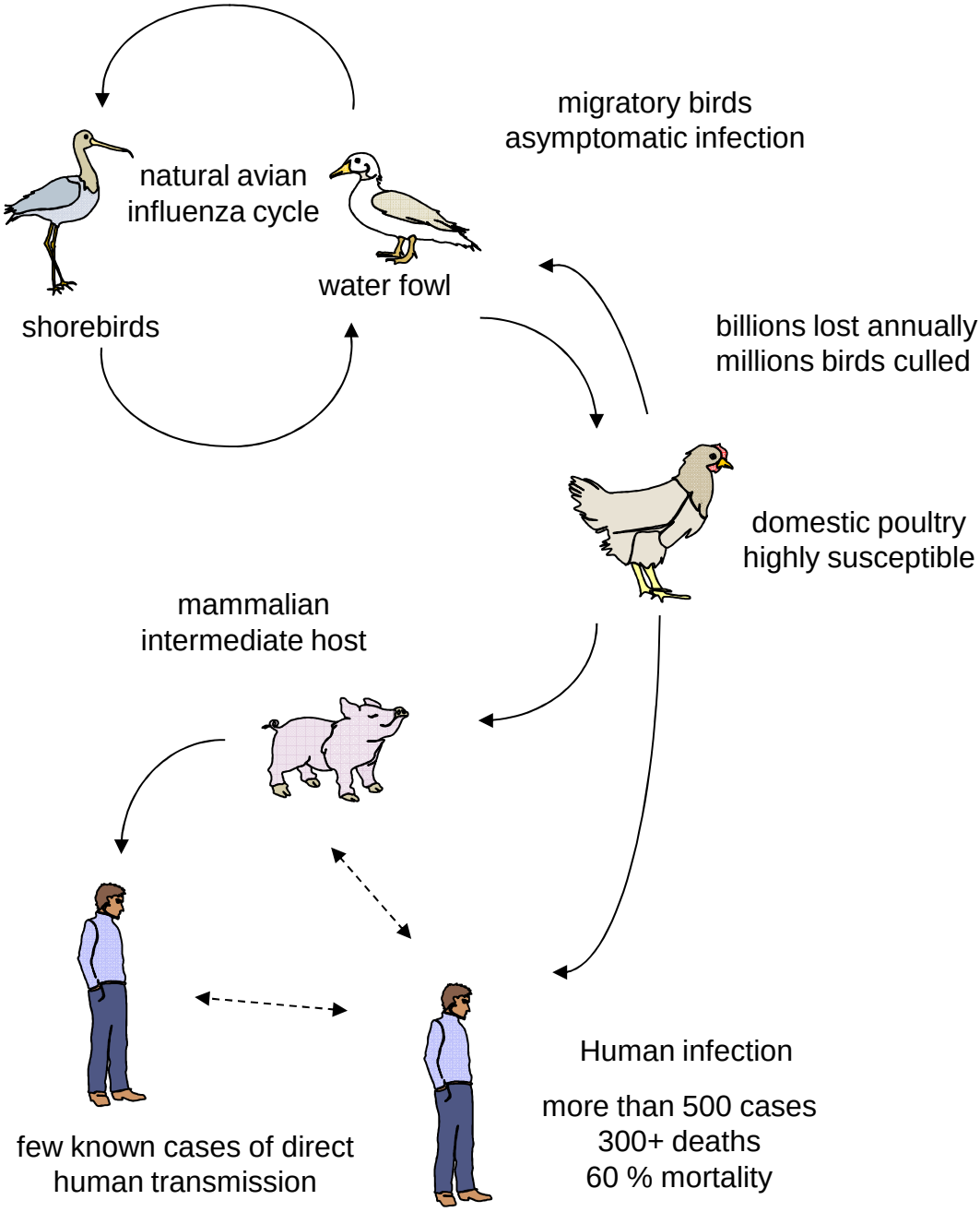

Figure 1.2. H5N1 avian influenza cycle is associated with varying pathogenicity in host species. H5N1 avian influenza viruses a thought to have a large natural reservoir in migratory birds which are typically asymptomatic to infection and have been implicated in the spread of the virus. Transmission of H5N1 influenza viruses into other species has been associated with increased pathogenicity and severe symptoms in chickens and humans.

excretion of high virus titres from the upper respiratory, and to a lesser extent, intestinal tracts was observed (Kishida *et al.,* 2005; Swayne *et al.,* 2006; Vascellari *et al.,* 2007). These new viruses caused disease and mortality in many different species of water birds and in some wild migratory birds (Swayne *et al.,* 2006).

Influenza viruses in wild aquatic birds were thought to have long been in a state of evolutionary stasis, with little evidence of clear evolution during the eighties and nineties (Gorman *et al.,* 1992). This balance has been changing, as new H5N1 viruses infect aquatic bird species. Influenza viruses reportedly replicate preferentially in the gastrointestinal tract of wild birds and are then excreted at high viral titres in faeces and are thought to spread to other wild birds and domestic poultry via contamination of water (Webster *et al.,* 1978; Webster *et al.,* 1992; Weber *et al.,* 2007). Nonetheless, some findings have suggested that ducks may shed virus particles primarily from the upper respiratory tract (Perkins *et al.,* 2002). From this reservoir, AI viruses are occasionally transmitted to other avian and mammalian hosts, including humans and can cause outbreaks of severe disease (Webster *et al.,* 1992). After the 1997 outbreak in Hong Kong it was determined that wild migratory birds, particularly ducks and geese, were responsible for the spread of H5N1 virus which was found to initiate from healthy birds in the Guangdong lake region of China (Chen *et al.,* 2004). In late 2002, new H5N1 outbreaks in two Hong Kong nature parks caused the death of many resident avian species, including waterfowl, flamingos, egrets, herons and other migratory birds (Ellis *et al.,* 2004). Concurrently, H5N1 viruses of a similar lineage were isolated from dead chickens in poultry markets and a local chicken farm. Shortly after, in February 2003, H5N1 influenza virus was isolated from two human patients suffering respiratory distress, one of whom died (Wuethrich, 2003). Fig. 1.2. highlights how the H5N1 avian influenza cycle can spread H5N1 viruses to intermediate hosts, such as chickens and then spill over into human populations.

The transfer of viruses between avian species can result in increased antigenic variation, particularly in the surface glycoprotein's, due to strong immune selective pressure of the new host (Matrosovich *et al.,* 1999). Interspecies transmission of AI had previously been considered to flow from waterfowl to terrestrial birds, since

many viruses isolated from domestic poultry contained genes of aquatic avian origin. However, analysis of emerging viruses in China suggested that virus lineages established in domestic poultry have now been transmitted back to ducks (Li *et al.,* 2003; Sturm-Ramirez *et al.,* 2004). It is currently thought that there is a two way flow of influenza viruses between aquatic birds and domestic birds in southern China and possibly Hong Kong (Fig. 1.2). Sturm-Ramirez *et al*., (2004) have found considerable antigenic variation in the H5 surface antigen of these viruses from the 2002 Hong Kong outbreak. The consequences of antigenic variation could directly affect the pathogenicity of H5N1 viruses via any biological mechanism that involves interaction with the HA protein, such as immune evasion or tissue tropism.

Early investigations indicated that HPAIV which were lethal in poultry could replicate in the internal organs of ducks but caused no disease signs (Kawaoka *et al.,* 1987). H5N1 infection tended to be pneumotropic, with mild lesions localized to the respiratory tract and some virus detected in the spleen and bursa (Perkins *et al.,* 2002). Yet, since the 2002 outbreak, studies with more recent H5N1 isolates, in a range of aquatic avian species, have resulted in severe disease and significant titres of virus isolated from organs. The tissue distribution of these 'new' H5N1 virus in ducks indicate that H5N1 HPAI viruses appear to have a high predilection for a wide range of cell types (Kwon *et al.,* 2005; Kishida *et al.,* 2005; Vascellari *et al.,* 2007; Pantin-Jackwood *et al.,* 2007; Keawcharoen *et al.,* 2008). There also appears to be a similarity in the general tissue tropism between various species of domestic and wild ducks, geese and swans, even if there is variability in the quantity of antigen in different tissues. This is confirmed by separate studies of infected swans, where H5N1 viruses appeared to target brain, liver and pancreatic tissue (Teifke *et al.,* 2007; Kalthoff *et al.,* 2008). Similar findings were reported in Canadian geese (Pasick *et al.,* 2007) and domestic ducks (Kishida *et al.,* 2005) suggesting that ducks may suffer a systemic form of infection from certain H5N1 influenza isolates.

One tissue type for which H5N1 influenza viruses appear to have a high predilection for in ducks, is myocardial, skeletal and smooth muscle. The presence of H5N1 virus in this tissue might be relevant to the more pathogenic nature of infection witnessed in

migratory birds (Mase *et al.,* 2005; Pantin-Jackwood *et al.,* 2007; Bingham *et al.,* 2009). The high pathogenicity of a Vietnamese strain isolated from ducks (Bingham *et al.,* 2009) was suggested to be due to the viral tropism to the heart and the brain. Infection of these tissues in conjunction with tissue degradation and inflammatory responses may be the cause of significant morbidity in this isolate of H5N1 (Pantin-Jackwood *et al.,* 2009). H5N1 viruses have also been reported to infect the CNS and cause histopathological changes in the brain of different avian species. Several of the newly emerging H5N1 viruses induced severe neurological signs and lesions in the brain of infected ducks (Sturm-Ramirez *et al.,* 2004). Bingham *et al*., (2009), observed pyrexia in the early stages of infection and mild persistent motor neurological disease later in the infection. Furthermore, H5N1 virus isolated from a wild duck in Hong Kong in 2002 caused systemic infection, neurological dysfunction and death in ducks. It was thought that the ability to transmit to the central nervous system was essential to the pathogenicity of the viral infection (Sturm-Ramirez *et al.,* 2004). Unfortunately, little is known about the host-to-pathogen immune response to H5N1 virus at a cellular level in ducks. In humans and chickens for example, studies have indicated a role for a hyper-innate immune response which may become dysregulated (Fig. 1.3). If we can identify the reasons for the differing immune response to H5N1 between chickens and ducks we may be able to use this information to develop new strategies to contain AI in domestic poultry.

1.7 H5N1 influenza in chickens

Since the re-emergence of H5N1, AI has become a zoonotic issue, prompting increased concern about the spread and transmission of virus from domestic poultry flocks. Most human infections seem to result from direct contact with sick or dead poultry, but other routes of transmission may also occur. It had been believed that HPAIV could not cross the species barrier to directly infect humans and that pigs were needed as an intermediate host or mixing vessel for viral reassortment (Webster *et al.,* 1992). However, it was reported in 1997 that 18 people become directly infected with H5N1 influenza viruses spread from poultry, resulting in 6 deaths (Subbarao *et al.,* 1998; Peiris *et al.,* 2007). Since September 2009, H5N1 outbreaks in

poultry have contributed to 556 reported human cases of H5N1 infection and 325 deaths have so far been recorded (WHO reports, June 2011). These figures represent an alarmingly high mortality rate of around 63%. The outbreaks in poultry farms in Hong Kong resulted in the slaughter of over 300 million birds, in order to control the spread of the virus and avert a human pandemic (Alexander, 2006). Nonetheless, mass culling proved an inadequate method of preventing influenza virus expansion, as it spread rapidly from its origins in China and is now thought to be endemic in south east Asia (Li *et al.,* 2010). Outbreaks of this nature are difficult to control and lead to increased rates of hospitalization and fatalities (Webby *et al.,* 2003; Lipatov *et al.,* 2005b). Despite numerous studies from *in vivo* and *in vitro* models, the basis of the unusual pathogenicity of the Hong Kong H5N1 influenza virus remains somewhat unclear (Webby *et al.,* 2003; Guan *et al.,* 2004). Therefore, a key to controlling influenza pandemics may lie in understanding and controlling AI in domestic poultry and more precisely understanding the immune responses and transmission of virus from chickens.

Unlike ducks, which are generally asymptomatic during HPAIV infection, chickens suffer a multisystemic disease which is associated with high morbidity and mortality (Hooper *et al.,* 1995; Swayne *et al.,* 2006). It is difficult to describe a set of clinical signs that are consistent indicators of an HPAIV outbreak as initial symptoms are not always apparent or varied depending on the viral strain (Alexander *et al.,* 1986; Nakatani *et al.,* 2005). It is concerning that recently re-assorted H5 and H7 HPAI viruses have shifted toward increased virulence for chickens as evidenced by shorter mean death times (MDT) and that these viruses which kill birds quickly tend to cause fewer clinical signs (Bean *et al.,* 1985; Perkins *et al.,* 2001). In experimentally infected chickens, early signs have included depression, ruffled feathers, decreased food consumption, dyspnea, and inflammation of the comb, legs and feet (Perkins *et al.,* 2001; Jones *et al.,* 2004; Tsukamoto *et al.,* 2007). Generally, H5N1 infection in the chicken is characterised by systemic infection and viremia. Severe haemorrhaging, particularly in the lungs and intestines, oedema, cutaneous ischemia and depleted neurological signs are present in HPAI infected chickens (Muramoto *et al.,* 2006).

After the initial infection, which is primarily detected in epithelial, macrophage and monocyte cell populations of the respiratory tract, viral replication spreads rapidly (Mo *et al.,* 1997; Muramoto *et al.,* 2006; Suzuki *et al.,* 2009; Mundt *et al.,* 2009). Endothelial cells are thought to be one of the major targets for HPAIV in chickens (Brown *et al.,* 1992; Mo *et al.,* 1997; Suarez *et al.,* 1998; Feldmann *et al.,* 2000). Viral infection may induce endothelial dysfunction, contributing to the systemic haemorrhaging witnessed in chickens (Muramoto *et al.,* 2006). The distribution of H5N1 antigen in infected chickens is widespread, with lung, heart, liver, kidney, spleen, brain, thymus, pancreas and intestinal tissue, regularly infected with high viral loads (Mo *et al.,* 1997; Muramoto *et al.,* 2006; Mundt *et al.,* 2009), although the majority of the damage inflicted in chickens appears to be centred on the respiratory system. Muramoto *et al*., (2006) have noted the formation of excessive blood coagulation in the lungs of infected chickens, which was associated with the viral antigen in endothelial cells. H5N1 infection also initiates lymphopenia and increased production of inflammatory cytokines in the lungs (Cheung *et al.,* 2002; Wong *et al.,* 2006; de Jong *et al.,* 2006b). This increased inflammatory response is not unique to chickens, as a number of studies have indicated high levels of cytokine induction in humans, macaques and mice (Kash *et al.,* 2006; Xu *et al.,* 2006; Kobasa *et al.,* 2007). The dysregulation of the cytokine response may also lead to necrosis and multiple organ failure (Cilloniz *et al.,* 2010), as shown in Fig. 1.3. Nevertheless, it is unclear what role cytokine dysregulation plays in contributing disease severity. Suzuki *et al*., (2009) observed that different H5N1 isolates induce a range of proinflammatory cytokines, but some of these are later suppressed in lung tissue. To fully understand the role of cytokines and inflammation, more investigations into the chicken immune response to H5N1 infection need to be carried out.

1.8 Host-pathogen interactions

In vertebrates, including avian species, there are two fundamental immune response systems which act to defend against pathogens (Takeda *et al.,* 2003). These are commonly known as the innate and adaptive immune systems. The innate immune system is able to identify a broad selection of non self elements which signify

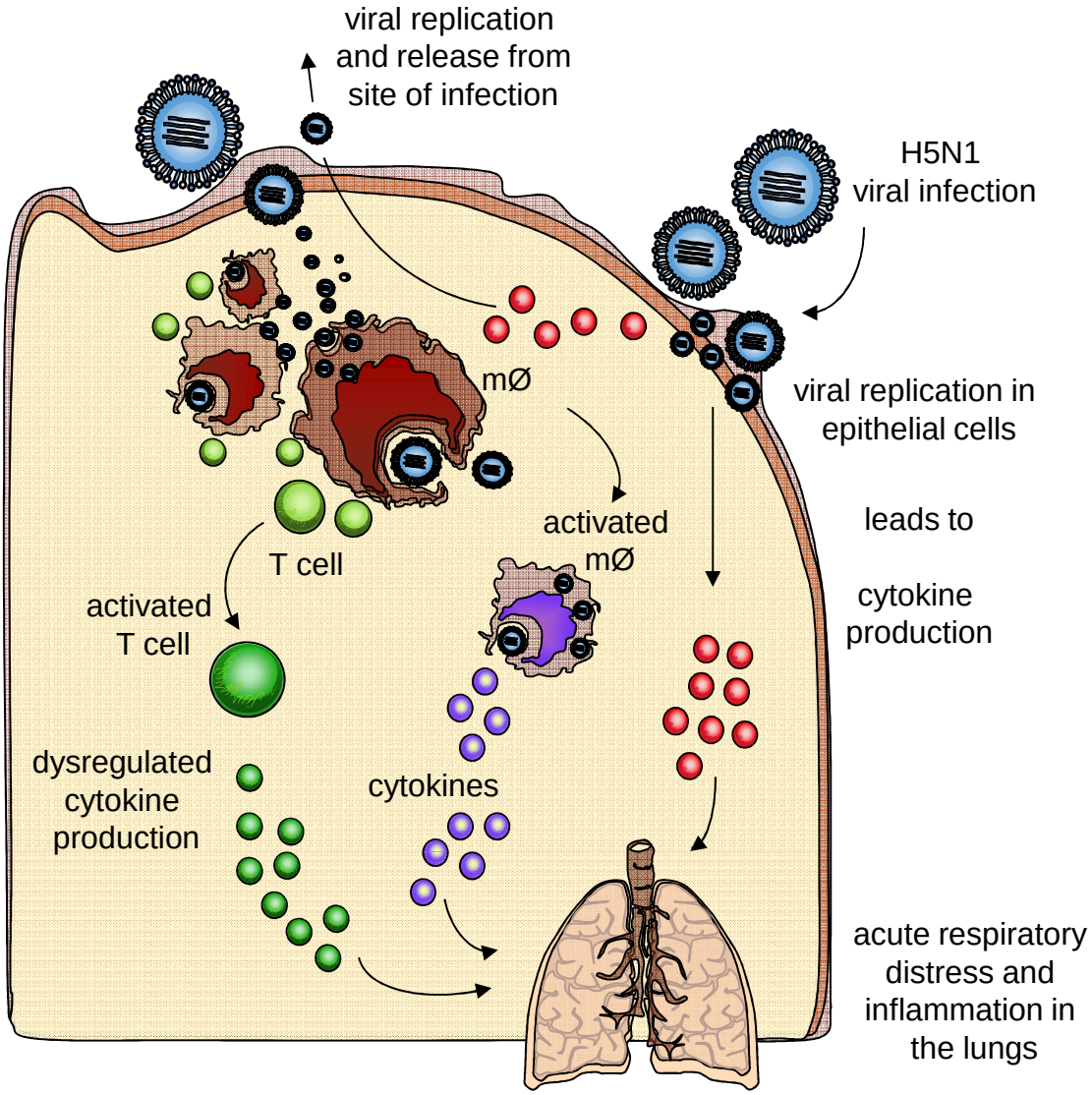

Figure 1.3. Hypercytokinemia or cytokine storm has been hypothesized to contribute to H5N1 influenza disease severity. Following H5N1 influenza infection, virus replication occurs rapidly in certain cell types, such as epithelial cells. This replication results in the activation of immune cells such as macrophages which are recruited to sites of infection. Macrophages then sequester the help of immune cells, such as CD4+ and CD8+ T cells, to produce an antiviral response. A cytokine storm appears to erupt when there is no resolution to this viral infection. During a cytokine storm, excessive amounts of cytokines are produced which lead to sustained inflammation and cellular damage. This typically takes the form of acute respiratory distress and multiple organ failure syndrome.

microbial intrusion of the host (Medzhitov, 2001). Early detection is critical for the ability of the host to mount a response prior to the significant progression of infection. The innate immune network triggers an initial host response to a panel of pathogen associated factors (Medzhitov, 2007) referred to as pathogen associated molecular patterns (PAMPS). The adaptive immune system is geared to recognise an ever increasing number of foreign antigens (Bogs *et al.,* 2010). This adaptive response is mediated through specialized cells, such as B and T cells which rearrange their immune associated genes leading to an extensive collection of immunoglobulin's and T cell receptors (Schatz, 1999; Bogs *et al.,* 2010) which afford the host a versatile system of protection.

1.8.1 *Pathogen / pattern recognition receptors*

The immune response to virus in mammalian studies is generally well characterised relative to that of the chicken and the duck (Puehler *et al.,* 2003). In mammals, following viral invasion, innate immune recognition is mediated by a series of germline encoded receptors, which belong to several distinct protein families, expressed primarily on antigen presenting cells (Janeway, 1992; Medzhitov *et al.,* 1997a). These receptors, originally coined pathogen recognition receptors (PRR), recognise conserved molecular patterns not produced by the host (Medzhitov *et al.,* 1997b). These antigens are better known as PAMPS and include the Toll-Like Receptor (TLR) family. TLR function as key sentinels in the first line of defence by detecting PAMPs and subsequently modulating the expression of immune functioning genes and mobilizing the wider immune response (Akira *et al.,* 2001; Kawai *et al.,* 2007). One key feature of some TLR is the ability to recognise viral RNA and mediate an antiviral response. The rapid nature of infections such as AI can be fatal in as little as 24 - 48 hours (Mase *et al.,* 2005),(Isoda *et al.,* 2006) further highlighting this reliance on an early host antiviral mechanism. Therefore, the activation of host responses by PAMPs and understanding these host-to-pathogen mechanisms may provide significant benefits for developing new approaches to protect against viral infection.

Other host PRRs, like RIG-I and MDA5 have also been identified as dsRNA detectors (Yoneyama *et al.,* 2004). RIG-I (retinoic-acid-inducible protein I, also known as Ddx58) and MDA5 (melanoma differentiation associated gene 5) contain two caspase recruitment domains (CARDs) which undergo a conformational change and initiate signal transduction (Thompson *et al.,* 2007). Both RIG-I and MDA5 lead to the production of type 1 IFN (Gitlin *et al.,* 2006) and have an important role in the host anti viral response (Gitlin *et al.,* 2006; Sabbah *et al.,* 2009). Additional studies suggest that MDA5 may be involved in the antiviral response in human epithelial cells following influenza infection (Siren *et al.,* 2006) and thus the MDA5 pathway may be critical in combating viral infections. However, further characterisation of both RIG-I and MDA5 is required to establish their role in the avian host response.

1.8.2 *Antiviral response of the TLR*

The Toll gene was originally discovered in Drosophila as being essential for normal embryonic development (Swayne *et al.,* 1988). Further genetic analysis reveals however, the cytoplasmic domain of Toll was similar to Interluekin1 (IL1) receptor (IL1R). This finding implicated a role for Toll in the innate immune response (Akira *et al.,* 2001). Viral infection may be detected by the host sensing viral nucleic acids through TLR3, 7, 8 and 9 which identify these receptors as a critical interface between pathogen and host (Lund *et al.,* 2003; Lund *et al.,* 2004). In mammals these TLR activate cellular pathways that lead to antiviral cytokines, such as IFN and interleukin (IL), which are critical for host viral control. TLR3 recognizes and mediates the dsRNA related host response (Alexopoulou *et al.,* 2001). TLR7, 8 and 9 functions from an endosomal location, which allows discrimination against self-nucleic acids that could otherwise initiate the same immune responses. TLR7 and 8 are activated by RNA ligands including ssRNA oligonucleotides rich in guanodine or uridine (Heil *et al.,* 2004). These types of RNA sequences have been proposed to repeat in sequences associated with viruses (Lund *et al.,* 2004). Additionally, both TLR7 and 8 recognise the synthetic compounds imiquimod (Levy *et al.,* 2006), resiquimod (Jurk *et al.,* 2002) and loxoribine (Hemmi *et al.,* 2002) which have been used to simulate viral infection in vitro. Chickens have a complete TLR7 gene, but TLR8 is only present in

a series of fragments which are disrupted by a retroviral, CR1-type insertion element (Philbin *et al.,* 2005). Duck TLR7 shares only 85% amino acid identity to chicken TLR7, differing primarily in the ligand-binding LRR domains (Macdonald *et al.,* 2008). However, it is proposed to function in the same fashion, mediating an antiviral host response. Duck TLR7 appears to be highly expressed in lymphoid tissues, such as the spleen and bursa. In addition, significant TLR7 expression is observed in the lung tissue of ducks, which is distinct from the expression pattern of chickens. The duck TLR7 expression pattern is comparable to that for human, which is predominantly expressed in spleen, and has significant expression in lung (Chuang *et al.,* 2000; Nishimura *et al.,* 2005; Macdonald *et al.,* 2008). High pulmonary expression of TLR7 could be significant in the context of highly pathogenic H5N1 avian influenza, which is primarily a lung infection (Pantin-Jackwood *et al.,* 2007). Cytokines, such as IL1 and IL18, produced in response to pathogens, activate target cells through receptors that are classified within the same family as TLR, inducing an amplified, yet regulated immune response as described in Fig. 1.4 (Boraschi *et al.,* 2006).

1.9 Cytokines

Cytokines play a critical role in the control of the innate and adaptive immune response. An important class of cytokines produced in response to virus are the interferons (IFN). IFN, particularly type I IFN, were initially discovered through their ability to inhibit the replication of influenza virus in chick chorioallantoic membranes (Isaacs *et al.,* 1957; Stark *et al.,* 1998). Since their discovery, a substantial amount of research has been directed at elucidating the antiviral properties of IFN, which extend to many aspects of the immune response (Theofilopoulos *et al.,* 2005). The IFN are typically characterised by their biological activity (the receptor through which they signal) and homology to each other (Pestka *et al.,* 2004). IFN are generally divided into three classes. Type I IFN, which includes IFNα and IFNβ, type II IFN or IFNγ and recently, a third type of IFN has been added to the fold. This new type III IFN has been labelled IFNλ and is proposed to be more closely related with the type I IFN (Pestka *et al.,* 2004).

The production and release of IFN from virally infected cells is vital in the induction of gene expression for key antiviral mechanisms in uninfected cells (Levy *et al.,* 2001). Furthermore, IFN stimulates the adaptive immune response by initiating the apoptosis of infected cells and assisting in the production of antibodies against viruses (Takizawa *et al.,* 1993). The antiviral properties of IFN also induce important pathways which may result in the production of the Mx proteins, protein kinase (PKR), RNase L and 2',5'-oligoadenylate synthase (OAS) (Sen, 2001). The Mx proteins are GTPases that associate with infecting viral proteins to either block nucleocapsid transport or RNA synthesis (Haller *et al.,* 2002). PKR is an inducible kinase that is important during various stages of influenza infection as it inhibits the viral replication through its role in phosphorylation (Garcia *et al.,* 2006). Alternate antiviral pathways stimulated by IFNs are directed towards degrading the viral RNA in order to inhibit viral replication. Two enzymes, OAS and RNase L are capable of eliminating the influenza virus through this approach (Player *et al.,* 1998). The strength of these antiviral pathways has been highlighted by animal studies, in which disruptions to either IFN gene expression or an antiviral pathway resulted in higher levels of susceptibility to viral infection (Huang *et al.,* 1993).

A second important class of cytokine are the interleukins (IL). Interleukins are secreted molecules, which, as their name suggests, are utilized by leukocytes for communication between cells without direct contact (Chen *et al.,* 2006). Interleukins can be any of a class of lymphokines that act to stimulate, regulate, or modulate lymphocytes such as macrophages, killer T cells, B cells and other components of the immune system. They can regulate a wide range of cellular processes, such as proliferation, cell activation, inflammation and cell differentiation. There are currently 35 well known ILs, however there are likely many more to be identified. Interleukins exert their effects through interactions with interleukin receptors (ILR), which are typically located in the outer membranes of many cell types. ILs are generally classed by family depending on the ligand and receptor used, such as the IL1 family which use the IL1 receptor and as its name suggests was the first IL to be described (Dinarello, 1987). However, ILs can also be described as either pro-inflammatory, or

anti-inflammatory depending on the role they play in the immune response. During viral infection there is a complex interplay between ILs and IFNs which work in synergy to activate a healthy immune response to curtail a viral infection (Fig. 1.4).

1.9.1 *IFNγ*

IFNγ, a type II IFN, is thought to promote both innate and adaptive responses to virus infection (Boehm *et al.,* 1997). The pleiotropic diversity of IFNγ is evident, as IFNγ specific receptors (IFNγR) are found on almost all lymphoid and non-lymphoid cells (Valente *et al.,* 1992; Pestka *et al.,* 2004) and are involved in antigen processing and presentation as well as anti microbicidal effecter functions (Schroder *et al.,* 2004). Several cell types can produce IFNγ, including NK cells, CD4+ and CD8+ T cells which, when primed by signalling from cytokines, most notably IL12 and IL18, secrete IFNγ. IFNγ shares some similar activity with IFNα and IFNβ, whereby they are able to induce major histocompatability complex (MHC) class I expression. However, only IFNγ can induce MHC II, which presents viral fragments to T helper cells, helping regulate the destruction of infectious virus (Loughlin *et al.,* 1993). Additionally, IFNγ plays a significant role in the differentiation of T helper cells and activation of macrophages (Leist *et al.,* 1988).

IFNγ binds to the IFNγ receptor complex (IFNGR), comprised of the R1/R2 chains, and exerts downstream actions (Bach *et al.,* 1997). The IFNγR complex utilises the janus activating kinase (JAK)/signal transducer activator of transcription (STAT) signal transduction pathway (Sakatsume *et al.,* 1995; Bach *et al.,* 1997). Once the signal is translocated into the nucleus of the cell, it initiates the transcription of IFNγ induced genes (Bach *et al.,* 1997). IFNγ induces the production of a variety of anti-viral proteins including reactive nitrogen intermediates (RNIs) and PKR from such cell populations as monocyte/macrophages (Malmgaard, 2004). These IFNγ induced factors give macrophages cytostatic and cytotoxic activity, allowing them to non-specifically kill a variety of intracellular and extracellular parasites (Schreiber *et al.,* 1985; Liew *et al.,* 1990). Critically, the role of IFNγ in protective immunity is illustrated in studies with gene knockout mice (KO), where the IFNγ, IFNγR1 and

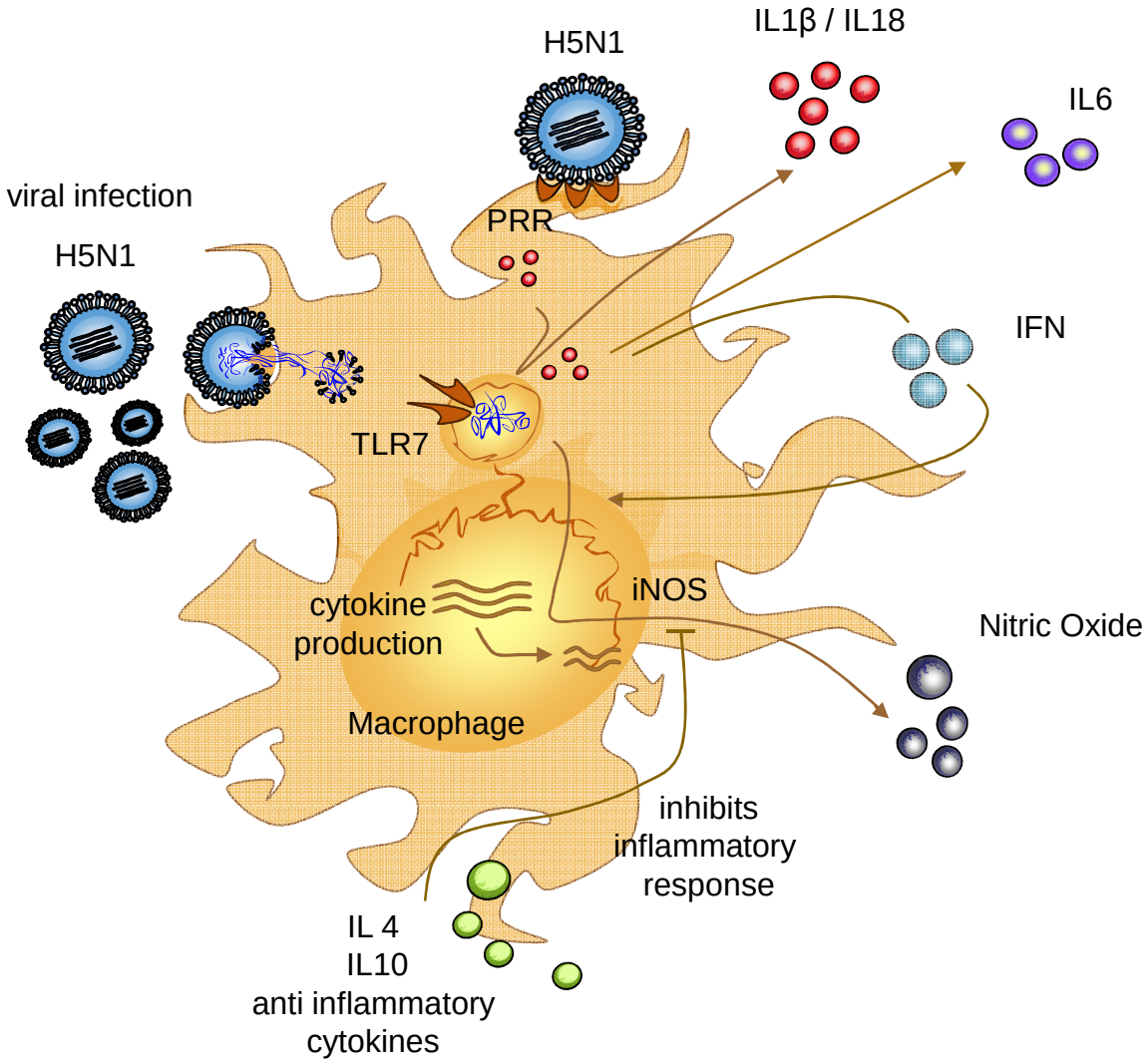

Figure 1.4. Inflammatory immune response following H5N1 influenza virus infection is characterized by an induction of cytokines, RNIs and APPs. When H5N1 influenza viruses infect a cell, the hosts initial response is through viral sensing receptors (pattern recognition receptors) such as TLR7. These receptors act to signal the innate antiviral response which culminates in the production of inflammatory molecules such as the ILs, IFNs, RNIs, and APPs. This proinflammatory response is usually kept in balance by anti-inflammatory mediation.

IFNγR2 genes have been deleted. When exposed to sub-lethal doses of viral and bacterial infection, KO mice die rapidly (Huang *et al.,* 1993; Lu *et al.,* 1998). The further understanding and regulation of these multiple IFN systems in the chicken and duck may help identify these molecules as antiviral candidates that could be used in strategies targeting AI.

1.9.2 *Interleukin1 / IL1α / IL1β*

IL1 and its related family members are primarily proinflammatroy cytokines with the ability to stimulate the expression of genes associated with inflammation. Mammalian IL1 was initially identified as a macrophage derived factor with lymphocyte costimulating activity. However, subsequent work revealed that IL1 acts not only on T lymphocytes and thymocytes but also on a wide variety of other cells and works in concert with a host of other proinflammatory cytokines, such as IL6 and tumor necrosis factor (TNF) (Krakauer *et al.,* 1999; Staeheli *et al.,* 2001). Three structurally related members of the IL1 cytokine family have been described in mammals (IL1, IL18 and IL33). IL1α and IL1β are biologically active cytokines that are specifically inhibited by the third family member, termed IL1 receptor agonist (IL1Ra). IL1β has been shown to strongly induce IL8, K60 and other CXC chemokines in both mammalian and avian cells (Introna *et al.,* 1993). Chicken IL1 was the first avian lymphokine to be identified when its activity was demonstrated in the supernatants of LPS stimulated adherent spleen cells (Hayari *et al.,* 1982). However, it was not until 1998 that chicken IL1β was expressed in HD-11 cells and cloned (Weining *et al.,* 1998). Chicken IL1β consisted of 267 amino acids and showed 25% similarity to human IL1β (Staeheli *et al.,* 2001). The exon:intron structure of the coding region of chicken IL1β corresponds almost exactly to that of mammalian IL1β (Kaiser *et al.,* 2004) and like its mammalian counterpart chicken IL1β lacks a signal peptide and seems to be synthesized as an inactive precursor molecule. Interestingly, the characteristic caspase-1 protease cleavage site may not be conserved in chicken pro-IL1β (Staeheli *et al.,* 2001). Recently, Wu *et al*., (2005), sequenced and analyzed the structural and functional homology of IL1β between five avian species, including chickens and ducks. This cross species study tested the reactivity of antisera against

each recombinant IL1β protein and found increased levels of K60 mRNA expression, indicating that chicken, duck, turkey and geese share a functionally active and structurally similar IL1β gene (Wu *et al.,* 2007). Sequence examination of the IL1β nucleotide encoding region in ducks revealed that it is nearly identical to that of chicken (94% identity). Given that IL1β activity increases in chickens subjected to a range of viral infections (Heggen *et al.,* 2000; Laurent *et al.,* 2001), its activity in ducks may be important. As ducks appear to be asymptomatic to many influenza viruses and since IL1β is an important factor in the pathogenesis of many diseases, further investigations into the role of IL1β may be beneficial (Dinarello, 1998).

1.9.3 *Interleukin18*

In 1989, the proinflammatory cytokine interleukin18 (IL18) was first described as an IFNγ inducing factor (IGIF) (Nakamura *et al.,* 1989). It was later purified from LPS stimulated mice liver cells and termed IL18 (Okamura *et al.,* 1995; Sims *et al.,* 2001). Mammalian IL18 is a product of activated macrophages, Kupffer cells and dendritic cells (DC), but is also expressed in osteoblats, keratinocytes, astrocytes and microglias (Okamura *et al.,* 1995). IL18 acts to enhance the cytotoxic activity of CD8 T cells (Dao *et al.,* 1996) and NK cells (Tsutsui *et al.,* 1996). IL18 is a potent inducer of IFNγ, but also induces GM-CSF, TNF and IL1, and functions to promote either TH1 or TH2 differentiation depending on the surrounding cytokine milieu (Nakanishi *et al.,* 2001). IL18 acts as a co-stimulant for TH1 cells to augment the production of IL2, GM-CSF, IL2R and induces IL1β and both CXC and CC chemokines (Puren *et al.,* 1998). However, the key mechanism that led to the discovery of IL18 was its ability to induce IFNγ from spleen derived T cells (Okamura *et al.,* 1995). There are a range of suggestions as to how IL18 induces IFN, but one of the major pathways is the synergistic partnership IL18 has with IL12, whereby, a higher level of IFNγ is induced than with either cytokine alone (Okamura *et al.,* 1995). IL18 is considered a member of the IL1 family because it shares similar signalling pathways with other IL1 family proinflammatory cytokines, such as IL1α and IL1β (Dinarello, 1998; Dinarello *et al.,* 1998). Although IL18 is structurally homologous to IL1 and its receptor belongs to the IL1R/TLR family, its function is quite different from that of

IL1. IL18 is important for the functional development of NK cells (Takeda *et al.,* 1998), augmenting the cytolytic activity of NK cells (Okamura *et al.,* 1995) and strongly inducing Fas ligand expression on NK and TH1 cells (Dao *et al.,* 1996). IL18 also activates the cytotoxicity of CD8+ T cells (Kohyama *et al.,* 1998) and with help, stimulates memory CD8+ T cells to proliferate, whereas naïve CD8+ T cells or CD4+ T cells do not proliferate. Furthermore, many of these functions of mammalian IL18 are observed in studies with chicken IL18.

Chicken IL18 cDNA was first identified and cloned by searching an EST database generated from a chicken bursa cell line (Schneider *et al.,* 2000). Chicken IL18 mRNA contains an ORF of 597 nucleotides that code for a 198 amino acid protein, approximately 30% identical to mammalian IL18 (Schneider *et al.,* 2000). Stimulation of chicken splenocytes with IL18 induces IFNγ secretion and proliferation, hence through detection of IFNγ, biological activity can be determined (Puehler *et al.,* 2003). Furthermore, IL18 mediated IFNγ secretion is dependant on TCR co-stimulation (Gobel *et al.,* 2003), closely resembling the observations made of mammalian IL18 (Nakanishi *et al.,* 2001). This indicates that naïve cells need co-stimulatory signals to become IL18 sensitive (Gobel *et al.,* 2003). IL18 is also a potent activator of TH cells. Following infection, cytokine secretion is induced as well as T cell proliferation and MHC II antigen up-regulation. In avian species, IL18 appears well conserved. The duck IL18 encoding sequence shares 85.3% similarity to the chicken equivalent at the nucleotide level. A His-duck IL18 fusion protein was recognized in western blots by mAbs against duck and chicken IL18, but not by mAb against human IL18 (Chen *et al.,* 2008). However, little work has been done to date on the biological activity of duck IL18, indicating a paucity of knowledge in what is a key cytokine within the inflammatory response.

1.9.4 *Interleukin6*

Interleukin6 (IL6) is prominent amongst proinflammatory cytokines for its diversity of biological activity. This is exemplified by the long list of alternative names given to IL6, such as B cell differentiation factor (BCDF), B cell stimulation factor (BSF2),

hepatocyte-stimulating factor (HSF), hybridoma/plasmacytoma growth factor (HPGF), cytotoxic T cell differentiation factor (CDF), 26K and IFNβ2. Uniquely, IL6 was cloned, almost inadvertently, long before the discovery of its major biological activities were confirmed. In an effort to clone IFNβ cDNA from human fibroblasts, Weissenbach *et al*., (1980) isolated a 26-kDa protein they termed IFNβ2 because of its antiviral activity. A second, more conventional path to IL6 started from the observation that activated T cells produce a late acting B cell differentiation factor capable of inducing immunoglobulin (Ig) production in activated B cells (Teranishi *et al.,* 1982). This molecule, termed BSF2 and cloned by Hirano *et al*., (1986) turned out to be identical to IFNβ2. The third line of research, which resulted in the identification of IL6, originated from the study of growth factors for B cell hybridomas. The existence of these factors had been known for quite some time but never successfully characterized (Van Snick, 1990). A cohort of separate researchers purified hybridoma/plasmacytoma growth factor (HPGF), and the cloning of HPGF in mouse and human cell lines established its identity as identical to the earlier discovered IFNβ2 and BSF2. This led to the renaming of IFNβ2, BSF2, HSF and HPGF as IL6, seemingly ending the saga of IL6 identification and characterization (Metcalf, 1974; Van Damme *et al.,* 1987; Van Snick *et al.,* 1988). However, later it was discovered that anti-IL6 antibodies (raised against human fibroblast-derived IFNβ) block the activity of the monocyte derived protein HSF, implicating IL6 in the regulation of the acute phase response (Gauldie *et al.,* 1987). Furthermore, IL6 has been shown to be active in hematopoises (Ikebuchi *et al.,* 1987; Wong *et al.,* 1988) and as a cytotoxic T cell differentiation factor (CDF) (Takai *et al.,* 1988). Nevertheless, whilst the role of IL6 appears mostly proinflammatory, IL6 may also promote the development of the TH2 immune response by inducing IL4 synthesis (Rincon *et al.,* 1997) and can inhibit TH1 differentiation by upregulating the suppressor of cytokine signalling 1 (SOCS-1) (Diehl *et al.,* 2000).

IL6 production has been documented in a wide variety of cells. A non-exhaustive list includes T and B lymphocytes (Hirano *et al.,* 1986), endothelial cells (Corbel *et al.,* 1984), monocytes/macrophages (Aarden *et al.,* 1987), fibroblasts (Weissenbach *et al.,* 1980) and a variety of tumor cell lines. IL6 is not usually produced constitutively by

normal cells, however, its expression is readily upregulated during viral infections (Frei *et al.,* 1989). In addition, a variety of cytokines, including IL1, TNF, either alone of in concert with IFNγ (Sanceau *et al.,* 1989), IL3, GM-CSF, and platelet derived growth factor (PDGF) induce IL6 production (Van Snick, 1990; Staeheli *et al.,* 2001). In the chicken, full length IL6 cDNA encodes a protein with 35% amino acid identity to human IL6 and recombinant chicken IL6 induced proliferation in the IL6 dependant murine hybridoma cell line 7TD1 (Van Snick *et al.,* 1986). In contrast only a part of the duck mRNA transcript for IL6 has been discovered. However, since the early discovery of IL6 in chickens, a number of studies have shown that avian cytokines exhibit conservation of genetic location, gene structure and regulatory sequences with their mammalian orthologues (Kaiser *et al.,* 1998; Kaiser *et al.,* 1999; Kaiser *et al.,* 2004). Therefore, even without the full duck genome, this conservation of IL6 across the species may help in the identification and characterisation of IL6 in the duck.

1.9.5 *Interleukin6 signalling*

IL6 signals through a cell surface receptor complex consisting of two distinct membrane bound glycoproteins, an 80 kDa cognate receptor subunit (IL6R) and a 130 kDa signal transducing element (gp130) (Yasukawa *et al.,* 1992). Expression of this trans-membrane spanning gp130 is found in almost all organs (Saito *et al.,* 1992), whilst IL6R is limited to predominantly hepatocytes and leukocytes populations. However, there is also a soluble form of the IL6R (sIL6R) that has been characterised in humans and mice which is binds gp130 with a similar affinity (Yamasaki *et al.,* 1988; Peters *et al.,* 1996). IL6 signalling is facilitated through the homodimerization of gp130 to the ligand receptor complex, with intracellular signalling then triggered by the activation of gp130 associated kinases (Gerhartz *et al.,* 1996). The Janus tyrosine kinases (JAK) and their binding partners, transcription factors of the STAT family are the major mediators of signal transduction (Heinrich *et al.,* 1998b). This signalling through the STAT pathway is a feature shared with the IFNs and many other cytokines and growth factors (Stahl *et al.,* 1994). The JAK activation causes the cytoplasmic tail of gp130 to become phosphorylated, and subsequently the STATs

also become phosphorylated, forming dimmers which translocate to the nucleus where they regulate transcription of IL6 (Heinrich *et al.,* 1998b).

IL6 is a pleiotropic cytokine, affecting the responses of other proinflammatory cytokines, acute phase molecules and reactive oxygen intermediates (Ma *et al.,* 1997; Luchtefeld *et al.,* 2011). The action of IL6 on a variety of immune pathways makes IL6 crucial to the host immune response against viral infection. During viral infection, IL6 is produced by many different cells, but endothelial cells, fibroblasts and monocyte/macrophages are probably the major sources during systemic inflammation (Akira *et al.,* 1992). The recent outbreaks of H5N1 influenza have also highlighted the role of IL6 in hypercytokinemia (Michaelis *et al.,* 2010; Hayashi *et al.,* 2011). In view of the wide range of biological activities IL6 has on various target cells, dysregulated overproduction of IL6 can cause severe inflammation through its promotion of inflammatory cytokines and the acute phase response. Furthermore, IL6 has been shown to be elevated in various cancers (Trikha *et al.,* 2003) and implicated in a variety of inflammatory conditions (Nishimoto *et al.,* 2004). Therefore, IL6 has become a target molecule whose actions could be blocked to help alleviate severe inflammation.

Most inhibitory molecules of IL6 seem to be targeted at either its receptor binding complex or the IL6 signalling pathway. Two drugs for the treatment of inflammatory diseases, Madindoline-A and Galiellalactone, have been used to reduce systemic levels of IL6. Omura *et al*., (1996) reported the isolation of an indole alkaloid diastereomer termed Madindoline-A (Mad-A) which works to block the cellular effects of IL6. Mad-A inhibits IL6 signalling by binding to gp130 and interfering with the homodimerization of its receptor complex (Fig. 1.5). Mad-A has been used in mice models investigating inflammation from arthritis and cancer. Furthermore, Mad-A has been shown to effectively inhibit the secretion of IL6 synthezised proteins, like Serum Amyloid A, an acute phase protein induced during inflammation (Hayashi *et al.,* 2002b). Galiellalactone (Gal), a tetrahydro-isobenzofuranone, has also been described as an inhibitor of IL6 signalling (Kushner, 1993; Weidler *et al.,* 2000). In contrast to Mad A, Gal has been identified as an inhibitor of the IL6 dependant

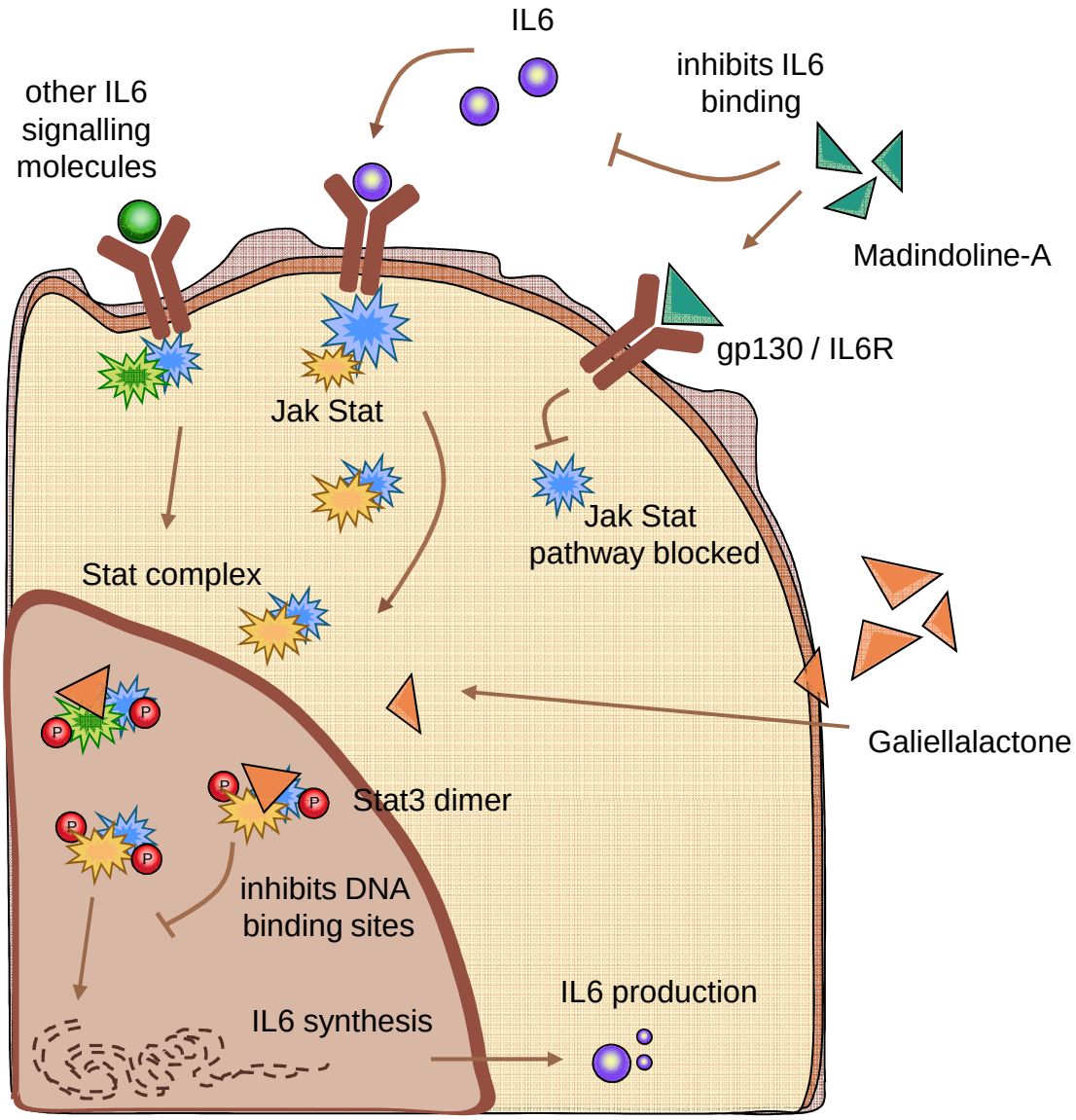

Figure 1.5. IL6 inhibition through the blocking of the IL6R and IL6 signaling pathway by Madindoline-A and Galiellalactone. The chemical compounds Mad-A and Gal block the signaling of IL6 through two methods. Mad-A competitively binds to the IL6R and gp130 complex to inhibit IL6 binding. Gal works through inhibiting the binding of the activated Stat3 dimer complex to its DNA binding sites which would normally signal RNA transduction.

JAK/STAT signalling cascade, whereby Gal binds to STAT (Weidler *et al.,* 2000) to suppress the signal cascade, as shown in Fig. 1.5. Gal has been used in numerous mouse models aimed at inhibiting tumorigenesis and for other STAT mediated diseases (Hellsten *et al.,* 2008). The effect these compounds may have in the chicken is unknown, but both Mad-A and Gal are possible therapeutics for targeting excessive inflammation during viral infection.

1.10 Acute phase reactants and reactive nitrogen intermediates

1.10.1 *Serum Amyloid A*

Serum Amyloid A (SAA) protein is an acute phase reactant in many species. The acute phase response (APR) comprises an increase in hepatic and plasma concentration of a group of proteins involved in the inflammatory process as mediators, inhibitors and immune regulators (Kushner *et al.,* 1981; Whicher *et al.,* 1985). SAA makes up part of the proinflammatory acute phase response, in concert with other molecules, such as C-reactive protein (CRP) and Alpha-1-acid glycoprotein (AGP). These act to restore physiological homeostasis at the site of injury and suppress any negative feedback of the acute reactants. SAA is secreted mainly by hepatocytes and its concentration significantly increases in the blood during an inflammatory response (Sipe, 1992; Nakayama *et al.,* 1993). Recent studies have also suggested the extrahepatic production of SAA from other cell types, such as macrophages (Upragarin *et al.,* 2005). SAA is a known precursor of amyloid A, which causes serious complications in chronic inflammatory diseases. This potent acute phase response is regulated by proinflammatory cytokines, such as IL1 and IL6 (Sztein *et al.,* 1981; Conti *et al.,* 1995), whereby elevated levels of these cytokines can induce SAA production. The first evidence for IL6 as an inducer of SAA came from work with human liver cells (Baumann *et al.,* 1987) which, when stimulated with a combination of IL1 and IL6 showed increased SAA production. Furthermore it has been observed that antiIL6 receptor monoclonal antibody can completely inhibit the induction of SAA, providing strong evidence for the induction of the APR by

increased IL6 expression (Hagihara *et al.,* 2004). The pathway through which SAA is induced may be dependant on the type of SAA being produced. SAA is encoded by several genes either induced or constitutively expressed and in humans four SAA genes have been described, SAA1 to SAA4 (Whitehead *et al.,* 1992). SAA1 and SAA2 encode the inducible form of SAA expressed mainly in the liver, whilst SAA4 encodes constitutive SAA expressed in a range of tissue (Benditt *et al.,* 1989; Meek *et al.,* 1994). In birds only one SAA gene has been described (Guo *et al.,* 1996), although it has high homology with SAA orthologues (Ovelgonne *et al.,* 2001).

The production of SAA during viral infections has been linked to the inflammatory response and also to the presence of fever and hypoxia, which leads to increased myocardial demands. Raised SAA concentrations have been found in acute serum samples from patients with cytomegalovirus, herpes simplex, rubella, measles, hepatitis and other viral infections (Shainkin-Kestenbaum *et al.,* 1982; Miwata *et al.,* 1993). Falsey *et al*., (2001) demonstrated that adults with influenza A have an increase in the levels of acute phase reactants. The inflammatory response in these patients may promote an increased induction of SAA, but there is also evidence that respiratory infection is linked with ischemic events. SAA was increased along with CRP concentration in infected influenza patients (Whicher *et al.,* 1985) and also in horses suffering equine influenza H3N8 (Hulten *et al.,* 1999). The plasma clearance of SAA is very quick in comparison to other acute phase proteins, and levels therefore, decrease soon after inflammation has subsided (Tape *et al.,* 1990). These characteristics make SAA well suited for real-time monitoring of inflammatory activity.

1.10.2 *Nitric oxide and inducible nitric oxide synthase*

Nitric oxide (NO) is an important reactive oxygen intermediate (ROI) which serves as a messenger molecule regulating immune function, blood vessel dilation and acts as a neurotransmitter in the brain and nervous system. NO was first appreciated as a mediator of macrophage function, whereby, macrophage-derived NO had an antimicrobial and tumoricidal effects (Nathan *et al.,* 1991). NO is generated by the

enzyme nitric oxide synthase (NOS), which catalyses the biosynthesis of NO and has been described in a range of tissues (Hom *et al.,* 1995; Wong *et al.,* 1996; Hickey *et al.,* 2002). Currently, a number of distinct NOS isoforms have been characterised in mammals. They are known as the neuronal isoform (nNOS), inducible isoform (iNOS) and endothelial isoform (eNOS) (Guan *et al.,* 2007). The two constitutive forms, nNOS and eNOS are activated by and dependant on changes in intracellular calcium, (Wong *et al.,* 2006) whereas iNOS is calcium independent due to the apparent tight binding of the calmodulin subunit in iNOS (Venema *et al.,* 1996). It is this iNOS form which serves as a key molecule in combating viral infection, acting as mediator of apoptosis and the acute phase protein response. Generally, in resting cells, iNOS is absent, however, it is strongly induced by cytokines and other immunological stimuli (Bogdan *et al.,* 2000). Regulation of iNOS takes place at the transcriptional and post-transcriptional level, involving a number of signal transduction pathways and molecules, including, STAT1/JAK1/IRF-1/NF-κB (MacMicking *et al.,* 1997b). A notable feature of iNOS is its prominent regulation by inflammatory cytokines such as IFNγ and IL6, or inhibitory cytokines, such as IL10 (MacMicking *et al.,* 1997a). More than 30 cytokines or cytokine-like factors have so far been described that increase or inhibit the expression of iNOS activity in immune cells (Diefenbach *et al.,* 1998; Bogdan *et al.,* 2000).

Although the antimicrobial activity of NO has been well documented for bacteria, NO may have the opposite effect during viral infections. During infection, iNOS can become dysregulated, producing an excessive amount of NO for long periods, which allows the generation of peroxynitrite through the coupling of NO with superoxide (Akaike *et al.,* 2000) and promotes inflammation (Stuehr *et al.,* 1992). In this manner iNOS is thought to be responsible for oxidative tissue injury and may also affect a host immune response with immunopathological consequences. Karupiah *et al*., (1998) observed that although iNOS mediates some antiviral effects of IFNγ, during influenza infection, over production of NO has a suppressive effect on Th1 derived immune responses. The activity of NO against other viruses remains unclear, however there are reports of an antiviral role for iNOS in HSV (Croen, 1993), Rabies (Karupiah *et al.,* 1993) and Sendai virus (Akaike *et al.,* 2000).

Whilst there has been considerable research on the biological functions of NO and the regulation of iNOS in humans and mice (Nathan *et al.,* 1994), little is known about iNOS in chickens and ducks. Chicken iNOS was cloned from a macrophage cell line and is highly conserved with respect to the critical cofactor binding sequences within the gene (Lin *et al.,* 1996). The recent cloning of duck iNOS from influenza infected spleen and liver tissue now allows the further investigation of the role of this immune gene (Burggraaf *et al*., 2011). Given the important role iNOS has in other species and its association with viral infection, whether iNOS contributes to an elevated inflammatory response during influenza infection in chickens and ducks warrants further study.

1.11 Hypercytokinemia

Hypercytokinemia or 'cytokine storm' is the manifestation of an inappropriate immune response in reply to a recognized pathogen. This systemic expression of a healthy but unbridled immune system results in the upregulation of a myriad of inflammatory mediators (Clark, 2007). Due to the pronounced elevation of cytokines during HPAIV infection, a cytokine storm has been widely suggested as a contributing factor for the fatal outcome of his disease. The multiple functions of cytokines potentially allow them to affect host survival both positively and negatively. During a cytokine storm damage to host cells occurs through either hyperacute production or persistent chronic over production of this immune response and is highlighted in a range of diseases (Henter *et al.,* 1991; Cheung *et al.,* 2002; Makhija *et al.,* 2002). In avian influenza a rapid degradation of the patient's organs, predominantly the lungs, systemic inflammation and intravascular coagulation are consequential of a cytokine storm. Hypercytokenemia and haemophagocytic syndrome, invokes the release of many different inflammatory mediators; primarily cytokines but, also free radicals and serum associated factors. Both proinflammatory cytokines, such as TNF, IFN, IL1β, IL6 and IL18 and anti-inflammatory cytokines, interleukin10 (IL10), and interleukin 1 receptor antagonist are elevated in the serum of patients experiencing a cytokine storm (Wong *et al.,* 2001; Takaoka *et al.,* 2006; de

Jong *et al.,* 2006b). An illustration of how a cytokine storm is triggered is shown in Fig. 1.3.

When the immune system is fighting pathogens, cytokines signal immune cells such as T cells and macrophages to travel to the site of infection. In addition, cytokines activate those immune cells, which in turn stimulate more cytokines (Cheung *et al.,* 2002; Chan *et al.,* 2005). Cytokines are activated through their highly specific cell surface receptors and most show multiple functional activities on a variety of target cells (Makhija *et al.,* 2002). Furthermore, many cytokines share similar biological effects and in the absence of any one cytokine, others may fill the void. Cytokines are produced in response to stimuli via receptor induced pathways. Under healthy circumstances cytokine secretion is a closely regulated process. In some instances when viral infection is severe, regulation of this signalling becomes uncontrolled, leading to a state of cytokine dysregulation, whereby too many immune cells are activated in a single place (Pang *et al.,* 2007). The precise reason for this is not entirely understood, but may be caused by an exaggerated response when the immune system encounters a new and highly pathogenic invader such as H5N1 influenza viruses (Cilloniz *et al.,* 2010). The resulting hypercytokinemia has the potential to do significant damage to body tissues and organs. Often sufferers of H5N1 disease present with a primary viral pneumonia, lymphopenia and syndromes of acute respiratory distress and multiple organ dysfunction (MODS) (Cheung *et al.,* 2002). If a cytokine storm occurs in the lungs of a patient for example, fluids and immune cells such as macrophages may accumulate and eventually block off the airways, potentially resulting in death (de Jong *et al.,* 2006b).

Some conjecture has arisen, however, over the blame attributed to cytokines in the pathogenesis of H5N1 infection. Investigations by Salomon *et al*., (2007) and to a lesser degree Szretter *et al*., (2006) suggest that cytokine inhibition does not protect against death and therefore, therapies that target the virus rather than cytokines may be preferable. Salomon *et al*., (2007), purport that the key proinflammatory cytokines, IL-6, TNF and the chemokine ligand CCL2, are not integral to the severity witnessed during infection (Salomon *et al.,* 2007), as mice deficient in hallmark inflammatory

cytokines succumb just as readily as wild type mice to H5N1 infection. The caveats of this study, however, are two fold. Initially, proinflammatory cytokines were only knocked out independently during the study. Given the redundancy of cytokine actions it is unrealistic to expect that inhibition of one single cytokine will have a profound effect on such a complex process as inflammation (Clark, 2007). Secondly, glucocorticoids were used to suppress the overall cytokine response and again the treatment did little to impede H5N1 pathogenicity. However, the authors themselves concede that there is only a narrow time frame in which these drugs are effective and research by Liberman *et al*., (2007) asserts that glucocorticoids only suppress the transcription of some of the proinflammatory cytokines. In separate studies there is also evidence that during pulmonary infections cytokine production is mainly restricted to the lung and it seems that diffusion of cytokines across the blood alveolar barrier is limited (Van Reeth *et al.,* 2002). As a result the most concentrated area of cytokine infiltration is in the lung, whilst plasma cytokine levels are generally low. Whether this is the case during H5N1 infection in chickens and ducks is yet to be investigated, although already some limited data suggests that ducks in particular have increased cytokine levels in their brain and heart. With these studies in mind it is clear that a better understanding of this complex interplay between cytokines, chemokines and the inflammatory response to influenza virus infection is needed. Knowledge of the interaction between cytokines and their mediators may give us insight into methods for controlling the appearance of complications and reduce the tissue damage associated with hypercytokinemia and influenza infection.

1.12 Vaccines and therapeutic approaches against influenza

New strategies to control AI are of paramount importance to help protect humans and poultry from influenza infection. Understanding the responses to viral infection in the avian immune system is of increasing importance and may offer new opportunities to intervene in the immune progression and potentially enhance an organism's ability to counter a viral challenge. Efficacious vaccines, when used in a comprehensive program can help reduce disease, viral shedding, and transmission to susceptible populations (Kapczynski *et al.,* 2009). However, conventional vaccines designed

around immunisation with HA and NA antigens may have limited routine use in chickens and ducks. This may be due to vaccine costs, efficiency and poor surveillance practices (Lee *et al.,* 2005a). Furthermore, despite protection, viral shedding may still be prevalent and spread to uninfected flocks, undermining the usefulness of this strategy (Lee *et al.,* 2005a).

The production of influenza vaccines also presents several challenges. As the influenza virus mutates frequently, vaccines require annual updates (Subbarao *et al.,* 2007) and the development of new vaccines depends upon responding to circulating strains, resulting in a considerable lag time (Kandel *et al.,* 2005). The current technique for producing influenza vaccine is by inoculation of live influenza virus into 10 day old embryonated chicken eggs. This inoculated vaccine virus is incubated for 2 days to allow viral replication (Hoffmann *et al.,* 2002). However, not all influenza viruses replicate in eggs in a manner which is conducive to efficient vaccine production (Horimoto *et al.,* 2006). Wildtype H5N1 viruses in particularly are virulent in poultry and therefore impede vaccine yields. Additionally, the availability of chicken eggs could be reduced during an outbreak of Influenza in poultry, creating supply problems for vaccine production (Subbarao *et al.,* 2007).

Current influenza antiviral agents reduce viral impact by blocking critical aspects of viral replication. Drugs such as amantadine and rimantadine, use adamantine compounds to interfere with the M proteins role in viral uncoating and genome release (Cox *et al.,* 1999). Other types of antivirals, such as sialic acid analogues, like relenza and tamiflu, are NA inhibitors and block the release of virus from infected cells reducing the spread of virus within the host and suppressing viral shedding (McKimm-Breschkin, 2005). These antivirals however may have limited use as AI resistance to the amantadine compounds (Bright *et al.,* 2005), particularly in China where it has been used considerably in chickens, is evident. Future possibilities for controlling AI may be through the immunomodulation of pathways associated with the innate immune response. Presently, the mechanisms that are associated with reduced pathology and protection against AI infection are poorly characterised. It is clear that activation of the immune system is necessary in order to get an anti-viral

immune response, however, too much activation may lead to immunopathology and progressive disease. There is increasing evidence to suggest that limiting immune activation may be associated with reduced viral replication (Droebner *et al.,* 2008; Zheng *et al.,* 2008; Reemers *et al.,* 2010).

Cytokines have been shown to augment immune activity across livestock species, laying the foundations for them as adjuvants for immuno-therapeutic research (Asif *et al.,* 2004). In the past, the development of effective anti-viral therapeutics for livestock or species which act as intermediate hosts for zoonotic disease, has been hampered due to the lack of effective specific reagents. Following the release of the chicken (Ren *et al.,* 2003) and bovine (Snelling *et al.,* 2007) genomes in 2004 and 2007 respectively, many new targets are now emerging. Researchers can now develop reagents and direct vaccine and/or immuno-therapeutic treatments at specific genetic targets which may impact on disease severity and spread. However, compared to mammals, there is a paucity of knowledge concerning the role of cytokines during influenza infection in the duck and chicken. Recently, more effort has been focussed on cytokine suppression in various human infections and diseases. Drug companies, such as Roche, announced the release of a human IL6 blocker for arthritis patients (Melton *et al.,* 2008), whilst Conaris have developed a gp130 fusion protein to suppress excessive IL6 induced inflammation (Waetzig *et al.,* 2010). The role of new drugs which suppress the inflammatory action of IL6 and IFN may be useful during severe viral infections (Ottolini *et al.,* 2003; Adam *et al.,* 2009) by helping to reduce the tissue damage associated with hypercytokinemia.

1.13 Objectives

Avian H5N1 influenza viruses are increasingly widespread in domestic poultry and wild migratory birds. These viruses are linked with a high rate of mortality and their mechanisms of pathogenesis are poorly understood. New strategies to control these zoonotic viruses and reduce the risk of human influenza are of critical importance. The infection of chickens and ducks varies dramatically in their clinical manifestations of HPAIV induced disease, whilst ducks appear asymptomatic,

chickens suffer a more severe disease. It is hypothesized that the intense hypercytokinemia resulting from H5N1 avian influenza infection is involved in the resultant fatality in chickens and humans. As such, understanding how and why cytokine dysregulation occurs may provide an insight into new strategies for the treatment of avian influenza. Many virulent H5N1 strains with high mortality in chickens and humans, such as Duck/ Indramayu/ BBVW/ 109/2006 isolated from Indonesia, show little in the way of adverse symptoms in other bird populations, such as ducks (Bingham *et al.,* 2009). With this diversity in response, it is critical to compare the host-pathogen relationship in both chickens and ducks and examine the immune response, highlighting the difference between the two. Furthermore, other more recent strains of avian influenza, such as the Muscovy Duck/Vietnam/453/2004 H5N1 strain, appear to be more lethal in ducks (Bingham *et al.,* 2009). It is similarly important to compare the response to these new wild-type viruses to understand the engendered differences in the immune response. The investigation of key cytokines, such as IFNγ, IL1β, IL6 and IL18 will provide an insight into the early proinflammatory response induced during H5N1 infection (Fig. 1.4.). However, other areas of the immune response are also critical in determining the outcome of disease. Viruses are thought to be recognised by pattern recognition receptors and as such it was thought that TLR7 could be an indicator of the initiation of immune genes. Additionally, acute phase response genes such as SAA and the inducible reactive oxygen intermediates like iNOS have a role in inflammation and the host response to infection. Therefore, a better understanding of how these genes are regulated and their role in the inflammatory pathway following H5N1 influenza infection is needed. A broader knowledge of these host-pathogen interactions will help in developing new strategies and approaches for modulating the outcomes of these infections in chickens and ducks.

Chapter 2

Materials and methods

2.1 Animals and husbandry

2.1.1 *Animal stocks*

Five-week-old Pekin ducks were purchased from Luv-a-Duck (Nhill, Victoria, Australia). Four to six-week-old commercial Ross broiler chickens were purchased from Barrters (Bannockburn, Australia). Specific pathogen-free (SPF) hyline white leghorn chicken eggs were purchased from SPAFAS (Woodend, Australia). Birds were individually identified with leg bands and randomly assigned to treatment groups. The ducks and chickens were fed with commercial grower chicken pellets, *ad libidum*. Water troughs that were deep enough for the ducks to float and dabble were placed in each animal biocontainment room. Animal rooms also had a partially enclosed dry retreat with wood shavings for the ducks and chickens to sit on. All animal work was conducted with the approval of the Commonwealth Science and Industrial Research Organisation (CSIRO) – Australian Animal Health Laboratory (AAHL) Animal Ethics Committee (AEC). Experiments using H5N1 virus infection were carried out under AEC applications 1211 and 1255, – "study of pathogenesis of H5N1 influenza in ducks and chickens." Experiments for the modulation of IL6 were carried out under AEC applications 1331 and 1332, - "characterization of the chicken response to H5N1 influenza following modulation of IL6 levels." All procedures were conducted according to the guidelines of the National Health and Medical Research Council as described in the Australian code of practice for the care and use of animals for scientific purposes (National Health and Medical Research Council , 2004).

2.1.2 *Animal rooms and biocontainment*

For H5N1 infection trials each of the groups of pekin ducks and broiler chickens were housed in separate rooms at microbiological security level 3 (PC3). The room

temperature was held at 22°C and the airflow was set at approximately 15 air changes per hour. At virus challenge and afterwards, staff wore disposable overalls, gloves, water-proof boots and breathing air protection. The latter consisted of full-head hoods ventilated with battery-driven filtered air. Seperate from the PC3 facility, SPF eggs were hatched in the small animal facility SPF hatchery and raised in positive pressure flexible plastic isolators. The cloacal temperatures of the ducks and chickens were measured using digital thermometers (Omron, Fishermans Bend, Australia), with a maximum readout of 43.9 °C. During infection the temperature of some ducks exceeded the maximum readout. All collection of tissues, blood and animal experiments were performed with CSIRO AEC approval.

2.2 Nucleic acid techniques

2.2.1 *Bacterial strains and plasmids*

The recombinant plasmids constructed in this study were transformed into *Escherichia coli* (*E. coli*) strain Top10F' or strain DH5α (Invitrogen, Carlsbad, USA). Polymerase chain reaction (PCR) products for sequencing were routinely ligated into the vector pGEM-T® Easy (Promega, Fitchburg, USA) which was prepared by *Eco*RV (Promega, Fitchburg, USA) digestion and the addition of 3' overhangs, allowing direct incorporation of polymerase chain reaction (PCR) products. Vector maps are included in Appendix 1.

2.2.2 *Isolation of genomic DNA*

Genomic DNA (gDNA) was isolated from SPF and commercial Ross chickens (Barrters, Bannockburn, Australia), as well as from Pekin ducks, using a gDNA purification kit (Qiagen, Venlo, Netherlands) in accordance to the manufacturer's instructions. For H5N1 influenza infected tissues, carbide beads were added to samples and with RLT (Qiagen, Venlo, Netherlands) buffer and homogenized, to ensure complete digestion of tissue and inactivation of viral particles.

2.2.3 *Isolation of RNA*

RNA was harvested from tissue samples (fresh or stored in RNA later) and from cell cultures using Tri-reagent (Sigma-Aldrich, St Louis, USA) according to the manufacturer's instructions. For H5N1 influenza virus infected tissues, carbide beads were added to ensure complete digestion of tissue and inactivation of viral particles.

2.2.4 *Purification of RNA samples*

Extracted RNA (2 µg) was made up to 8 µL using diethylpyrocarbonate (DEPC) H_2O and subjected to deoxyribonuclease (DNase) treatment using a DNase-1 kit (Sigma-Aldrich, St Louis, USA) according to the manufacturer's instructions. The DNase-treated RNA was then checked for concentration and purity using a Nano drop (Thermo Scientific, Waltham, USA).

2.2.5 *Reverse transcription PCR (RT-PCR)*

DNase treated RNA was reverse transcribed to complimentary DNA (cDNA) using a reverse transcription kit (Promega, Fitchburg, USA) according to the manufacturer's recommendations. Breifly, to the 2 µg of DNase treated RNA was added: random hexamers (2 µL), 10x reaction buffer (4 µL), $MgCl_2$ (4 µL), RNasin ribonuclease inhibitor (2 µL), dNTP's (4 µL), AMV Reverse transcriptase (1 µL) and nuclease free water to the volume of 30 µL. cDNA was then incubated at 42°C for 1 h followed by a 10 min, 95°C inactivation step. Finally, cDNA was diluted 1:2 or 1:5 with DEPC treated H_2O and stored at -20°C for subsequent analyses.

2.2.6 *Polymerase Chain Reaction (PCR)*

PCR amplification of DNA was routinely performed using an Applied Biosystems DNA Thermocycler 480 (Perkin Elmer, Waltham, USA), in 0.5 mL conical, thin walled EasyStart® PCR tubes (Molecular Bio Products, Waltham, USA) following the manufacturer's guidelines**.** To these tubes, 2 µL of template cDNA (approx 100ng), 1 µL of diluted (100ng/mL) forward and 1 µL of diluted reverse primers were added (Table 2.1.) with 1 µL RED Taq™ DNA polymerase (Sigma-Aldrich, St Louis,

USA). The forward and reverse primer combinations typically flanked the 5' regions of the duplex DNA region of interest. The PCR cycling conditions were as follows:

Step 1	94°C	5 min	
Step 2	94°C	1 min	Step 2 – 4 repeated 35 cycles
Step 3	55°C	1 min	
Step 4	72°C	1 min	
Step 5	72°C	15 min	
Step 6	4°C	stored	

Where expected PCR products were greater than 1 kb, modification of the extention cycle (Step 4) was increased to 1 min multiplied by the number of expected kb. The PCR products were then analysed on a 1.0% (w/v) agarose gel (Section 2.2.8).

2.2.7 *RACE (rapid amplification of cDNA ends) PCR*

The cloning of complete gene sequences occasionally required the use of a RLM 3-prime RACE amplification kit (Ambion, Austin, USA). Nested PCR primers were designed within the gene of interest and synthesis of cDNA for 3' RACE and PCR amplifications was as per manufacturer's instructions except Qiagen Omniscript and Proofstart DNA polymerase were substituted for cDNA synthesis and PCR amplification as described previously (Tachedjian *et al.,* 2006).

2.2.8 *Electropheretic separation of DNA*

DNA samples were routinely resolved on 1 - 1.5% (w/v) agarose in tris-acetic acid, ethylenediaminetetracetic acid (EDTA) (TAE) (Appendix 2) gels. DNA grade agarose was dissolved in 1x TAE buffer with either 0.25 µg/mL ethidium bromide (Bio-Rad, Hercules, USA) or Gel Red™ (Biotium, Hayward, USA) 10000x in DMSO, with 10.0 µL diluted into 100 µL buffer to visualise bands. The molten agarose was poured into gel moulds, using a comb to form wells and allowed to set. After the combs were removed, gels were submerged in a mini gel tank (Owl Seperation Systems, Waltham, USA) and filled with TAE buffer. DNA samples were combined wth 6x loading buffer (Invitrogen, Carlsbad, USA) when required and loaded into gel lanes. To measure band separation, 7 µL of either 1 kb or 1 kb Plus, DNA marker (Fermentas,

Waltham, USA) were used to compare DNA fragments. Samples were electropheresed at ~ 85 V for approximately 45 min using a power supply 200/400 power unit (Pharmacia Gene, St Louis, USA). DNA were visulaised under either UV or filtered blue light.

2.2.9 *PCR product purification*

PCR products were purified by initially separating the DNA bands using gel electropheresis (as above). DNA bands of interest were then excised from the gel (by scalpel) and purified using a microcentrifuge and purification kit according to the manufacturer's instructions (Promega, Fitchburg, USA).

2.2.10 *DNA and RNA quantification*

The quantification of DNA or RNA was determined using a Nano drop® ND-1000 spectrophotometer (Thermo Scientific, Waltham, USA). Routine analyses were carried out by applying a 1 µL sample of DEPC treated H_2O as a blank reference and then measuring 1 µL of each DNA or RNA sample. Acceptable RNA contamination was defined as samples with a 260:280 nm absorbance ratio greater than 1.9.

2.2.11 *Restriction endonuclease digestion*

Restriction enzyme digests were prepared by combining 1 µg of DNA with 10% (v/v) of the appropriate buffer as recommended by the manufacturer (Promega, Fitchburg, USA) into 1.5 mL sterile microcentifuge tubes (Eppendorf, Hamburg, Germany). Volumes were made up to 10 µL by adding milliQ (mQ) H_2O (Millipore, Billerica, USA) after the addition of 0.5 µL of the specific restriction enzyme (Promega, Fitchburg, USA). Digests were incubated at 25°C or 37°C depending on enzyme for 3h. For double digests the volume was increased to 15 µL after addition of 0.5 µL of the second restriction endonuclease. Digested DNA was resolved on a 1% agarose gel (Section 2.2.8) and excised as required (Section 2.2.9).

2.2.12 *Ligations of PCR products into plasmid vectors*

PCR products for cloning or permanent storage were ligated into the plasmid vector pGEM-T® Easy (Promega, Fitchburg, USA) (Section 2.2.1). Typically, a 3:1 insert to vector molar ratio was used and carried out as follows: DNA of interest was combined with 1 µL of vector, 5 µL of ligation buffer and 0.5 µL of T4-DNA ligase (Promega, Fitchburg, USA), made up to a final volume of 10 µL using mQ H_2O. The ligation mixture was then incubated overnight at 4°C ready for sequence analysis or transformation into electrocompetant cells.

2.2.13 *Transformation of E. coli – (electroporation)*

Aliquots of competent *E. coli* cells, (section 2.2.1) stored at -80°C were thawed at 4°C. To individual cell aliquots, 2-2.5 µL of plasmid DNA was added. The cell aliquot was then transferred to a electroporation cuvette (Bio Rad, Hercules, USA) at 4°C and immediately electroporated in a Gene Pulser (Bio Rad, Hercules, USA). Electroporation parameters used were 2.25V, 25µFD capacitance and 200 Ω resistance. Imediately following electroporation 800 µL of LB broth (Appendix 2) pre-warmed to 37°C was added to the cuvette and the total cell suspension was transferred to an eppendorf tube then incubated at 37°C for 1 h with shaking at 250 RPM in a shaking incubator. To select pGEM-T® Easy (Promega, Fitchburg, USA) vector transformants, a 50-100 µL aliquot was plated onto LB agar (Appendix 1) plates containing 100 µg/mL of Ampicillin (Amp) (Progen, Brisbane, Australia), 200 µg/mL of IPTG (Sigma-Aldrich, St Louis, USA) and 20 µg/mL of X-gal (Promega, Fitchburg, USA) and incubated overnight at 37°C. Remaining electroporated cells were stored at 4°C. After overnight incubation, transformants with inserts in pGEM-T® Easy were able to be distinguished as white colonies compared to transformants without inserts which turned blue, selected colonies were then isolated and screened as follows (2.2.14).

2.2.14 *Isolation of plasmid DNA from E. coli*

Small scale DNA isolation was based on an alkali lysis method using QIAprep Spin Miniprep kit (Qiagen, Venlo, Netherlands). Briefly, single white colonies were

isolated and grown in 5 mL LB broth supplemented with Amp 100 μg/mL and incubated at 37°C overnight with 250 RPM shaking. Three mL of bacterial suspensions were transferred in 2 aliquots to microcentrifuge tubes (Eppendorf, Hamburg, Germany) and centrifuged for 1 min at 10,000 RPM in a biofuge (Heraeus, Newport, UK) and supernatants removed by vacuum aspiration. Cell pellets were then resuspended in 250 μL of P1 buffer (Qiagen, Venlo, Netherlands) and plasmid DNA was isolated according to the manufacturer's recommendations (Qiagen, Venlo, Netherlands). The plasmid DNA was eluted firstly in 20 μL of dH_2O and then repeated a second time in 10 μL dH_2O and DNA quantified by Nano drop (Thermo Scientific, Waltham, USA) (Section 2.2.10).

2.3 Sequencing and bioinformatics

2.3.1 *Sequencing*

Plasmid DNA was sequenced at the Micromon DNA sequencing facility, Monash University (Clayton). The method used dye-terminator reactions performed on an ABI integrated Thermal Cycler and then run on an ABI 377 sequencer (Applied Biosystems, Foster City, USA).

2.3.2 *Bioinformatics*

Sequence data was stored and analysed using Clone Manager Professional 9 (Version 9.0). Sequencing comparisons were carried out using Align Plus and Clustal W. To identify existing homologous sequences a queried sequence was loaded into the NCBI BLAST database (National Centre for Biotechnology Information, National Library of Medicine, USA) which enables the alignment of sequences of varying homology.

2.3.3 *Phylogenetic analyses of iNOS*

The iNOS gene from various species was compared and the similarity displayed using a phyogenetic tree. The tree was constructed using BioManager version 2, Australian National Genomic Information Service (ANGIS). The various iNOS sequences were

uploaded to ANGIS and multiple sequence alignments were carried out using the ClustalW program. Subsequently, a tree was produced using the program PROTDIST and this was further analysed with the program SEQBOOT and CONSENSE with this dataset depicted graphically using TREEVIEW. The organisation of the iNOS duck gene was predicted using the SMART analyses software version 4 (Schultz *et al.,* 1998). This program predicts and identifies domains of a given sequence based on other known and characterised protein motifs.

2.4 Virology methods

2.4.1 *Influenza H5N1 virus strains*

Ducks and chickens were challenged with either one of two strains, both originally isolated from ducks. The Vietnamese strain, A/Muscovy duck/Vietnam/453/2004, was isolated from a Muscovy duck in 2004, the Indonesian strain, A/Duck/Indramayu/BBVW/109/2006, was from West Java from 2006 (Bingham *et al.,* 2009). They were both received directly from the country of origin, the former as a Madin-Darby Canine Kidney (MDCK) cell culture supernatant and the latter 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. A total volume of 0.5 mL was inoculated per animal, with each duck and chicken dose containing approximately $10^{7.2}$ median egg infectious doses (EID_{50}) for the Vietnamese strain and $10^{6.0}$ EID_{50} for the Indonesian strain. The LPAIV used was of the H5N3 subtype, A/duck/Victoria/1462/2008. Chickens were similarly inoculated with a dose containing $10^{6.0}$ EID_{50}

2.4.2 *Virus titration assays*

Heart, brain, lung and spleen tissues were ground by mortar and pestle or homoginized by bead beating with carbide beads and 10% weight/volume homogenates in phosphate-buffered saline were prepared. These were titrated in Vero cells from American Type Culture Collection (ATTC CRL-1586). In a previous study (Lowther *et al*., unpublished data) the sensitivity of Vero cells for influenza H5N1

(A/Vietnam/1203/2004) was examined and results were compared with both MDCK cells and eggs, which are the commonly used substrates for the culture of influenza viruses. Vero cells were found to be slightly less sensitive than eggs, but more sensitive than MDCK cells for this H5N1 strain. Flat-bottomed 96-well micro-titre plates (Nunc, Roskilde, Denmark) were seeded with a Vero cell suspension (10^6 cells per mL). 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 CO_2 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 per 0.1 mL ($TCID_{50}$/0.1 mL).

2.4.3 *Calculation of $TCID_{50}$*

The mathematical derivation of viral infectious units ($TCID_{50}$) is based on the method of Reed and Muench (Reed, 1938). This method requires calculating the percentage of positive wells (typically, out of four) for each dilution step. The dilution at the 50% endpoint can then be determined using the Karber formula (Karber G., 1931), mathematically expressed (Log ID_{50} = L-*d* (S-0.5)), where L = negative log lowest dilution, d = difference between log dilution steps and S = the sum of proportions of positive tests. For example if a viral supernatant dilutes out to 10^{-5}

Virus dilution	proportion infected wells
10^{-1}	10/10
10^{-2}	10/10
10^{-3}	10/10
10^{-4}	7/10
10^{-5}	3/10
10^{-6}	0/10

Then Log ID_{50} = L-d (S-0.5) = -1 -1 (3.7 – 0.5)

= - 4.2

The $TCID_{50}$ is normally expressed as log units per inoculum volume. Thus, a 50 µL inoculum has $10^{4.2}$ $TCID_{50}$ or 5.5 log units per mL.

2.4.4 *Virus swabs*

Swabs were thawed from minus 80°C storage and 0.1 mL undiluted swab media was inoculated into the allantoic cavity of three 9-12 day-old Ross commercial chicken eggs (Barrters, Bannockburn, Australia). The embryos were observed for death between 1 and 3 days; allantoic fluids from dead eggs were tested for influenza virus by hemagglutination (HA) test using chicken red blood cells. The swabs were placed back into minus 80°C storage for later titration of positive swabs.

2.5 Cell culture and bioassays

2.5.1 *Primary and continuous cell lines*

Cells were cultured in 75 cm^2 flasks (Corning, Lowell, USA) in a 37°C humidified incubator supplemented with 5% CO_2. The chicken embryonic fibroblast cell line DF1 (ATCC CRL-12203) was maintained in Dulbecco's modified Eagles media (DMEM) (Thermo Scientific, Waltham, USA) supplemented with 10% foetal calf serum (FCS), 2mM glutamine, 1.5% sodium bicarbonate, penicillin (100 U/mL), streptomycin (100 µg/mL) and 10 mM N-2hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), (DMEM-DF1-10 media). The chicken marcrophage cell line HD11 was maintained in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 5% FCS, 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL) and 10 mM HEPES (RPMI 5 media). Cells were routinely passaged as required (approximately every 2-3 days) to maintain optimal growth conditions and harvested by by washing three times in phosphate buffered saline-A (PBSA) (Appendix 2) and then incubating for 5 min at 37°C with 3 mL of trypsin (Gibco Invitrogen, Carlsbad, USA) and collected into approximately 3 mL of cell specific media. Cells were then centrefuged at $800g_{max}$ then resuspended in 1 mL of media and counted (Section 2.5.4). New 75 cm^2 flasks were seeded with 3 x 10^6 cells

for future use. Cells were seeded 4 x 10^5 into 12-well plates (Nunc, Roskilde, Denmark) or 1 x 10^6 for 6-well plates and settled 24 h prior to treatment.

2.5.2 *Preparation of peripheral blood mononuclear cells (PBMCs) and splenocytes*

The blood and spleen were removed from 4-6 week old animals. The spleen was placed on a sterile 200 μm sieve and minced using a syringe plunger into 10mL of DMEM (Appendix 2) containing 5% (v/v) FCS to prepare single cell suspensions. The single cell suspensions of leukocytes were dispersed through a 70 μm strainer into Petri dishes containing DMEM. Suspensions were layered over lymphoprep (Nicomed Pharma, Oslo, Norway) and centrifuged at $1500g_{max}$ for 15 min. Mononuclear cells at interface were collected, washed twice in 25 mL PBSA and then resuspended in 10 mL of DMEM media supplemented with 10% FCS. Following trypan blue staining to confirm viability, cells were counted and seeded, typically 1 x 10^6 into 12-well plates (Nunc, Roskilde, Denmark) and settled overnight prior to activation.

2.5.3 *Cell activation*

Mitogens for cytokine activation and TLR stimulation included Poly(I:C) (Invitrogen, Carlsbad, USA), which was used to mimic dsRNA, IFNγ, which was use to simulate an innate cytokine responses and *E. coli* LPS (Sigma-Aldrich, St Louis, USA), used to stimulate IL6 and mimic bacterial infection. All compounds were stored at -80°C and thawed on ice prior to use. The various compounds were applied following overnight seeding of cells and growing to 80-90% confluence (section 2.5.1).

2.5.4 *Cell counting*

Cells were counted using a haemocytometer following trypan blue staining (Sigma-Aldrich, St Louis, USA) to exclude stained dead cells.

2.5.5 *NO bioassay*

NO production in sera was measured using a NO colorimetric assay kit (Roche, Basel, Switzerland) (cat No. 11756281001). Chicken and duck sera was irradiated and then filtered in a 10000 molecular weight cut off (MWCO) column (Satorious, Gottingen, Germany) (Vivascience cat No. 13239-E). The NO bioassay was conducted per manufacturer's instructions using a positive NO control with a standard curve plotted and samples were measured at 550 nm wavelength (OD_{550}). This assay requires an equal volume of N-1-naphthyl-ethylenediamine 0.1% in distilled water and sulphanilamide 1% in 5% H_3PO_4 with sample sera, in a 96-well plate. Duplicate samples were averaged and compared to a standard curve generated using $NaNO_2$ as a standard.

2.5.6 *Nitrate production Greiss bioassay*

The continuous chicken macrophage-like cell line (HD11) can be used to determine LPS stimulated NO production by measuring the accumulation of nitrite in the supernatant of HD11 cell cultures (Green *et al.,* 1982; Sung *et al.,* 1991). The accumulation of nitrite was measured by culturing HD11 cells with LPS for 24 or 48 h followed by Greiss assay (Migliorini *et al.,* 1991). This assay requires the removal of 100 µL of the supernatant of interest and combining with an equal volume of Griess reagent (1:1 v/v of N-1-naphthyl-ethylenediamine 0.1% in distilled water and sulphanilamide 1% in 5% H_3PO_4) into a 96-well plate and then measuring the absorbance (OD_{540}). Duplicate samples were averaged and compared to a standard curve generated using $NaNO_2$ as a standard.

2.5.7 *IL6 dependant 7TD1 bioassay*

IL6 activity in chicken and duck plasma was determined by the ability to stimulate the proliferation of 7TD1 murine hybridoma cells in a 3H-thymidine incorporation assay based on previously described methods (Frei *et al.,* 1989). Briefly, 7TD1 cells were cultured in DMEM supplemented with 5% FCS, glutamine, 0.55 mM argiarginine, 0.24 mM asparagine, penicillin (100 U)/streptomycin (100µg/mL), and recombinant chicken IL6 (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 tissue culture plates (Nunc, Roskilde, Denmark), then starved 7TD1 cells were added to each well, and plates were incubated at 37° C in a humidified CO_2 incubator for 48 h. For the last 5 hours 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, USA). Filters were placed in plastic pouches with 5 mL scintillant, and radioactivity was measured on a MicroBeta TriLux 1450 β counter (EG&G Wallace, Turku, Finland). The presence of IL6 protein in chicken plasma was further confirmed by inhibition of its activity in the 7TD1 bioassay by the addition of rabbit anti-ChIL6 antisera. Pre-immunization sera were used as a negative control serum.

2.5.8 *Alpha-1-acid glycoprotein (AGP) immunodiffusion assay*

Chicken sera samples were collected 24 h.p.i, and AGP analyzed using a commercially available radial immunodiffusion test (Carditech Services, Hampshire, UK). Agarose trays were impregnated with two standard samples, a low of 250 µg/mL of AGP and a high of 1000 µg/mL of which 5 µL was placed into individual wells, whereby results could provide data for generating a standard curve. The other wells were filled with 5 µL of test sample and incubated overnight in a humid environment at 25°C. The interaction of migrating AGP with anti AGP in the matrix is indicated by the formation of a precipitation ring and concentrations of AGP correlated with the diameter of the ring using the standards and measuring gauge provided.

2.5.9 *Madindoline-A and Galiellalactone administration*

Mad-A (ALX-350-328) and Gal (ALX-350-336) (Alexis biochemicals, Plymouth, USA) were emulsified in ethanol and diluted in PBS for the desired concentration. Chickens were administered a combination of Mad-A/Gal at 0.1, 0.3 or 0.8 mg/kg (0.2 mL total volume) through an intra-peritoneal injection just prior to challenge.

2.6 Quantitative gene analysis

2.6.1 *Quantitative-RTPCR (Q-RTPCR)*

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 (C_T) method to derive fold change gene expression, according to the manufacturer's instructions (Applied Biosystems, Foster City, USA). Primers and probes (Table 2.2) were designed using Primer express software and where possible were designed across intron:exon boundaries. Probes were labelled with the reporter dye carboxyfluorescein (FAM) and the quencher tetramethyl-6-carboxyrhodamine (TAMRA). Briefly, triplicate or more samples were each measured in 25 µL reactions. PCR cycling was performed as follows: 95°C for 15 sec, 61°C for 30 sec and 68°C for 30 sec. 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 $\Delta\Delta C_T$ (Applied Biosystems, Foster City, USA) as follows. Target gene C_T values were normalized to the endogenous control glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), a house-keeping gene, to derive the ΔC_T. This was compared with an untreated calibrator to derive the $\Delta\Delta C_T$ and relative gene quantitation, or fold expression relative to the untreated control (calibrator), was derived using $2^{-\Delta\Delta CT}$.

2.6.2 *Q-RTPCR calculations*

Q-RTPCR data was analysed according to the ABI Prism 7700 recommendations using the comparative C_T method. This method requires that the primer and probe efficiencies are similar for target and control genes. Therefore each primer and probe set was compared for efficiency by plotting C_T scores from five 10 fold dilutions of a relevant sample to generate standard curves. Each standard curve was compared to the endogenous normaliser, GAPDH and then plotted against the log input amount of template. To determine relative gene expression levels the mean C_T score for duplicate samples was first obtained and then a ΔC_T score was calculated by subtracting the mean C_T score of the endogenous normaliser from the mean target

gene scores. The derived scores were then used to calculate a $\Delta\Delta C_T$ score by subtracting the mean ΔC_T experimental reference control from the ΔC_T experimental treatment conditions. Error was expressed as a standard error (SE) of the mean. These values were then plotted using Microsoft Excel.

2.6.3 *Statistical analyses*

Data means and standard errors where calculated using the program Excel (Microsoft, USA). Further statistical analysis was performed using the program GraphPad Prism version 3.03 (GraphPad Software, USA). Two-tailed Mann-Whitney 'U' tests and unpaired, two-tailed 't' tests were used to determine differences between two groups of data. Bonferroni post-hoc tests were carried out when F values obtained in ANOVA were of statistical significance. Experimental values differing significantly from control values are indicated by different levels of significance, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

2.6.4 *Histopathology and immunohistochemistry*

After euthanasia tissue pieces from chickens and ducks were placed into 4% formaldehyde in neutral-buffered saline (Appendix 2). After no more than 2 days of formalin fixation for soft tissues and 21 days for bony tissues, tissues were trimmed and processed into paraffin wax by routine histological methods. Bony tissues were decalcified prior to processing by 7-14 days immersion into a solution of EDTA in neutral-buffered formalin. Sections of tissues were cut onto silanised slides and stained by an immunoperoxidase test. The steps in this process included endogenous peroxidise blocking with 10% H_2O_2 (30% w/v) for 10 min, digestion of the section with 5-7 µg/mL proteinase K for 5 min, application of a specific primary antibody for 1 h, application of horseradish peroxidase-conjugated (HRP) secondary antibody (DAKO Envision, Glostrup, Denmark) for 45 min, colour development using AEC+ substrate chromogen (DAKO Envision, Glostrup, Denmark) for 5-6 min, and finally counterstaining with Mayer's haematoxylin.

2.6.5 *Antibodies used for immunohistochemistry*

For H5N1 viral antigen staining the primary antibody was an anti-H5N1-influenza virus nucleoprotein rabbit polyclonal antiserum, the nucleoprotein antigen used to prepare the antiserum was expressed in a recombinant *E. coli* and purified by transfer to a sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). For iNOS a rabbit polyclonal directed against iNOS (Abcam, Cambridge, UK), ab3523, was used with a secondary (goat anti-rabbit) HRP conjugated antibody (DAKO Envision, Glostrup, Denmark). Duplicate sections were stained with haematoxylin and eosin for visualisation of tissue morphology.

2.6.6 *Microscopy*

After sections were stained and cover slipped, slides were observed using a Leica model DM LB (Leica Microsystems, Wetzlar, Germany) microscope at a range of magnifications (10X, 20X, 40X, 100X). Photographs were taken by attaching a digital camera (Kodak DX7630) to the microscope and scale bars were either = 50 μm or 100 μm, as indicated in the bottom right corner of images.

2.7 Fluorescence-activated cell sorting (FACS) analysis

2.7.1 *Antibody staining for flow cytometry*

Briefly, chicken splenic leukocytes isolated as in Section 2.5.2, were washed in PBSA and resuspended in 200 μL of FACS buffer (0.01 % sodium azide and 2 % FCS in PBSA) (Appendix 2). Approximately 1 x 10^6 cells were aliquoted into 96-well, round bottom plates (Dynex, Sussex, UK). Plates were centrifuged in a Rotina 48R centrifuge at $380g_{max}$ for 1 min and supernatants discarded. Primary antibody solution (25 μL) diluted FACS wash was added to wells and plates were incubated at 4°C for 15 min. FACS wash (200 μL) was then added to each well and samples washed by centrifuging again at $380g_{max}$ for 1 min. Where required, a secondary antibody incubation step was performed as above. Suspensions were analysed by flow cytometry FACS AriaI (Becton Dickinson, Franklin, USA) with a total of 3 x 10^4

cells analysed for each sample. Chicken cells were stained with CD4 (IgG_1, FITC, Clone CT-4), CD8 (IgG_1, R-PE, Clone CT-8), IgM (IgG_{2b}, FITC, Clone M-1) and MHCII (IgG_1, FITC, Clone 2G11) (Southern Biotechnologies, Birmingham, USA).

2.7.2 *Analysis of flow cytometry*

Analysis of data was performed using DiVa Software (Becton Dickinson, Franklin, USA). For the analysis of flow cytometry data, gates were set on chicken lymphocytes in the spleen. Dead and highly granular cells were excluded from data acquisition as determined by forward against side light scatter measurements. A mixture of secondary conjugate antibodies (isotype control) were used as negative controls for each cell population to measure non-specific background antibody staining levels.

2.8 Study designs for animal infection trials

2.8.1 *H5N1 pathogenicity in chickens and ducks with Vietnam453 and Indramyu109 isolates*

The ducks and chickens were each placed into three groups each in separate rooms. The first group, of 15 ducks, was challenged with the Vietnamese strain, the second with the same number of ducks were challenged with the Indonesian strain, the third, of 6 ducks, were uninfected controls. In a similar fashion, 15 chickens were challenged with the Vietnamese strain, 15 chickens challenged with the Indonesian strain and 6 chickens, were uninfected controls. Birds were assigned numbers randomly as shown in Table 2.3. Challenge was administered by placing two drops of inoculum into each eye and nostril, and the remainder was instilled into the mouth. Three birds from each infected group were euthanized, according to a pre-designated schedule, chickens at day 1 or when severe symptoms developed, ducks on each of days 1, 2, 3, 5 and 7 after challenge and tissue samples were taken. The Vietnamese strain induced clinical signs severe enough to warrant euthanasia of some of the ducks prior to their designated dates. Uninfected controls were euthanized coincident with 0

and 7 d.p.i. Cloacal temperatures were taken from each of the challenged ducks, starting before challenge and every day afterwards. Oropharyngeal and cloacal swabs were taken before challenge and every day after challenge; they were placed into 2 mL of transport media (PBS), containing penicillin, streptomycin and gentamycin) and placed into storage at minus 80^{o}C.

2.8.2 *Modulation of IL6 with Mad-A/Gal in chickens administered with E. coli*

The chickens were separated into four groups and housed in two positive pressure isolators. Birds were assigned numbers randomly as shown in Table 2.4. Chickens in the first three groups received 0.1, 0.3 or 0.8 mg/kg of Mad-A/Gal administered by i.p. injection in concert with *E. coli* LPS (2 mg/kg). Chickens in the fourth group were set as a control and received PBSA in conjunction with *E. coli* LPS (2 mg/kg) administered through i.p. injection (0.2 mL total volume). After 24 h all chickens were euthanized through CO_2 asphyxiation and sera, lung, liver, spleen and heart samples were harvested for a range of gene expression analysis experiments.

2.8.3 *Modulation of IL6 with Mad-A/Gal in chickens infected with the Vietnam453 H5N1 influenza isolate*

Chickens were placed into three groups, in separate rooms. The first group, of 6 chickens was administered 0.8 mg/kg of Mad-A/Gal and then challenged with the Vietnamese H5N1 influenza strain. The second group, with the same number of chickens, were not administered Mad-A/Gal, but instead injected with PBS and similarly challenged with the Vietnamese H5N1 influenza strain. The third group, of 6 chickens, were uninfected controls. Birds were assigned numbers randomly as shown in Table 2.5. Challenge was administered by placing two drops of inoculum into each eye and nostril, and the remainder was instilled into the mouth. Chickens were monitored closely during the infection period and mean survival times recorded. Following euthanasia chicken sera, lung, liver, spleen and heart samples were harvested for a range of gene expression analysis experiments.

Table 2.1. List of oligonucleotide sequences used in the cloning of chicken and duck genes

Primer name	Primer type	Sequence 5’ - 3’
ChIFN 1F	Sequencing fwd	ATGACTTGCCAGACTTACAACTTG
ChIFN 2R	Sequencing rev	TTAGCAATTGCATCTCCTCTGAG
DkIFN D1F	Sequencing fwd	AATGACTTGCCAGACCTACTGCTTG
Dk IFN D2R	Sequencing rev	ACAGTAATTGTAGAGAAAC
ChIL1 1F	Sequencing fwd	GAGATGGCGTTCGTTCCCGACCTG
ChIL1 2R	Sequencing rev	AGTCAGCGCCCACTTAGCTTGTAGG
DkIL1 D1F	Sequencing fwd	GAGATGGCGTTCGTTCCCGACCTG
DkIL1 D2R	Sequencing rev	AGTCAGCGCCCACTTAGCTTGTAGG
ChIL6 1F	Sequencing fwd	ATGAACTTCACCGAGGGCTGCGAG
ChIL6 2R	Sequencing rev	TCAGGCACTGAAACTCCTGGTCT
DkIL6 D1F	Degenerative fwd	ATGA**W**C**B**T**NR**CCGA**N**GGC**W**GCGA
DkIL6 D2R	Sequencing rev	TCAGACACTGACACTCCTGGTGT
ChiNOS 1F	Sequencing fwd	AATGCTGTGCCCATGGCAGTTTGCA
ChiNOS 2R	Sequencing rev	TGTTGCCAACATGCTCCTTGAGGTG
ChiNOS 4R	Sequencing rev	TTATATTCTTTTGACTTCATGTGGGA
DkiNOS D5F	Sequencing fwd	ATGCTGTGCCCATGGCAGTTTG
DkiOS D32R	Sequencing rev	GTGGAGTAGTGATATCAAGCAA
DkiNOS D7F	Sequencing fwd	GAATGGTTTAAGGAGTTAGATCTG
DkiNOS D34R	Sequencing rev	TTAATTTGTGCTTGGACTGATGGG
DkiNOS RACE1	RLM-RACE out	GCGAGCACAGAATTAATACGACTCACT
DkiNOS RACE2	RLM-RACE in	CGCGGATCCGAATTAATACGACTCACT
ChSAA 1F	Sequencing fwd	ATGAGGCTCTGTATCTGCTTCGTG
ChSAA 2R	Sequencing rev	TTAGTATTTGCTGGGAAGGCCCG
DkSAA D1F	Sequencing fwd	ATGAGGCTCTGTATCTGCTTCGTG
DkSAA D2R	Sequencing rev	TTAGTATTTGCTGGGAAGGCCCG

Table 2.2. List of probes and primers for Q-RTPCR detection of cytokine mRNAs

Duck cytokine primers

Target RNA	Probe or primer sequence	Exon boundary	Accession no	Ref
GAPDH	Forward primer: 5'-CATGACAACTTTGGCATTGTGGA-3' Reverse primer: 5'- TGGCTGTGATGGCATGGA-3' Probe: (FAM) -CAGTGGTCATAAGACCC	ND	AF339357 AY436595	(Van Tuinen *et al.*, 2001)
IL1β	Forward primer: 5'-AGCGGCACCGAGCC-3' Reverse primer: 5'-GGGTCAGCTCGACGCT-3' Probe: (FAM) -CAGCTGGAGGAAGCC	4/5	DQ393268	(Wu *et al.*, 2007)
IL6	Forward primer: 5'-GGATAGAGAAAATCACCACACACCT-3' Reverse primer: 5'-CCCTCACGGTTTTCTCCATAAATGA-3' Probe: (FAM) -CATCCTCCGAGACTTT	1/2	AB191038	(Kaiser *et al.*, 2003)
IL18	Forward primer: 5'-TGAACATGGCAGCTTTTGAAGATG-3' Reverse primer: 5'-TGTAACAGTGCATGCAGAAGTTCAT-3' Probe: (FAM) -TCCGCTGCCAGATTT		AF336122	(Chen *et al.*, 2008)
IFNγ	Forward primer: 5'-TGTAGCTGATGGCAATCCTGTTTT-3' Reverse primer: 5'-ACAATCTGGCTCAGTATGATCCTTTTTT-3' Probe: (FAM) -CTGGACAGAGAGAAATG	3/4	AF100929	(Huang *et al.*, 2001)
TLR7	Forward primer: 5'-ACTCTGGATTTAAGCAGAAACAACATCT-3' Reverse primer: 5'-GGCATCTCAGGAAATCAAGATCCT-3' Probe: (FAM) - CCCCTCAGACTTCC	1/2	DQ888645 AY940195	(Macdonald *et al.*, 2008)
SAA	Forward primer: 5'-AGAGGACCTGGAGGTGCTT-3' Reverse primer: 5'-CTCACCCTGCTCTGCCA-3' Probe: (FAM) -CCGGGCATCGCTGATC	ND	U59908	(Guo *et al.*, 1996)

Chicken cytokine primers

Target RNA	Probe or primer sequence	Exon boundary	Accession no	Ref
GAPDH	Forward primer: 5'-CCCCAATGTCTCTGTTGTTGAC-3' Reverse primer: 5'-CAGCCTTCACTACCCTCTTGAT-3' Probe: (FAM) -CTTGGCTGGTTTCTCC		K01458	(Panabieres *et al.*, 1984)
IL1β	Forward primer: 5'-AGCGGCACCGAGCC-3' Reverse primer: 5'-GGGTCAGCTCGACGCT-3' Probe: (FAM) -CAGCTGGAGGAAGCC	4/5	NM204524 Y15006	(Weining *et al.*, 1998; Downing *et al.*, 2009)
IL6	Forward primer: 5'-CCTGGCGGCCACGAT-3' Reverse primer: 5'-CGAGTCTGGGATGACCACTTC-3' Probe: (FAM) -CAGATGGTGATAAATCC	2/3	EU170468 GU230779	(Kaiser *et al.*, 2003; Kaiser *et al.*, 2004)
IL18	Forward primer: 5'-TCGACATTCACTGTTACAAAACCA-3' Reverse primer: 5'-ACCTGGACGCTGAATGCAA-3' Probe: (FAM) -CGCGCCTTCAGCAGGGATGC	2/3	NM204608 AJ276025	(Schneider *et al.*, 2000; Xu *et al.*, 2005)
IFNγ	Forward primer: 5'- GGCGTGAAGAAGGTGAAAGATATC-3' Reverse primer: 5'- GCTTTGCGCTGGATTCTCAAG -3' Probe: (FAM) -CAAGCTCCCGATGAACG	3/4	NM205149	(Digby *et al.*, 1995; Kaiser *et al.*, 2003)
TLR7	Forward primer: 5'-TCAGAGGTGGCTGCACAC-3' Reverse primer: 5'-CAACAGTGCATTTGACGTCCTT-3' Probe: (FAM) -CATGATGTACCATTTTCC	1/2	AJ627561	(Philbin *et al.*, 2005)
SAA	Forward primer: 5'-AGAGGACCTGGAGGTGCTT-3' Reverse primer: 5'-CTCACCCTGCTCTGCCA-3' Probe: (FAM) -CCGGGCATCGCTGATC	2/3	AW198579 GU929209	(Ovelgonne *et al.*, 2001)

FAM, 6-carboxyfluorescein; quencher dye (Applied Biosystems).

Table 2.3. Chicken and duck numbers and clinical and pathological signs of infection in H5N1 infection with Vt453 or Ind109 influenza strains.

A/Muscovy duck/Vietnam/453/2004 (Vt453 strain)

A/Duck/Indramayu/BBVW/109/2006 (Ind109 strain)

Chicken identity	Day of death/ euthanasia	Shedding at euthanasia (egg)	Shedding at euthanasia (vero)	Severity of infection
Vt453				
85	1 (18 h)	pos	<0.5	Severe
90	1 (18 h)	pos	<0.5	Severe
80	1 (19 h)	pos	<0.5	Severe
88	1 (20 h)	pos	<0.5	Moderate to severe
76	1 (24 h)	neg	<0.5	Moderate
94	1 (20 h)	pos	<0.5	Moderate to severe
Ind109				
83	1 (24 h)	pos	2.0	Severe
77	1 (24 h)	pos	3.5	Severe
95	1 (24 h)	pos	1.0	Severe
82	1 (24 h)	pos	3.0	Severe
86	1 (25 h)	pos	<0.5	Moderate
93	1 (24 h)	pos	3.25	Moderate to severe
91	1 (25 h)	pos	<0.5	Mild
87	1 (24 h)	pos	4.0	Severe
78	1 (25 h)	pos	1.0	Severe
84	1 (25 h)	pos	2.75	Severe
92	1 (24 h)	pos	1.25	Moderate to severe
89	1 (26 h)	pos	<0.5	Moderate

Egg inoculation - 0.2 mL/egg x 3 of neat swab media

Vero inoculation - 0.1 mL/well x 4 of neat to 10^6

A/Muscovy duck/Vietnam/453/2004 (Vt453 strain)

Duck identity	Day of death/ euthanasia	Maximum temperature (day after challenge)	Temperature at euthanasia	Summary of clinical and pathological signs
1	1	42.9 (1)	42.9	Normal
2	1	42.6 (1)	42.6	Normal
5	1	42.7 (1)	42.7	Normal
6	2	43.3 (2)	43.3	Normal
9	2	43.7 (2)	43.7	Normal
10	2	43.3 (2)	43.3	Normal
42	3	43.7 (2)	43.0	Depressed
44	3	43.4 (2)	43.0	Depressed
80	3	43.1 (2)	42.7	Depressed, weeping eyes
81	5	>43.9 (2)	42.5	Depressed. Pale spots on pancreas
84	5	43.0 (3)	41.9	Depressed
85	4 (D)	43.7 (2)	na	Found dead 4 days after challenge. Pallor of heart
86	4	43.5 (2)	43.2	Depressed, euthanased 3 days before designated time
93	5	>43.9 (4, 5)	>43.9	Depressed, euthanased 2 days before designated time. Pallor of heart
94	5	>43.9 (3)	43.0	Depressed, euthanased 2 days before designated time

A/Duck/Indramayu/BBVW/109/2006 (Ind109 strain)

Duck identity	Day of death/ euthanasia	Maximum temperature (day after challenge)	Temperature at euthanasia	Summary of clinical and pathological signs
3	1	42.8 (1)	42.8	Normal
4	1	42.1 (1)	42.1	Normal
8	1	42.7 (1)	42.7	Normal
41	2	42.6 (2)	42.6	Normal
45	2	42.9 (1)	42.3	Normal
76	2	42.4 (1)	42.3	Normal
79	3	43.6 (3)	43.6	Normal
83	3	42.4 (1)	42.0	Normal
89	3	42.4 (1, 3)	42.4	Normal
90	5	42.9 (1)	42.4	Normal
91	5	42.4 (5)	42.4	Normal
96	5	43.2 (3)	42.9	Normal
97	7	42.6 (1)	41.4	Normal
98	7	>43.9 (4)	41.4	Normal
99	7	42.2 (3)	41.7	Normal

Table 2.4. List of chicken numbers for Mad-A/Gal administration and LPS treatment

Chicken No	Mad-A/Gal treatment	LPS dose	Day of euthanasia
014	none	2.0 mg/kg	day 1
015	none	2.0 mg/kg	day 1
115	none	2.0 mg/kg	day 1
117	none	2.0 mg/kg	day 1
013	0.10 mg/kg	2.0 mg/kg	day 1
019	0.10 mg/kg	2.0 mg/kg	day 1
111	0.10 mg/kg	2.0 mg/kg	day 1
116	0.10 mg/kg	2.0 mg/kg	day 1
010	0.30 mg/kg	2.0 mg/kg	day 1
011	0.30 mg/kg	2.0 mg/kg	day 1
018	0.30 mg/kg	2.0 mg/kg	day 1
114	0.30 mg/kg	2.0 mg/kg	day 1
012	0.75 mg/kg	2.0 mg/kg	day 1
110	0.75 mg/kg	2.0 mg/kg	day 1
118	0.75 mg/kg	2.0 mg/kg	day 1
119	0.75 mg/kg	2.0 mg/kg	day 1
001	none	none	day 1
002	none	none	day 1
003	none	none	day 1
004	none	none	day 1

Table 2.5. List of chicken numbers for Mad-A/Gal treatment during H5N1 infection

Chicken No	Mad-A/Gal	H5N1 virus	Euthanasia	Symptom level
60	none	$10^{5.5}$ EID_{50}	died 24 h.p.i	severe
62	none	$10^{5.5}$ EID_{50}	euth 27 h.p.i	medium
64	none	$10^{5.5}$ EID_{50}	died 26 h.p.i	severe
65	none	$10^{5.5}$ EID_{50}	died 24 h.p.i	severe
68	none	$10^{5.5}$ EID_{50}	died 24 h.p.i	severe
72	none	$10^{5.5}$ EID_{50}	euth 30 h.p.i	mild
47	0.80 mg/kg	$10^{5.5}$ EID_{50}	died 24 h.p.i	severe
51	0.80 mg/kg	$10^{5.5}$ EID_{50}	euth 31 h.p.i	mild
55	0.80 mg/kg	$10^{5.5}$ EID_{50}	euth 27 h.p.i	medium
57	0.80 mg/kg	$10^{5.5}$ EID_{50}	euth 28 h.p.i	medium
63	0.80 mg/kg	$10^{5.5}$ EID_{50}	died 25 h.p.i	severe
73	0.80 mg/kg	$10^{5.5}$ EID_{50}	euth 32 h.p.i	mild
48	none	none	36 h	healthy
49	none	none	36 h	healthy
56	none	none	36 h	healthy
58	none	none	36 h	healthy

Chapter 3

H5N1 avian influenza is associated with elevated cytokine expression and pathogenicity in chickens compared to ducks.

3.1 Introduction

Host-pathogen interactions are critical to the outcome of disease. These interactions set the tone for the subsequent immune response against pathogens through receptors and their downstream regulation of immune genes (Kato *et al.,* 2006; Wang *et al.,* 2007; Loo *et al.,* 2008; Ehrhardt *et al.,* 2009). Recent studies have provided a broader understanding of the immune response to viral infection and the important role cytokines play in response to diseases such as highly pathogenic avian influenza (HPAI) virus (Le Goffic *et al.,* 2007; Kobasa *et al.,* 2007). Moreover, it has been recognised that the cytokine response generated by viral recognition often leads to inflammation at sites of infection (Wang *et al.,* 2008; Ichinohe *et al.,* 2009). It appears that H5N1 influenza infection causes the induction of proinflammatory cytokines, such as ILs, as well as antiviral cytokines like the IFNs (Cheung *et al.,* 2002). These cytokines usually function to abrogate viral replication, (de Jong *et al.,* 2006b; Szretter *et al.,* 2007) however, they may also be associated with disease manifestation (Cheung *et al.,* 2002). Nevertheless, the production of cytokines is essential to the amelioration of viral infection (Wang *et al.,* 2007). Furthermore, an aberrant or dysregulated, overproduction of cytokines may lead to hyperacute inflammation (Cheung *et al.,* 2002).

The contribution of a cytokine storm in causing disease is somewhat unclear, however infections such as dengue fever (Leong *et al.,* 2007; Clark, 2007), malaria (Liao *et al.,* 2004; Clark *et al.,* 2008) and severe acute respiratory syndrome (SARS) (Cameron *et al.,* 2008) appear to promote an aberrant inflammatory cytokine response during

disease onset (Ohno *et al.,* 1996; Huang *et al.,* 2005; Leong *et al.,* 2007; Pang *et al.,* 2007). This cytokine storm may be responsible for the cellular and organ damage associated with infection. In human H5N1 virus infection an increased inflammatory response has been reported and may contribute to the severity of virulence and associated mortality (Tran *et al.,* 2004; de Jong *et al.,* 2005; Chan *et al.,* 2005). Although it is still poorly understood, the clinical signs caused by H5N1 virus in chickens are widespread viremia and systemic inflammation, suggesting that the chicken may also manufacture an overproduction of cytokines in response to H5N1 virus infection (Suzuki *et al.,* 2009).

Outbreaks of H5N1 virus in chickens have proven difficult to control, particularly due to the spread of virus by migratory birds, such as ducks. A high viral load with extensive tissue tropism and the associated immune response appear to be key aspects in the rapid onset of infection (Cheung *et al.,* 2002; Lee *et al.,* 2005b; Isoda *et al.,* 2006; de Jong *et al.,* 2006b; Suzuki *et al.,* 2009). The extremely acute nature of infection in chickens, however, is not commonly shared in ducks. Ducks often appear asymptomatic when infected with identical H5N1 viruses as chickens (Kishida *et al.,* 2005; Jeong *et al.,* 2009) and little is understood about the duck immune response. Nevertheless, it has been observed that H5N1 viruses activate an inflammatory cytokine cascade in many species (Isoda *et al.,* 2006; de Jong *et al.,* 2006b; Suzuki *et al.,* 2009) which may allow virus propagation in an uncontrolled manner (To *et al.,* 2001). Furthermore, cytokines which are upregulated by H5N1 infection, such as IL6 and IFN, are responsible for increasing the levels of acute phase proteins and reactive nitrogen intermediates, which have been shown to contribute to cellular damage during prolonged inflammation (Akira *et al.,* 1992; Wasilenko *et al.,* 2009). These observations have prompted the suggestion that the differing immune response in chickens and ducks is critical for determining the severity of H5N1 influenza infection.

The infection of chickens and ducks varies dramatically in their clinical manifestations with regard to HPAI induced disease. Given that the mechanisms for differences in the disease pathogenesis are still unclear, we investigated the immune

responses in chickens and ducks infected with H5N1 influenza virus. By utilising an infection model with two H5N1 strains with varied pathogenicity, we sought to characterize and compare the cytokine and acute phase response in these birds. The levels of IFNγ, SAA and the interleukins, IL1β, IL6 and IL18 were examined by Q-RTPCR following infection. Similarly, a comparison of inflammatory molecules following infection of chickens with a low pathogenic avian influenza virus (LPAIV) H5N3 virus (Vc1462) was also made. In addition, we aimed to investigate the levels of the pattern recognition receptor, Toll-like receptor 7 (TLR7), as it has been reported that H5N1 viral RNA is recognized by TLRs, which play an important role in activating the innate immune response (Geeraedts *et al.,* 2008). It is hoped that a better understanding of these host-pathogen interactions may be helpful in determining immune modulation strategies aimed at reducing HPAIV induced illness in chickens and humans.

3.2 Results

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

To investigate the pathogenicity of the Vt453 and Ind109 H5N1 viruses in chickens and ducks an analysis of time until mortality was undertaken. Chickens inoculated with Vt453 were the first to succumb to infection, with all chickens dying (or euthanized due to severe symptoms) between 18 to 24 hours post infection (h.p.i,) (Fig. 3.1). Similarly, chickens inoculated with Ind109 died or were euthanized between 24 to 28 h.p.i. Chickens infected with either Vt453 or Ind109 showed clinical signs of infection including weakness, depression, ataxia, swelling of the head, laboured breathing, fever and seizures. In contrast, ducks infected with Vt453 and Ind109 initially showed no signs of disease, except for a mild fever (data not shown). However, between 48 to 72 h.p.i, some ducks infected with the Vt453 strain showed signs of depression and weeping eyes. By 72 to 96 h.p.i, with Vt453, severe disease was observed in some ducks, with neurological symptoms, ataxia and in some cases death (or ducks requiring euthanasia). Ducks infected with the Ind109 strain showed

no signs of infection except for a mild fever. No ducks died from the Ind109 infection and by 168 h.p.i, body temperature had returned to normal.

3.2.2 Chickens appear to have rapid and systemic H5N1 viral replication compared to ducks

To compare the viral replication efficiency between chickens and ducks and to investigate the organs targeted by the virus during infection, tissues were harvested for viral analysis. In chickens, both strains of H5N1 replicated and disseminated to a range of organs. Within 24 h.p.i, chicken lung, spleen and heart tissue had the highest viral titres, between 6 and 7 $\log_{10}$ tissue culture infectious doses ($TCID_{50}$). Furthermore, the brain of chickens contained large amounts of virus, between 5 and 6 $\log_{10}$ $TCID_{50}$ (Fig. 3.2). In contrast, ducks had lower levels of virus over a longer infection time-course and also responded differently to the H5N1 strains. In ducks, the H5N1 Vt453 strain was present in the lung, spleen and heart (4, 2 and 7 $\log_{10}$ $TCID_{50}$ titre values, respectively) between 48 to 72 h.p.i. Both strains of H5N1 were present in the brain, with the highest replication at 72 h.p.i, (between 1 to 2 $\log_{10}$ $TCID_{50}$ of virus). The Ind109 strain, however, had considerably lower titres than the Vt453 in the spleen and heart of ducks with only high replication of virus, between 2 to 3 $\log_{10}$ $TCID_{50}$ in the lung tissue (Fig. 3.2).

3.2.3 H5N1 viral antigen appears systemic in chicken tissues in comparison to duck tissues

In chickens, the histopathology caused by the two viruses appeared to be widespread and systemic in nature. Both Vt453 and Ind109 caused foci of acute necrosis and haemorrhage in a range of tissues. While these lesions were most prevalent in the red pulp of the spleen (Fig. 3.3A), they were also found sporadically in the parenchyma of the liver, lung and kidney, and in the lamina propria of a variety of epithelial tissues (Fig. 3.3). These necrotic foci were particularly prevalent in lymphoid follicles of the bronchioles, turbinates and intestine. In chickens, the bulk of antigen staining was in the endothelium, where it was present in dense quantities in blood vessels of all sizes (Fig. 3.3). Therefore, antigen was present in all vascularised tissues examined.

Antigen was also present in the parenchyma of various tissues. Occasional foci were present in neurons and glial tissue (Fig. 3.3D). In the myocardium antigen was generally present in copious amounts (Fig. 3.3J), while in smooth and skeletal muscle, antigen was less common. In the lung parenchyma, antigen was very dense and, for this reason, it was not clear which cell types were involved. Acinar infection of the pancreas was sparse and when it was found was generally in small foci that spread across acini, antigen staining was predominantly nuclear. In kidneys, glomerular tufts contained dense endothelial antigen; in tubular epithelium it occurred as small foci within neighbouring cells, or occasionally as single whole tubules. Viral antigen was also present in the lamina propria and sub-epithelial layers of various epithelial tissues. Antigen was present in capillary endothelium and in many cases had spread, to varying degrees, to the surrounding connective tissues. However, in chickens, the predominant tissue type infected is the vascular endothelium, ensuring the presence of antigen throughout all vascularised tissues. The origin of this antigen is most probably due to haematogenous spread of virus from an initial site of local replication. Although not obvious histologically due to its peracute progression, endothelial infection would have caused blood vessel damage and acute circulatory collapse.

In ducks, viral antigen staining appeared restricted to fewer sites of infection when compared to chickens, with both virus strains showing similar patterns of tissue distribution. However, the quantities of viral antigen were generally considerably more when ducks were infected with the Vt453 strain. Furthermore, in several of the ducks infected with the Ind109 strain no viral antigen was detected, including all three ducks euthanized at 24 h.p.i and 168 h.p.i.

The Vt453 strain appeared to have a particular predilection for muscle cells of all types. Myocardium was one of the most consistently infected, causing severe, acute, diffuse myocarditis, which was associated with heavy antigen staining (Fig. 3.4J). It also had a significant predilection for skeletal muscle including not only the major muscle masses, but also muscles in the trachea and oesophagus. Antigen was also found in single fibres of smooth muscles of the walls of the gastrointestinal tract and blood vessels (Fig. 3.4C, H, L). When infecting the brain the Vt453 strain caused

random foci of antigen staining in neurons and neuropil. Often these foci were not associated with lesions. Only small to moderate quantities of viral antigen was present in lung tissue, and this was present in single cells scattered within the lung parenchyma. Feather follicles also frequently contained dense viral antigen (Fig. 3.4K). Staining was present in the feather and follicle epithelial layers and in the feather pulp.

The tissue responses associated with Ind109 viral antigen were noticeably more mild than those in ducks infected with the Vietnamese strain (Fig. 3.2 and Fig. 3.4). Ind109 viral antigen was present in most birds in single cells in myocardium, skeletal muscle and smooth muscle, the latter mainly in blood vessel walls (Fig. 3.4J and 3.4L). It was also present in most ducks in connective tissues, including fibrous tissue, chrondocytes and osteoblasts (Fig. 3.4I and 3.4H). Generally, viral antigen was not apparent in duck lymphoid tissues, antigen was found in small amount in epithelial reticular cells of the thymus and bursa, it was found in single cells in one spleen and in a caecal lymphoid follicle of another duck. In the brain, viral antigen occurred in occasional foci in neurons and in neuropil (Fig 3.4D). Occasional foci of antigen were present in pancreas tissue. No antigen was detected in parenchyma of lung, liver and kidney tissues during Ind109 infection of ducks.

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

To determine the expression of cytokines in chickens infected with H5N1 influenza, cytokine levels in the spleen, brain, lung and heart were examined using Q-RTPCR. In chickens infected with H5N1 Vt453 rapid increases in mRNA expression levels of the proinflammatory cytokines (IFNγ, IL1β, IL6 and IL18) were detected 24 h.p.i, when compared to levels in uninfected chickens (Fig. 3.5). IL6 had the largest increases in mRNA expression, with 100 fold increases in heart, 80 fold increases in spleen and 70 fold increases in lung tissues, which were significantly (*$p<0.05$ or **$p<0.01$) increased over the levels of IL6 in infected ducks. The acute phase protein SAA increased in a profile similar to that of IL6, increasing in heart 80 fold, spleen 40 fold and lung 40 fold. Intriguingly, chicken TLR7 levels were significantly (*$p<0.05$)

lower than infected duck TLR7 levels 24 h.p.i in a range of tissues. Chickens infected with Ind109 also had increases in proinflammatory cytokine levels (IFNγ, IL1β, IL6 and IL18) at 24 h.p.i. IL6 levels were increased between 20-40 fold, although not as pronounced as the levels of IL6 observed in chickens infected with Vt453 (Fig. 3.5). Similarly, SAA was increased in heart, brain and lung tissue (40 fold, 10 fold and 20 fold, respectively) of Ind109 infected birds. TLR7 expression did not appear increased in any tissues from chickens infected with the Ind109 strain.

3.2.5 H5N1 infection increases TLR7 expression in ducks, however, inflammatory cytokine levels are higher in infected chickens

Compared to chickens, the initial (24 h.p.i) proinflammatory cytokine gene levels observed in H5N1 infected duck tissues were significantly lower (*$p<0.05$ or **$p<0.01$). Both Vt453 and Ind109 strains of H5N1 induced little change in the levels of IFNγ, IL1β, IL6 and IL18 expression when compared to uninfected ducks at 24 h.p.i. Furthermore, the acute phase gene SAA, showed no apparent change in duck spleen, brain, lung or heart tissues 24 h.p.i (Fig. 3.5). However, TLR7 levels were increased in spleen, brain and heart tissue (between 2-4 fold, *$p<0.05$) at 24 h.p.i. As seen in Fig. 3.6, 24 after H5N1 infection, ducks show increased TLR7 expression in spleen, brain and heart tissue (more than 80 fold increase in heart) when compared to chicken TLR7 levels. As virus titres in ducks increased approaching 72 h.p.i, significant increases (*$p<0.05$ or **$p<0.01$) in some proinflammatory cytokine levels were also observed in duck spleen, brain and heart tissue. In ducks infected with Vt453, at 72 h.p.i, levels of IFNγ were increased 30-40 fold in brain tissue, along with increases of 5-10 fold in IL18 (Fig. 3.7). Both Vt453 and Ind109 strains of H5N1 induced increases of IFNγ, IL1β and IL6 in heart tissue (2-5 fold, 5-15 fold and 15-20 fold, respectively). In spleen tissue IL1β was increased 10 fold compared to the levels observed in uninfected ducks. Importantly, these cytokine levels were still lower than those observed in chickens 24 hours after H5N1 infection.

3.2.6 Elevated IL6 in sera of chickens and ducks infected with H5N1

Since increases in IL6 mRNA levels were observed across a range of chicken tissues during infection, an analysis of the levels of IL6 protein in the sera of infected birds was made. Blood was collected from birds and serum IL6 levels measured in an IL6 dependant cell proliferation bioassay. In the sera of H5N1 Vt453 influenza virus infected chickens, IL6 protein levels were significantly elevated by between 8-10 fold when compared to uninfected chickens (*$p<0.05$). In contrast, IL6 protein levels in serum from Vt453 infected ducks were not increased at 24 h.p.i, and only increased by 2 fold at the apparent peak of infection (72 h.p.i), when compared to levels in uninfected ducks (Fig. 3.5A). To confirm that IL6 protein was responsible for the increased cell proliferation, a neutralising IL6 antibody assay, whereby anti-IL6 antibody was added to infected chicken or duck sera, was conducted. Both chicken and duck IL6 sera levels were lowered in a dose dependant fashion by the addition of the anti IL6 antibody, however, chicken IL6 levels were considerably higher than those observed in the duck (Fig. 3.5B).

3.2.7 Following H5N1 influenza infection, serum IL1β levels are increased in chickens when compared to ducks

In concert with increased serum IL6 levels, the concentration of IL1β protein is also increased in chickens following H5N1 influenza infection. Following the collection of blood, IL1β levels were measured using an ELISA bioassay and at the relative peak of infection, chickens appeared to have 3 fold more IL1β expression than in infected ducks. In ducks, IL1β levels were modestly increased 72 h.p.i, (Fig 3.9).

3.2.8 Immunohistochemistry shows co-localisation of IL6 and H5N1 antigen in chickens

As IL6 levels were significantly increased in a range of tissues and in the sera of H5N1 influenza infected chickens, we sought to assess the expression of IL6 with respect to H5N1 virus localisation. Immunohistochemistry (IHC) for H5N1 (Vt453) antigen (Fig. 3.10B and E) revealed staining in a range of chicken tissues at 24 h.p.i. Typically, H5N1 antigen was prevalent in a wide range of tissues and often associated

with necrotic lesions. In lung tissue (Fig. 3.10B), H5N1 antigen appeared throughout the parenchyma and around the epithelial cells of blood vessels. Serial sections of lung tissue were stained with anti-IL6 antibody (Fig. 3.10C) and showed co-localisation of IL6 with H5N1 antigen. Liver hepatocytes (Fig. 3.10D) and sinusoidal cells (presumably endothelial cells) appeared necrotic and were positive for H5N1 antigen, particularly around the lesions observed (Fig. 3.10E). In serial sections of liver tissue, IHC for IL6 showed a prevalence for staining in the foci of infected lesions confirming elevated IL6 expression being co-localised with H5N1 infected tissue.

3.2.9 H5N3 Vc1462 influenza infection induces cytokine expression in chickens, however, at lower levels than H5N1 infection

To determine if all influenza viruses of the H5 subtype increase proinflammatory cytokine levels in a similar fashion to that observed during HPAIV H5N1 infection, chickens were infected with the LPAI H5N3 Vc1462 virus. Lung and spleen tissues were sampled at the peak of Vc1462 infection (72 h.p.i) and examined using Q-RTPCR for a range of inflammatory cytokines. In the spleen, levels of the inflammatory genes, IFNγ, IL1β, and IL6 were significantly (*$p<0.05$) increased by 5-8 fold when compared to uninfected chickens. Likewise, in the lung of Vc1462 infected chickens IL1β and IL6 levels were significantly increased compared to uninfected birds (Fig. 3.11). A comparison at the peak of influenza infection for the levels of IFNγ, IL1β, IL6 and IL18 in H5N3 infected chickens to levels in H5N1 infected chickens shows there are 5-15 fold higher gene expression levels in H5N1 infected chickens (Fig. 3.12). Similarly, the levels of the acute phase gene, SAA were also 25 fold higher (**$p<0.01$) in the lung during H5N1 virus infection compared to the levels observed during H5N3 virus infection.

3.3 Discussion

Avian H5N1 influenza infections have been associated with a high rate of mortality and a dysregulated cytokine response (de Jong *et al.,* 2006b). However, the

mechanisms of disease pathogenesis are still poorly understood. Furthermore, different H5N1 influenza viruses appear to have significant variation in the severity of disease they cause in different species (Perkins *et al.,* 2003) and are unique from other H5 subtype influenza viruses which have low pathogenicity. It is unclear how this varying pathogenicity occurs, but it may be hypothesised that differing host-pathogen immune interactions generated during H5N1 influenza infection may play a role in disease outcome. In chickens, viral replication occurs rapidly and the immune response appears to culminate in a high production of inflammatory molecules throughout a range of organs. In contrast, ducks appear to suffer a milder disease, with only some strains of H5N1 causing a gradual pathogenesis and a lower rate of fatality (Swayne, 2007; Kim *et al.,* 2009; Brojer *et al.,* 2009; Bingham *et al.,* 2009). We have shown that when chickens are infected with either of two HPAIV (Vt453 or Ind109) strains, an earlier and increased production of proinflammatory molecules are produced in comparison to ducks. Moreover, the infection of chickens with LPAIV, such as the H5N3 (Vc1462) strain induced a cytokine response, however, this response was significantly lower compared to the H5N1 virus. In contrast, ducks infected with H5N1 appear initially to not respond with an increase of proinflammatory molecules, although the levels of the viral sensing receptor TLR7 are increased. Furthermore, ducks appear to produce only a moderate cytokine response later in their infection period corresponding to when viral replication levels reach their peak. These differences in the immune response between chickens and ducks may be critical in the outcome of disease manifestation during H5N1 infection.

Infections with H5N1 typically begin in the upper respiratory tract and lungs of human patients (Kobasa *et al.,* 2007; Mizuguchi *et al.,* 2007). Although different H5N1 isolates may have varying tissue tropism in humans, it would appear that these viruses become systemic in their nature of infection (Gu *et al.,* 2007; Zhang *et al.,* 2009). Similarly, dissemination of virus in chickens is thought to occur rapidly (Katz *et al.,* 2000) after the early infection is established (Das *et al.,* 2008). The mechanism allowing influenza virus to propagate in such an uncontrolled fashion has not been definitively identified, however, it is believed to be multigenic in nature. Virulence studies using H5N1 reassortment viruses have shown that multiple basic amino acids

and the enzymatic cleavage of the HA protein, as well as the presence of a lysine at position 627 of the PB2 protein, are associated with highly virulent H5N1 influenza strains (Bogs *et al.,* 2010). However, other mechanisms may assist viral dissemination, such as vascular leakage and inflammation generated by an excessive immune response. The highest viral loads in infected chickens and therefore greatest inflammatory response, is likely to occur in the lungs, the initial site of infection. We observed that lung tissues of infected chickens demonstrated the highest levels of viral titres, in excesses of 7 $\log_{10}$ $TCID_{50}$, with both strain of H5N1. Nevertheless, virus titres in the spleen, heart and brain tissue were almost as high as those found in the lung. This intense viral replication may be promoted by excessive inflammation, which impacts on the porosity of blood vessels allowing greater viral spread. Swayne *et al.,* (2007) have suggested that this altered permeability may be responsible for edema and hemorrhage, particularly in cardiovascular endothelial cells and is followed by viral replication in multiple organs (Swayne, 2007). This efficient virus replication results in multiple organ failure and death. In chickens, the predominant tissue type infected is the vascular endothelium, ensuring the presence of antigen throughout all vascularised tissues. The origin of this antigen is most probably due to haematogenous spread of virus from an initial site of local replication and this endothelial infection would have caused blood vessel damage and acute circulatory collapse. This is probably one of the likely mechanisms of death in infected chickens. Furthermore, studies of other infectious diseases such as SARS, Hepatitis and Mareks, have identified that altered permeability of blood vessels contributes to pathogenesis, however, whether the mechanism behind this weakening of cellular membranes is driven by inflammation or viral proteins is unclear (Swayne *et al.,* 1988; Liao *et al.,* 2004).

Conversely, ducks had little viral replication early (24 h.p.i) in the infection. This may be due to either the inability of the virus to bind cell surface receptors efficiently and establish an initial infection or due to an effective immune response which limits early viral replication and dissemination. It was not until 48-72 h.p.i that viral titres increased in duck tissues. The H5N1 Vt453 strain in ducks demonstrated a propensity to replicate to higher titres than the Ind109 strain. Vt453 viral titres replicated to their

highest level in duck heart tissue, where replication levels were similar to that in chickens (Fig. 3.2). Furthermore, both the Vt453 and Ind109 strain replicated to similar levels in tissue culture using Vero cells. This data suggests that a high viral load in the heart and lung may be associated with an increase in mortality as only ducks infected with the Vt453 strain died or showed severe symptoms, whilst ducks infected with Ind109 were able to recover from infection. Similarly, recent studies of various H5N1 influenza strains in chickens have suggested that heart failure and the associated inflammation in heart tissue may be a reason why chickens die so quickly following infection (Karpala *et al.,* ; Nakamura *et al.,* 2008).

To help understand this differing pathogenicity generated by H5N1 infection in chickens and ducks, we investigated the expression of key inflammatory molecules in the innate immune response. The levels of cytokines, pattern recognition receptors and acute phase reactants were all investigated following infection. In chickens, both H5N1 viruses caused an intense production of cytokines and acute phase reactants. The proinflammatory cytokines IL1β, IL6 and IL18 were all increased during infection with either H5N1 strain in chickens (Fig 3.5). IL1β and IL6 impact on the regulation of immune molecules produced by epithelial and vascular endothelial cells during infection and have been implicated in the influenza induced pathogenesis of mammals (Skoner *et al.,* 1999; To *et al.,* 2001). During infection in chickens, IL1β and IL6 appear to be systemically increased, with heart, brain, lung and spleen tissues showing elevated levels. This increase in key inflammatory cytokines prior to death has been noted in humans and primates which appear to have a similar symptom severity (Kaiser *et al.,* 2001; Kobasa *et al.,* 2007) during infection. In addition, IL6 has been shown to be released by macrophages and epithelial cells during lung injury (Hierholzer *et al.,* 1998b) and the effects of IL6 have been shown to be synergistic with IL1 and TNF. Furthermore, studies of other zoonotic viruses which induce hypercytokinemia, such as SARS, suggested that IL6 in the lung environment may contribute to disease severity (Cheung *et al.,* 2002; Cheung *et al.,* 2005). Confirming the mRNA expression, levels of IL6 were observed to be increased 8-10 fold in the sera of H5N1 infected chickens (Fig 3.8) in comparison to uninfected chickens. Moreover, IHC supported the observation that IL6 was often present in chicken

tissues with the highest degree of H5N1 antigen, such as the lung. It is tempting to suggest that some of the clinical manifestations associated with H5N1 infection in chickens may be partially due to excess IL6 production as this was often associated with necrotic lesions, such as in liver tissue, which showed a co-localization of IL6 with H5N1 antigen staining (Fig 3.10).

In contrast to chickens, the same proinflammatory genes did not appear to be upregulated during the early infection period of ducks with either H5N1 strain. It was not until 120 h.p.i, that infected duck tissues began to display some increases in their expression of inflammatory cytokines. Ducks infected with the Vt453 H5N1 strain showed the most pronounced changes in cytokine production, however, levels were still lower than those observed in chickens. In the brain of Vt453 infected ducks, IFNγ and IL18 were increased (Fig 3.7), suggesting an increased inflammatory response in this tissue and may be associated with the neurological disorders observed in some ducks. Intriguingly, the levels of viral replication in the brain are comparatively low, suggesting that a direct relationship between high viral levels and high cytokine production is not always the case. In the spleen and heart, IL1β expression levels are elevated in infected ducks, yet, in comparison to chickens these levels are also relatively low. Similarly, with the exception of duck heart tissue, IL6 levels are low in ducks infected with either H5N1 influenza strain. The increased IL6 production in the heart of infected ducks may contribute to the severity of infection in some ducks, however, the levels of IL6 in the sera of infected ducks indicates only a 2-fold increase over that of uninfected ducks. This contrasts markedly to chickens, in which IL6 levels are raised 10-fold in infected sera. The disparity in cytokine production could potentially impact on the downstream regulation of IL6 stimulated genes, contributing to the hyperinflammatory immune response observed in chickens.

High levels of cytokines, such as IL6 and IL1, promote the induction of the acute phase response, which consequently increases inflammation (Ramadori *et al.,* 1988; Nakayama *et al.,* 1993). In chickens, SAA was observed to be elevated between 20 to 70 fold (Fig 3.5). Furthermore, SAA levels correlate with the increased expression of IL6 in tissues during infection with either H5N1 strain. Excessive SAA production

has been shown to have deleterious effects in a number of diseases (Huang *et al.,* 2005), whilst suppression of SAA family members and their receptors, TLRs, can impact accelerated inflammation (Hiratsuka *et al.,* 2008). Conversely, a high production of SAA and an associated increase in IL6 is not observed in infected ducks. This suggests that a combination of increased IL6 and SAA may promote a deleterious inflammatory response in the host following H5N1 infection. However, it is not known through what mechanisms IL6 is being upregulated following H5N1 infection. It is suspected that IL6 may be induced by pattern recognition receptors, which detect influenza viruses (Koyama *et al.,* 2007), such as the TLRs and RIG-like helicases (Lund *et al.,* 2004; Kato *et al.,* 2006; Kobasa *et al.,* 2007; Cilloniz *et al.,* 2009; Barber *et al.,* 2010). Intriguingly, the levels of TLR7 were observed to be increased in H5N1 infected ducks but not in chickens. This may be crucial for both the generation of an antiviral response and the regulation of inflammatory cytokines. Furthermore, studies by Barber *et al*., (2010) suggest that ducks are indeed benefitted by the antiviral action of RIG-I, which the chicken lacks. The over expression of RIG-I in chicken cells improved the response to H5N1 influenza infection. However, the action of RIG-I alone may not be sufficient to prevent severe pathogenesis given that mammals and other avian species with functional RIG-I are still susceptible to many HPAI H5N1 viruses. Therefore, it is likely that other factors may contribute to the increased severity of H5N1 infection observed in chickens. One possible explanation for the comparatively low TLR7 levels in chickens may be due to virus induced suppression of this gene. It has also been observed that the products of immune receptors, such as TLRs, are the target of NS1 gene immunosuppressive activity (Karpala *et al.,* 2008). As chickens had higher viral levels they may also suffer from increased NS1 gene activity (Matikainen *et al.,* 2006; Kato *et al.,* 2006; Kobasa *et al.,* 2007).

Since H5N1 influenza infection causes such high levels of cytokine production in chickens and may contribute to disease severity, we compared this immune response to chickens infected with a low-pathogenic H5N3 influenza virus (Vc1462). Chicken lung and spleen tissue showed modest increases in IFNγ, IL1β, IL6 and SAA gene

expression levels when compared to uninfected chickens (Fig 3.11). However these cytokines appear decreased when compared to the relative increases observed in chickens infected with H5N1 influenza (Fig 3.12). This highlights how quickly chickens are overwhelmed by intense viral replication and the associated cytokine influx following H5N1 influenza infection. Conversely, the cytokine response in ducks, which suffer a less systemic infection, is similar to that of chickens infected with the Vc1462 H5N3 influenza virus. The production of proinflammatory cytokines is low in infected ducks and this lessened inflammatory response may be important in the amelioration of influenza infection. Furthermore, recent studies in H5N1 infected mice treated in a delayed fashion (48 h.p.i) with the antiviral zanamivir showed IL6 levels reduced by 10 percent compared to infected control mice, however, at this dose survival was not improved. In contrast, when H5N1 infected mice were treated with a combination of an antiviral and immunomodulators of cyclooxygenase-2 (COX-2) survival was improved significantly and inflammatory markers decreased by almost half (Zheng *et al.,* 2008). Similarly, Okada *et al.,* (2009), suggested that the administration of ETR-P1, an anti-inflammatory, could protect 10 day old chickens from H5N1 influenza infection (Okada *et al.,* 2009). With this in mind, future work, directed at modulating the levels of IL6 following H5N1 influenza infection may be beneficial for developing potential immunotherapies.

This data provides an insight into the different immune response observed in two avian species and supports the association of high virulence and cytokine production with the mortality in chickens. Understanding the role of these proinflammatory cytokines during influenza infection and the different immune response generated in chickens and ducks may help provide new strategies for the treatment and prevention of avian influenza.

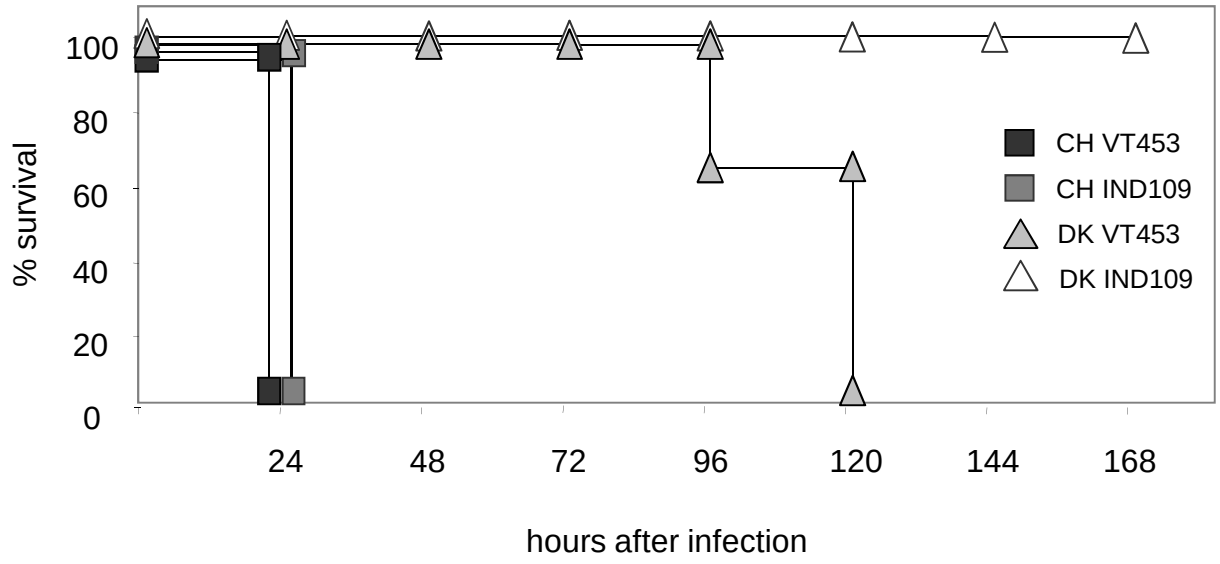

Figure 3.1. H5N1 influenza has increased severity and mortality in chickens compared to ducks. Survival of chickens and ducks after oral-intranasal inoculation with one of two highly pathogenic H5N1 influenza viruses. Graph depicts the percentage survival of chickens (CH) shown as squares or ducks (DK) shown as triangles, inoculated with H5N1 Vt453 or Ind109 influenza virus. Chickens infected with either H5N1 strain needed to be euthanized or succumbed to viral infection within 24 hours of infection. Ducks infected with the Ind109 isolate appear asymptomatic to infection and were observed up to 168 hours post infection. Ducks infected with the Vt453 isolate succumbed to infection between 96 and 120 hours post infection .

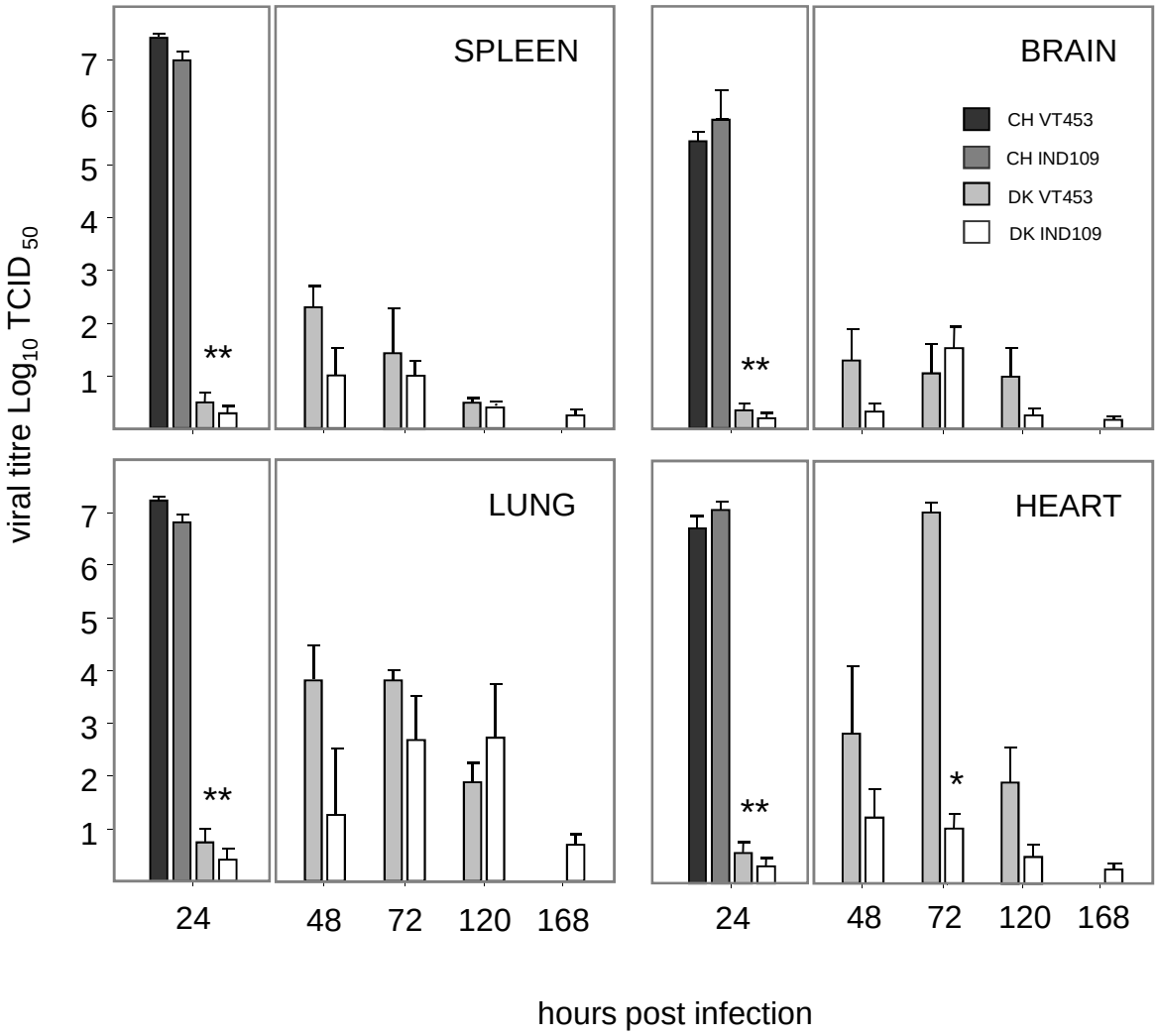

Figure 3.2. Chickens appear to suffer a rapid and systemic H5N1 viral infection compared to ducks. Viral titres in chicken and duck tissues following infection with either H5N1 Vt453 or Ind109 influenza virus. Titres of individual lungs, hearts, spleens and brains were measured in Vero cells. The graph shows the mean virus titres from four birds (log_{10} $TCID_{50}$) at 24, 48, 72, 120 and 168 hours post infection. Chickens have higher levels of both Vt453 and Ind109 H5N1 influenza virus in comparison to ducks. Uninfected controls showed no virus. Data shows mean of 4 birds in each condition; error bars show SE. Single asterisk indicates statistically significant differences of means with $p<0.05$, double asterisk indicates statistically significant differences of means with $p<0.01$.

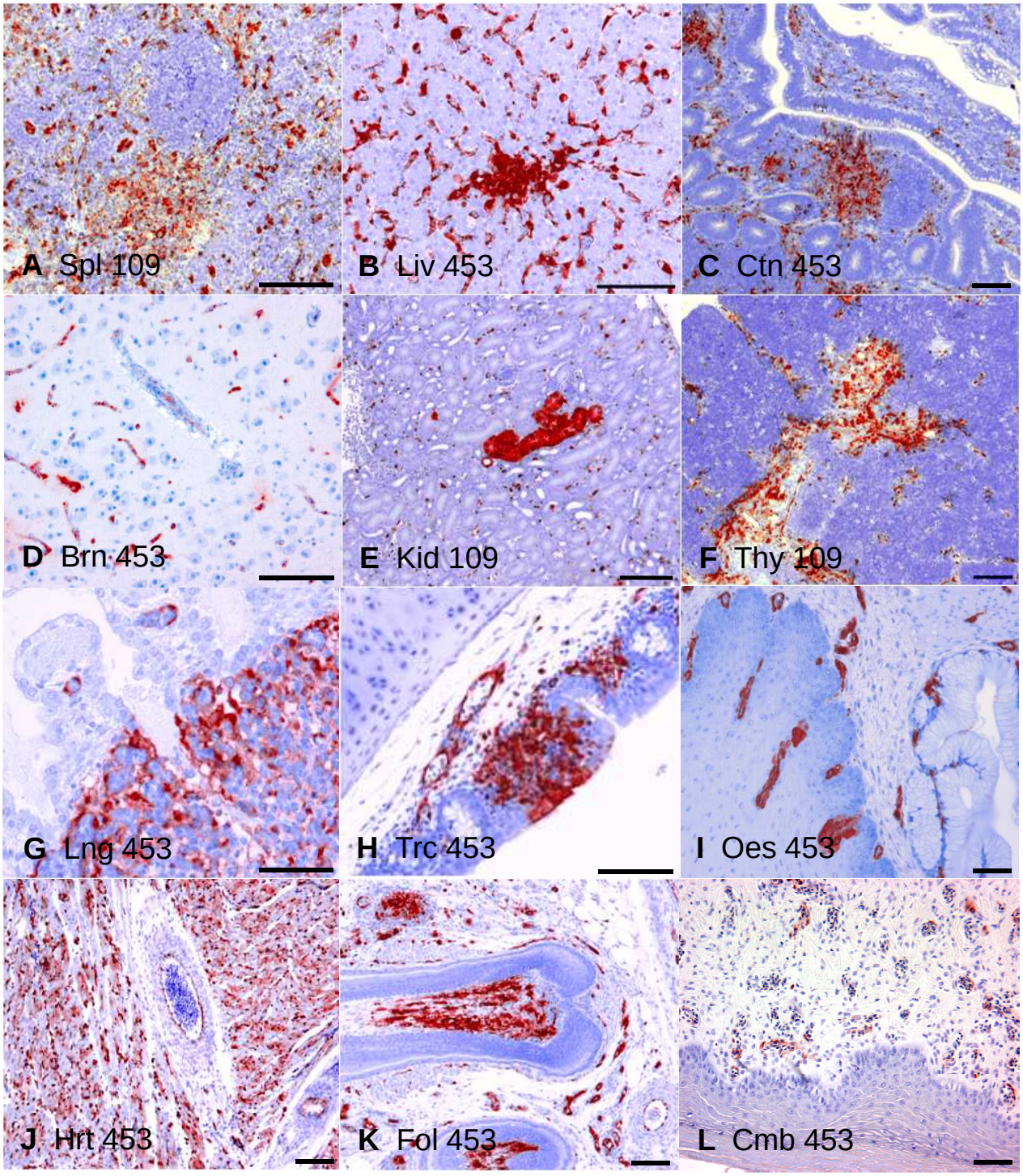
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Figure 3.3. H5N1 viral antigen appears systemically spread in chicken tissues in comparison to duck tissues. Immunohistochemistry (IHC) for H5N1 antigen staining in chicken tissues 24 h.p.i, with either H5N1 Ind109 or Vt453 strain. The histopathology caused by the two viruses was similar in chickens with foci of acute necrosis and haemorrhage. **(A)** Spleen, chicken 77 Ind109 strain, with IHC stain showing H5N1 viral antigen as red/brown colour prevalent in the red pulp and necrotic tissue. **(B)** Liver, chicken 76 Vt453 strain, with staining of sinusoidal endothelial cells and dense focal staining corresponding to necrotic hepatocytes. **(C)** Caecal tonsil, chicken 94 Vt453 strain, showing staining of viral antigen in caecal lymphoid tissues and lamina propria. **(D)** Brain, chicken 94 Vt453 strain, antigen staining in capillaries and in a foci of neurons. **(E)** Kidney, chicken 77 Ind109 strain, with severe H5N1 antigen staining. **(F)** Thymus, chicken 77 Ind109 strain, showing antigen located predominantly in the reticular epithelium.**(G)** Lung, chicken 94 Vt453 strain, where H5N1 viral antigen was present in blood vessel endothelium and dense antigen staining in respiratory tissue of the lung. **(H)** Trachea, chicken 88 Vt453 strain, showing viral antigen in sub mucosa and in a foci of the lamina propria and overlying epithelium. **(I)** Oesophagus, chicken 85 Vt453 strain, showing widespread viral antigen within capillaries and epithelial cells at the dermal-epidermal junction. **(J)** Heart, chicken 94 Vt453, showing viral antigen in capillaries and myocardium. **(K)** Feather follicle, chicken 76 Vt453 strain, showing dense H5N1 antigen at the base of the feather follicle. **(L)** Comb, chicken 76 Vt453 strain. All scale bars = 100 μm.

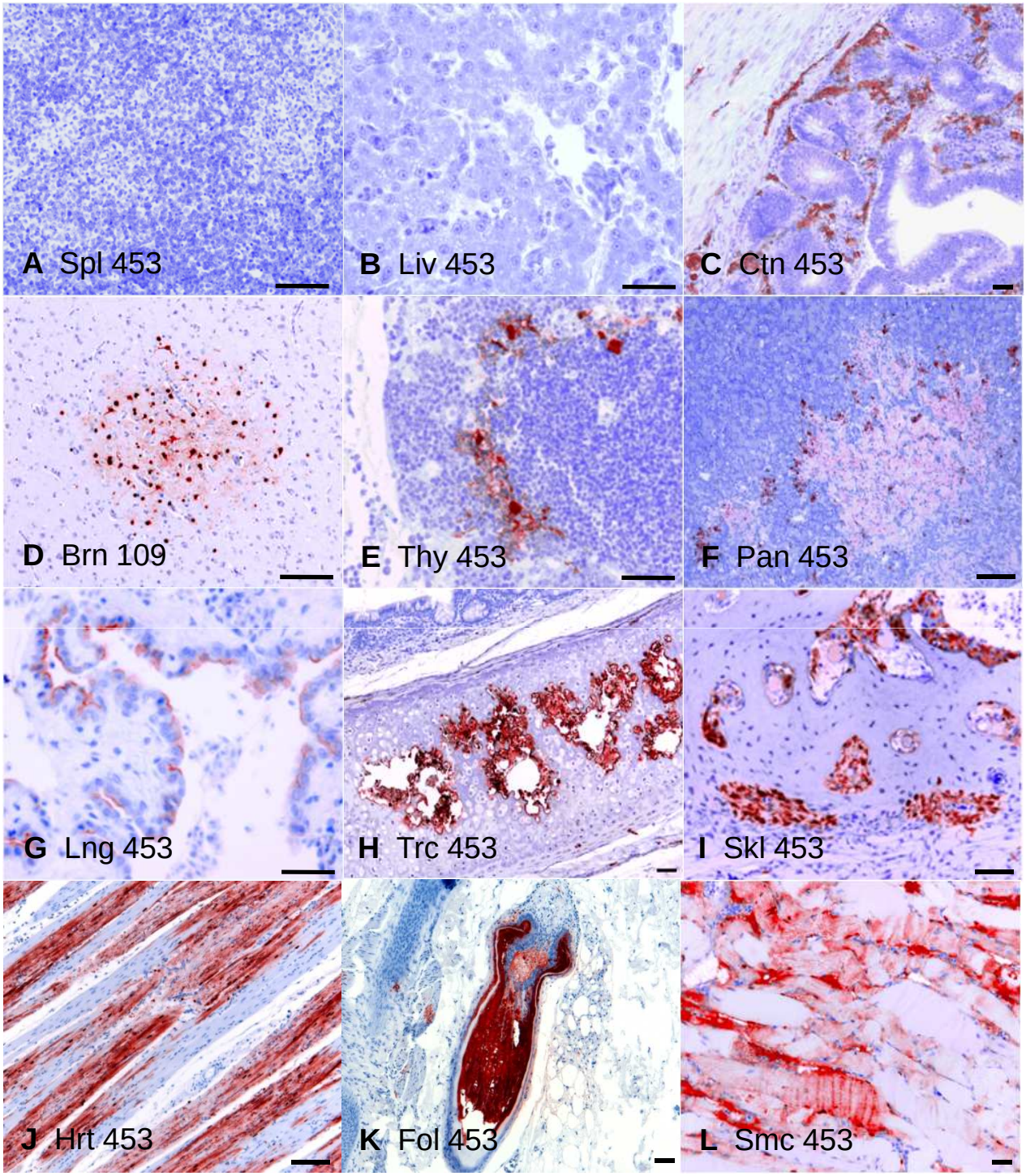
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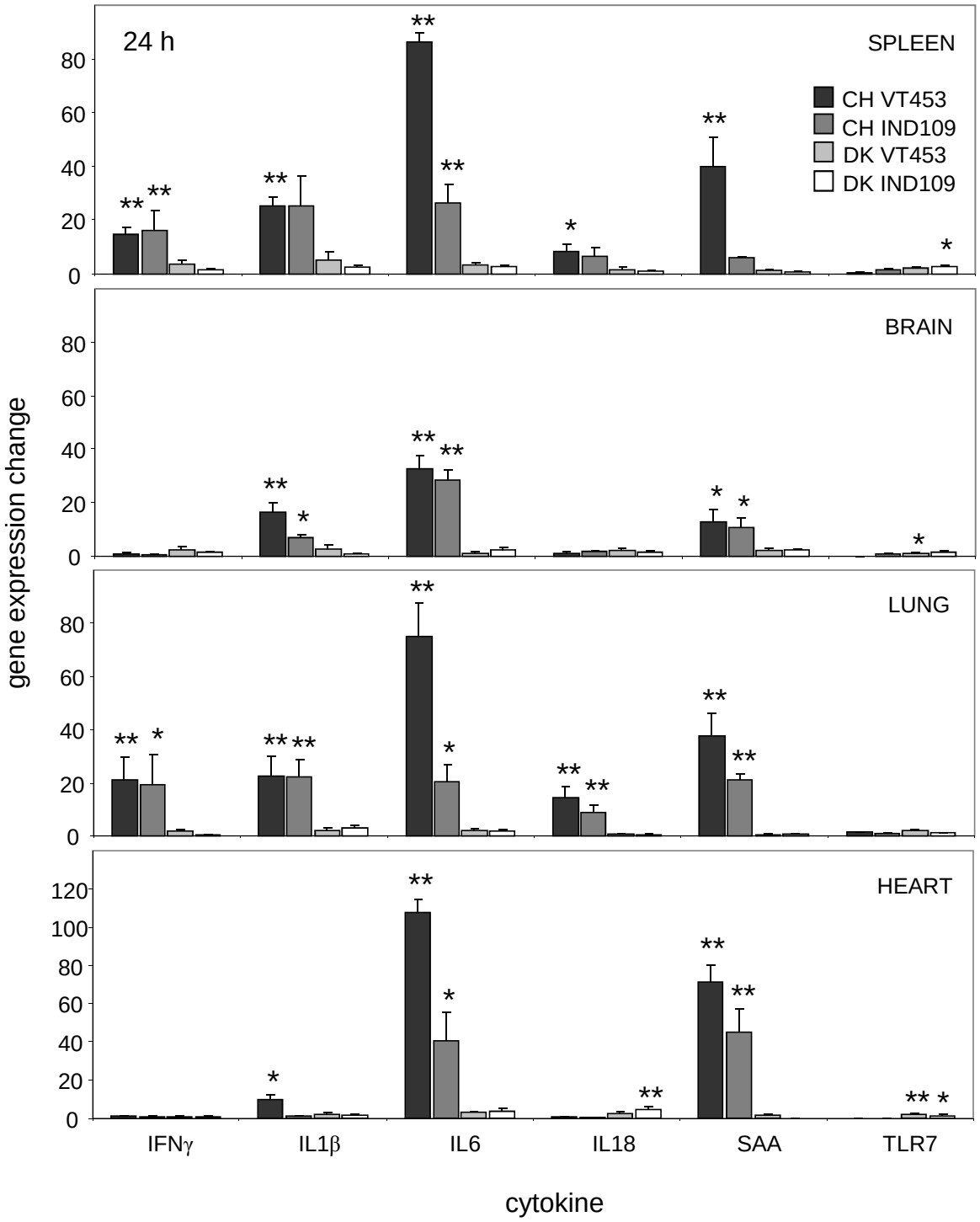
Figure 3.4. H5N1 viral antigen has a predilection for muscle tissue in ducks, with less widespread antigen staining compared to chickens. IHC for H5N1 antigen staining in duck tissues between 24 to 72 h.p.i, with either H5N1 Ind109 or Vt453 strain. Lesions and antigen were detected in all but one of the ducks infected with the H5N1 Vt453 strain. The major tissue types affected appeared to be heart, muscle, brain and pancreas. The histopathology caused by the H5N1 Ind109 strain showed a generally similar tissue distribution, however the quantities of viral antigen appeared considerably lower. In several birds, no viral antigen was detected in any tissues. Virus was rarely detected in most lymphoid tissues. **(A)** Spleen, duck 44 Vt453 strain, 72 h.p.i, with no apparent IHC staining for H5N1 viral antigen. **(B)** Liver, duck 80 Vt453 strain, 72 h.p.i, with no apparent staining for viral antigen. **(C)** Caecal tonsil, duck 44 Vt453 strain, 72 h.p.i, showing staining of viral antigen primarily in lamina propria. **(D)** Brain, duck 89 Ind109 strain, 72 h.p.i, showing antigen staining in a focus of neurons and the neuropil. **(E)** Thymus, duck 80 Vt453 strain, 72 h.p.i, with H5N1 antigen staining primarily in epithelial cells and connective tissue. **(F)** Pancreas, duck 80 Vt453 strain, 72 h.p.i, showing antigen located around outer edge of acinar necrotic lesion. **(G)** Lung, duck 10 Vt453 strain, 48 h.p.i, with antigen staining in the pleural surface of air sacs. **(H)** Trachea, duck 44 Vt453 strain, showing viral antigen in chondrocytes of the tracheal rings. **(I)** Bone and surrounding connective tissue of the skull, duck 44 Vt453 strain, 72h.p.i, showing widespread viral antigen within capillaries and epithelial cells at the dermal-epidermal junction. **(J)** Heart, duck 44 Vt453 strain, 72 h.p.i, showing viral antigen in heart muscle tissue. **(K)** Feather follicle, duck 44 Vt453 strain, 72 h.p.i. **(L)** Smooth muscle tissue, duck 80 Vt453 strain, 72 h.p.i. All scale bars = 50 μm.


24 h
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IL1β
IL6
IL18
SAA
TLR7

Figure 3.5. H5N1 Vt453 and Ind109 infection is associated with increased cytokine and acute phase protein expression in chickens when compared to ducks. Q-RTPCR was used to measure the cytokine profile of chickens and ducks 24 h.p.i, with either Vt453 or Ind109. The mRNA expression of IFNγ, IL1β, IL6, IL18, SAA and TLR7 was analyzed in spleen, brain, lung and heart tissue of infected and non infected animals. Chickens had higher levels of proinflammatory cytokines in contrast to ducks. Of the two H5N1 viruses, the Vt453 strain consistently induced the highest levels of IL6 and SAA in chickens at the peak of their infection. Data represents the mean fold expression of either duck or chicken genes relative to each uninfected tissue type with GAPDH used as the housekeeping gene. Data shows mean of 4 birds in each condition; error bars show SE. Single asterisk indicates statistically significant differences of means with $p<0.05$, double asterisk indicates statistically significant differences of means with $p<0.01$.

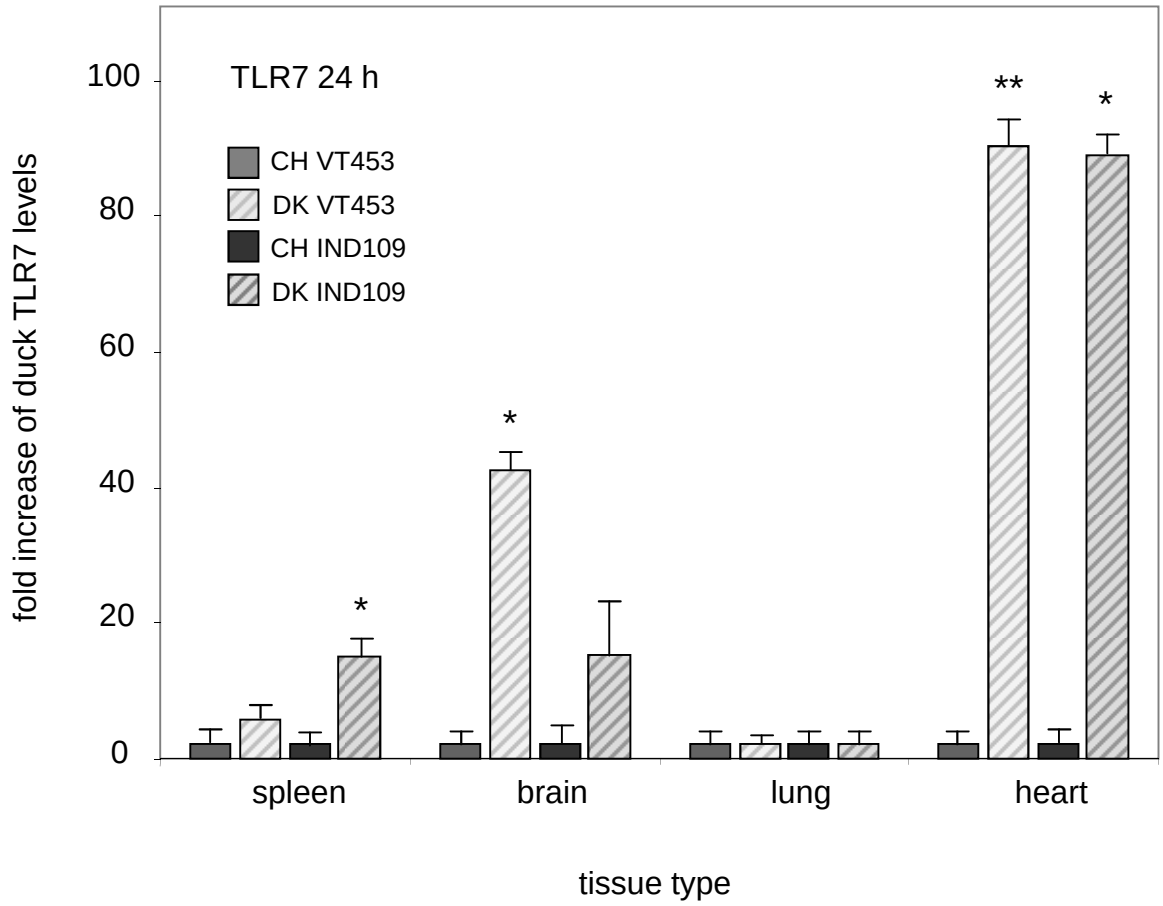

Figure 3.6. Duck TLR7 levels in brain and heart tissue are increased following H5N1 infection when compared to chickens. Q-RTPCR was used to measure the cytokine mRNA expression of TLR7 in ducks and chickens 24 h.p.i. The gene expression levels of TLR7 in ducks appears to be increased in comparison to chicken. Data represents the mean fold expression of duck TLR7 levels relative to chicken TLR7 levels with GAPDH used as the housekeeping gene (n=4 birds, error bars show SE). Single asterisk indicates statistically significant differences of means with $p<0.05$, double asterisk indicates statistically significant differences of means with $p<0.01$.

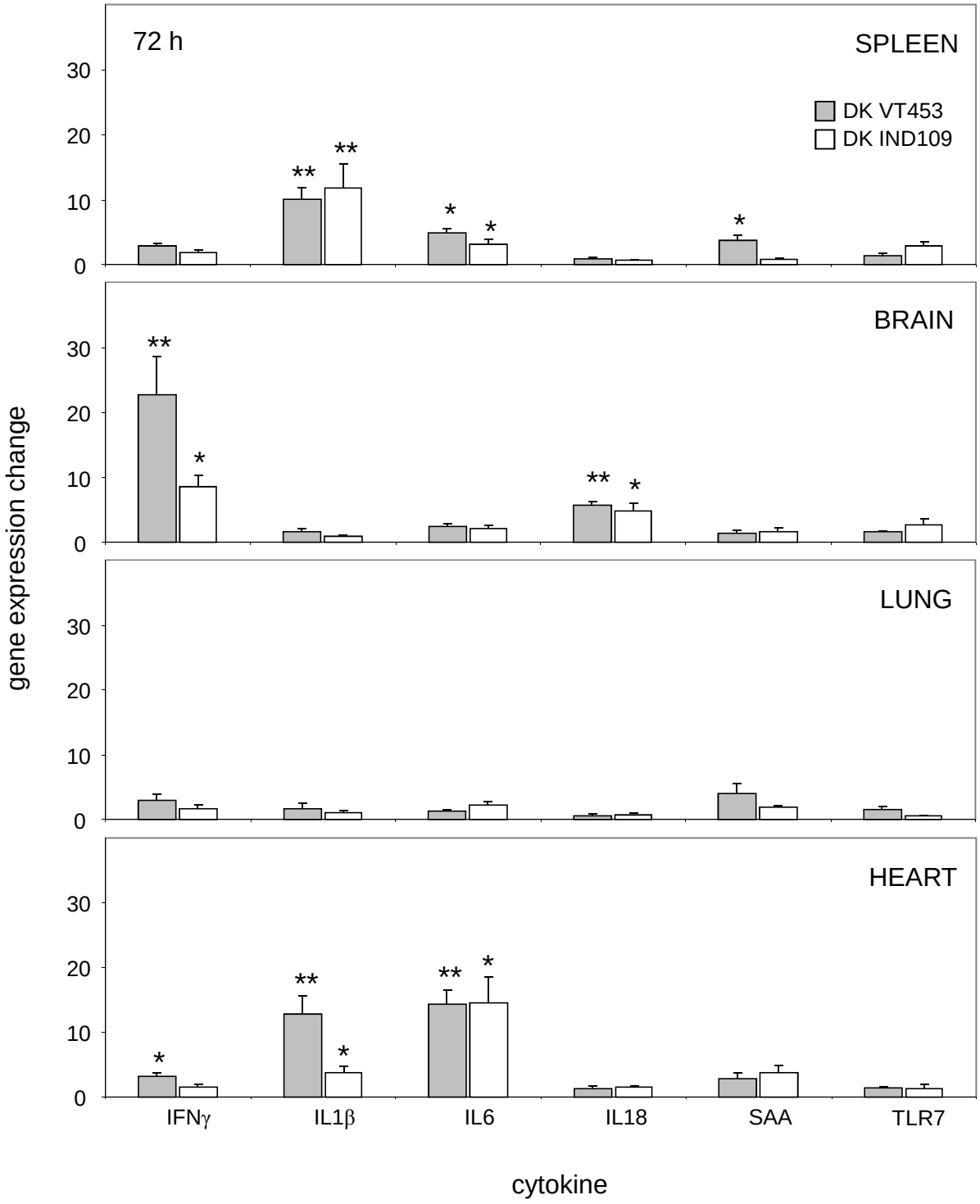
72 h
SPLEEN
DK VT453
DK IND109
BRAIN
LUNG
HEART
gene expression change
cytokine
IFNγ
IL1β
IL6
IL18
SAA
TLR7
0
10
20
30
*
**

Figure 3.7. Ducks show increases in cytokine levels in brain and heart tissue 72 hours after H5N1 infection. Q-RTPCR was used to measure the cytokine mRNA expression of IFNγ, IL1β, IL6, IL18, SAA and TLR7 in ducks during the peak of their infection, 72 hours after H5N1 inoculation. The gene expression levels of IFNγ, IL1β and IL6, 72 h.p.i, begin to increase compared to uninfected ducks. However, increases in duck cytokine genes appear more tissue specific than chickens and are comparatively less than that observed in chickens 24 h.p.i. Data represents the mean fold expression of duck gene expression relative to each uninfected tissue type with GAPDH used as the housekeeping gene (n=4 birds, error bars show SE). Single asterisk indicates statistically significant differences of means with $p<0.05$, double asterisk indicates statistically significant differences of means with $p<0.01$.

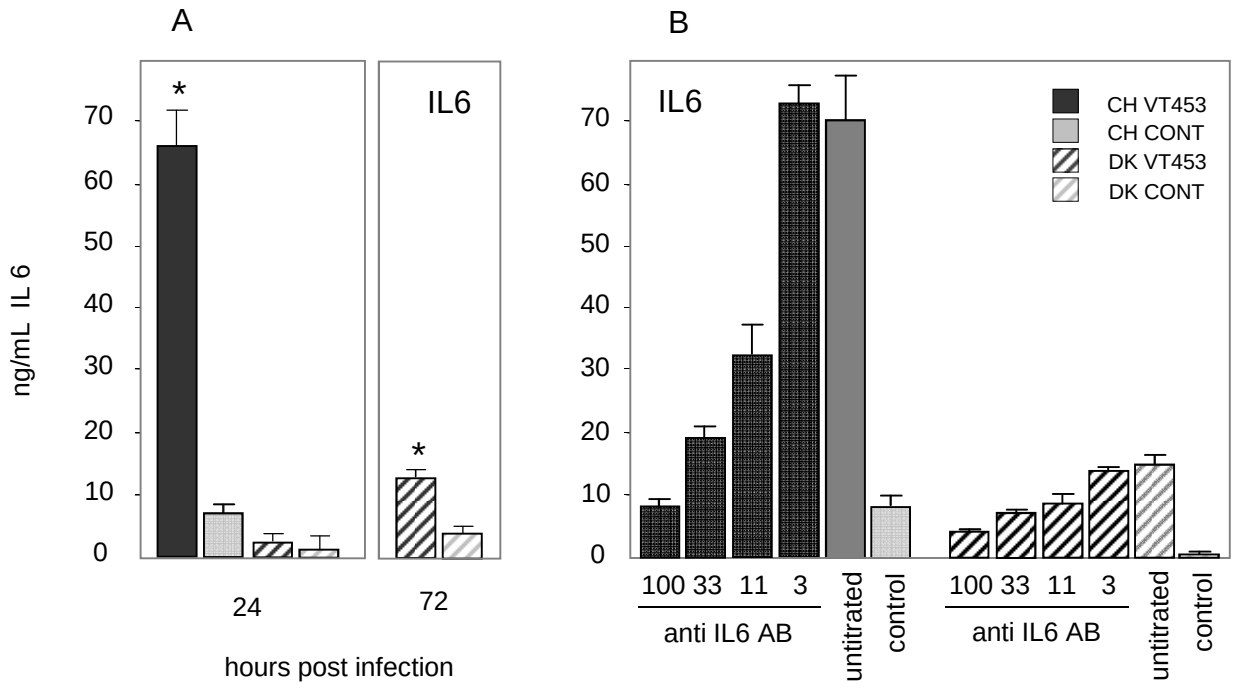

Figure 3.8. Following H5N1 influenza infection, levels of IL6 are increased in chickens but not in infected ducks. (A) Levels of IL6 protein measured in the sera of chickens and ducks. IL6 was measured using an IL6 dependant 7TD1 cell line and examined at the peak of infection in either animal. In chickens, 24 h.p.i, with H5N1 Vt453 IL6 is increased 8-10 fold, whilst ducks, 72 h.p.i, with Vt453, have a 2 fold increase in IL6. **(B)** Anti IL6 antibodies (AB) were used at a range of concentrations to neutralize the levels of IL6 protein, with higher concentrations of anti IL6 AB needed to reduce IL6 in sera from H5N1 infected chickens, confirming the higher levels of IL6 in these animals. Data represents mean IL6 concentration of 4 birds; error bars show SE. An asterisk indicates statistically significant differences of means with $p<0.05$.

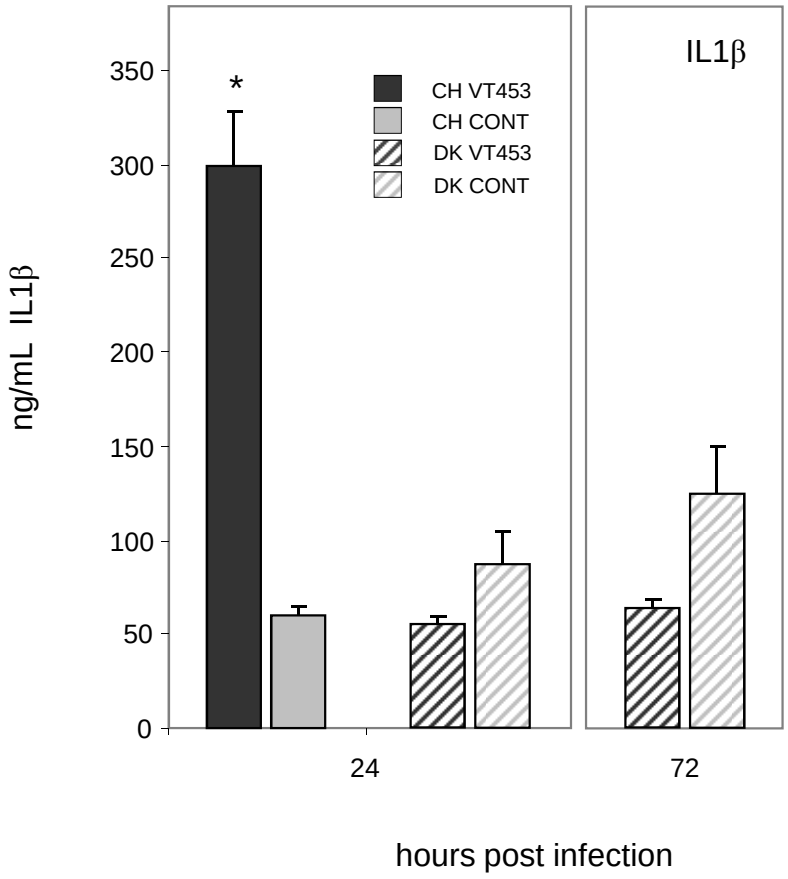

Figure 3.9. Following H5N1 influenza infection, levels of IL1β are increased in chickens when compared to ducks. Levels of IL1β protein measured in the sera of chickens and ducks using an IL1β ELISA (Invitrogen). Examining IL1β levels at the relative peak of infection in either animal indicated that chickens, 24 h.p.i, with Vt453, induce 5-6 fold increases of IL1β in their blood, whilst ducks have much lower levels of IL1β at their relative peak of infection (72 h). Data represents mean levels of IL1β concentrations of 4 birds, error bars show SE. An asterisk indicates statistically significant differences of means with $p<0.05$.

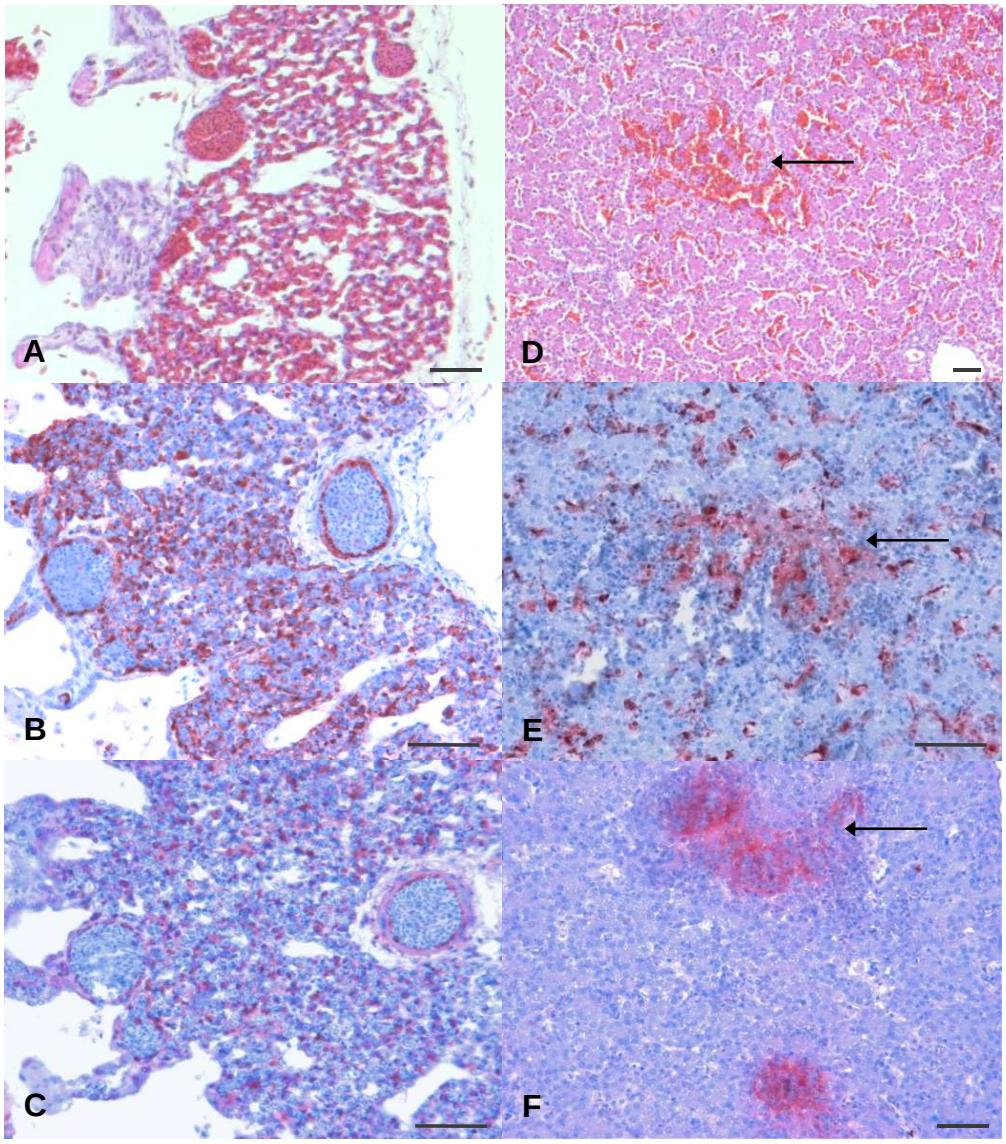

Figure 3.10. Immunohistochemistry of chicken tissues shows colocalisation of H5N1 antigen with IL6 staining. Left hand panel shows H5N1 infected chicken (94) lung tissue, right hand panel shows H5N1 infected chicken (76) liver tissue. **(A)** Lung tissue with blood vessels and lesions in parenchyma (haematoxylin and eosin) . **(B)** Lung tissue showing H5N1 viral antigen staining in proximity to blood vessels and throughout the parenchyma. **(C)** Lung tissue with IHC staining for IL6 surrounding blood vessels and throughout the parenchyma. **(D)** Liver tissue with arrow identifying necrotic lesion (haematoxylin 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 μm.

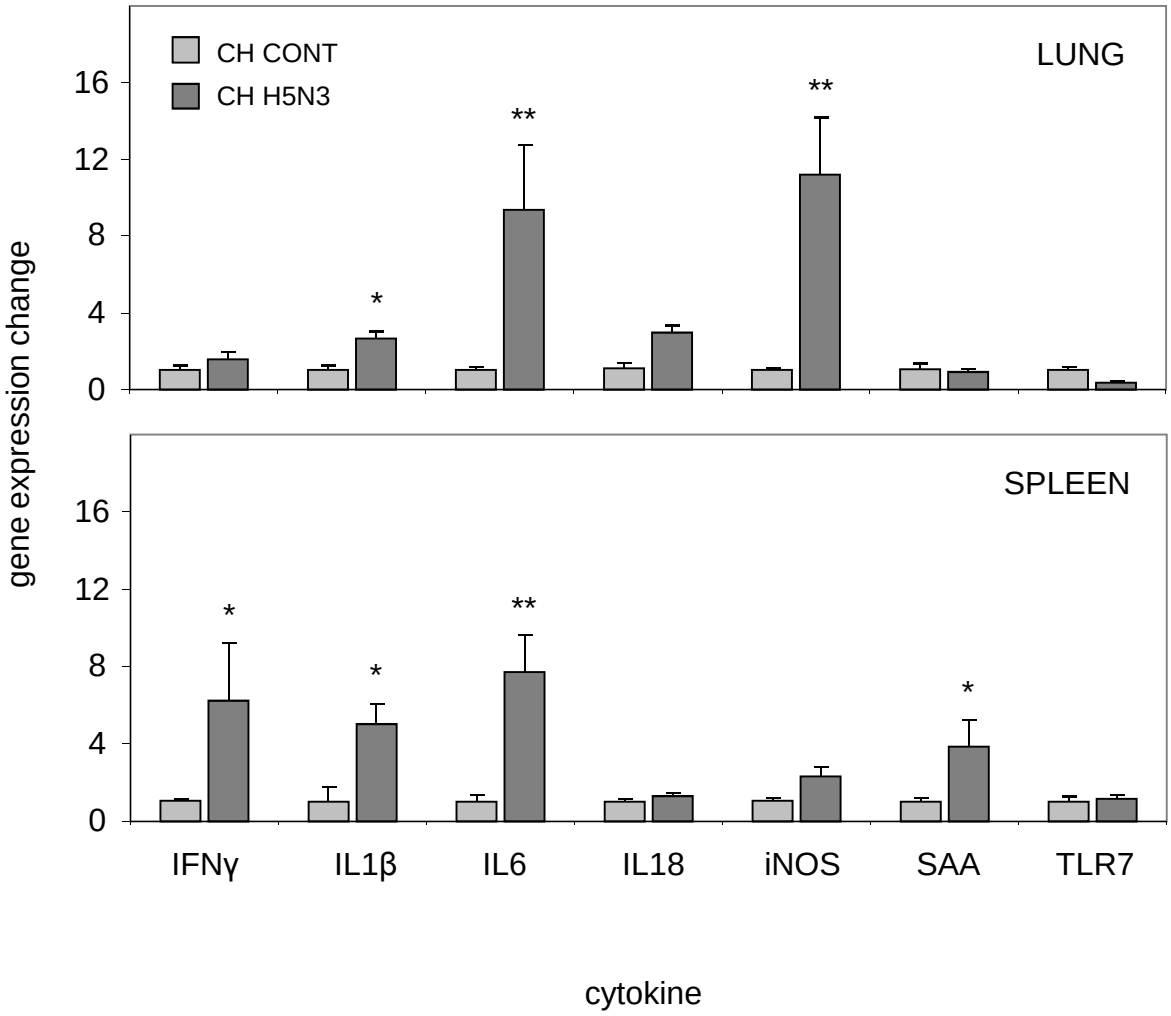

Figure 3.11. H5N3 influenza infection only modestly increases cytokine levels in chicken lung and spleen tissues. Chickens were infected with H5N3 Vc1426 influenza virus and were sampled 72 h.p.i, by Q-RTPCR for the gene expression levels of a range of cytokines. Following H5N3 infection, levels of cytokine gene expression were compared to uninfected control chickens and determined in conjunction with the house keeping gene GAPDH. Data represents the mean fold expression relative to unstimulated controls with 4 birds in each group, error bars display SE. Single asterisk indicates statistically significant differences of means with $p<0.05$, double asterisk indicates statistically significant differences of means with $p<0.01$.

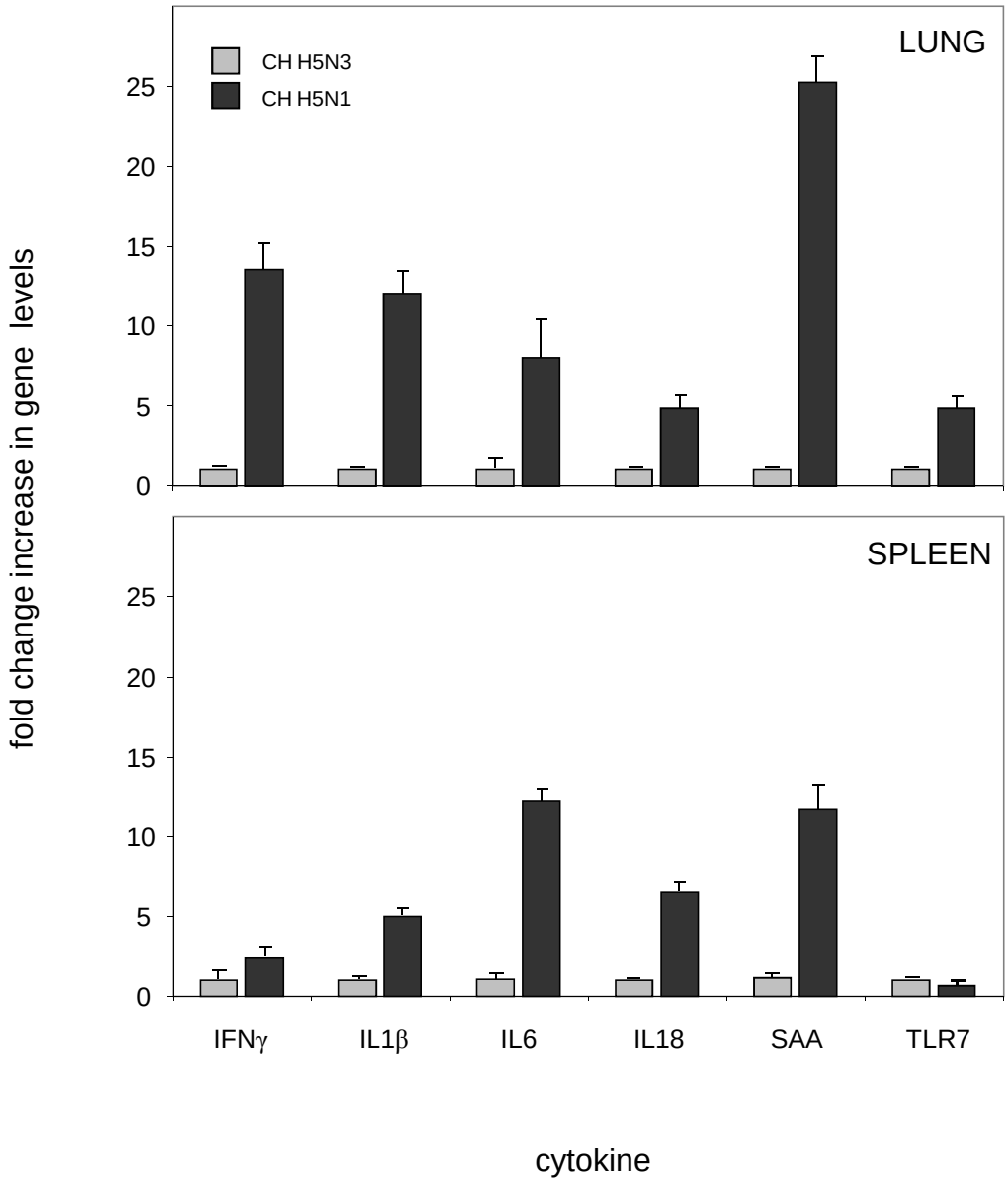

Figure 3.12. H5N1 influenza infection increases cytokine levels when compared to H5N3 influenza infection in chicken lung and spleen tissues. Chicken lung and spleen tissues infected with the H5N1 Vt453 virus have higher cytokine levels than tissues infected with the low-pathogenic H5N3 influenza virus. Data is shown as gene expression levels (measured by Q-RTPCR) with H5N1 infected samples relative to H5N3 infected samples, normalized against the housekeeping gene GAPDH (n=4, error bars show SE).

Chapter 4

Increased iNOS expression in organs is associated with a higher severity of H5N1 influenza virus infection.

4.1 Introduction

H5N1 influenza virus strains have been prevalent and highly pathogenic in gallinaceous birds, specifically chickens, causing acute systemic disease (Perkins *et al.,* 2001; Perkins *et al.,* 2001; Isoda *et al.,* 2006; Nakamura *et al.,* 2008; Nakamura *et al.,* 2008). The severity of infection, however, varies dramatically between chickens and other avian species, such as ducks (Swayne *et al.,* 1988; Perkins *et al.,* 2001; Mase *et al.,* 2005; Mase *et al.,* 2005; Mase *et al.,* 2005; Isoda *et al.,* 2006; Isoda *et al.,* 2006; Pantin-Jackwood *et al.,* 2007; Pantin-Jackwood *et al.,* 2007; Bogs *et al.,* 2010). Infection of ducks is often asymptomatic, whereby H5N1 viruses, which are categorised as highly pathogenic in chickens, appear to display reduced clinical signs in the duck (Mo *et al.,* 1997; Mo *et al.,* 1997; Kwon *et al.,* 2005; Kishida *et al.,* 2005). Furthermore, little is known about the immune response generated following H5N1 influenza infection in both chickens and ducks. Viral replication may be associated with an increased proinflammatory response (Guan *et al.,* 2004; de Jong *et al.,* 2005; Suzuki *et al.,* 2009), as chickens exhibit widespread viremia and an associated increase in cytokines, leading to inflammation. This suggests that H5N1 influenza virus may be a potent inducer of proinflammatory cytokines, chemokines and free radicals in chickens (Akaike *et al.,* 1996; Cheung *et al.,* 2002; de Jong *et al.,* 2005; Suzuki *et al.,* 2009). Therefore, key questions that remain unanswered in the field are what drives the pathogenicity associated with H5N1 influenza infection and does the virus induced cytokine response contribute to increased virulence?

The free radical NO is an important messenger molecule linked to an array of immune responses (Moncada, 1993). NO plays a role in mediating macrophage cytotoxicity and antiviral activity (Nathan, 1992; Nathan *et al.,* 1994; Mayer *et al.,* 1997). NO is

generated by the enzyme NOS, which catalyses the biosynthesis of NO in a range of tissues. Currently, a number of distinct NOS isoforms have been characterised in mammals: the neuronal isoform (nNOS), inducible isoform (iNOS) and endothelial isoform (eNOS) (Stuehr *et al.,* 1992; Adams *et al.,* 1998; Adams *et al.,* 1998; Knudsen *et al.,* 2003; Knudsen *et al.,* 2003). The two constitutive forms, nNOS and eNOS, are activated by, and dependant on, changes in intracellular calcium (Venema *et al.,* 1996; Alderton *et al.,* 2001), whereas iNOS is calcium independent (Forstermann *et al.,* 1995). It is this inducible NOS (iNOS) form which serves as a key molecule in combating viral infection, through NO production and acting as a mediator of apoptosis and the acute phase protein response (Arstall *et al.,* 1999; Sandri *et al.,* 2008; Sandri *et al.,* 2008). Excessive production of iNOS, however, may have negative effects reacting with other damaging oxidants and promoting inflammation (Evans, 1995). Recently the iNOS gene was cloned in the chicken (Lin *et al.,* 1996), which has allowed investigations of iNOS activity and the role of this gene in a range of tissues following influenza virus infection of chickens. However, at present there is a paucity of information surrounding the duck iNOS gene and its role in the immune response to influenza virus infection.

To explore the role of iNOS and NO production and contrast this in the immune response of chickens and ducks during H5N1 influenza infection, we identified and cloned iNOS in ducks. We showed that levels of NO were elevated in both infected chickens and ducks when compared to uninfected birds. Given the severity of pathology associated with H5N1 infection varies in the organs of chickens and ducks, we also examined the pathways leading to NO production at the prominent sites of infection. Our results show that infected chickens have an earlier and more prevalent expression of iNOS in lung, spleen, caecal tonsil and liver tissue as compared to ducks. The high expression of iNOS in chicken organs may contribute to the increased severity of disease associated with H5N1 infection in this species.

4.2 Results

4.2.1 Elevation of NO in the sera of chickens and ducks infected with H5N1 influenza

To establish if NO expression was associated with the immune response during H5N1 influenza virus infection, assays were carried out to determine the concentration of this molecule in the sera of chickens and ducks. Serum was isolated from chickens at the peak of infection, 24 h.p.i, whilst from ducks serum was isolated after 24 h.p.i, and 72 h.p.i, with 72 h.p.i, being the relative peak of infection in ducks and NO levels measured (Fig. 4.1). H5N1 virus infected chickens showed a 4 fold increase in NO in sera when compared to uninfected chickens. Ducks initially showed no change in NO levels at 24 h.p.i, during H5N1 virus infection when compared to uninfected birds. After 72 h.p.i, ducks showed only a 2 fold increase in NO levels compared to uninfected ducks and lower levels of NO in comparison to H5N1 virus infected chickens.

4.2.2 Molecular cloning and bioinformatic analyses of duck iNOS

As increased levels of NO were observed in the sera of H5N1 infected animals we wanted to further characterise the levels of iNOS, as a potential indicator of NO, in the tissues of infected animals. In order to determine the relationship between NO production and iNOS expression, we first had to identify iNOS in the duck. iNOS is typically produced as part of the oxidative burst in macrophages. Duck splenic leukocytes were therefore stimulated with LPS for 24 hours and PCR amplification with degenerative primers directed at duck iNOS was performed. A partial iNOS isoform (II NOS) cDNA product was obtained which was approximately 2500 bp and showed 89% sequence identity with chicken iNOS (chiNOS) cDNA (Fig. 4.2). RLM 3 prime RACE (Invitrogen) was then used to capture the complete iNOS sequence which was deposited in GenBank (accession No. FJ966247). This cloned sequence of 3447 bp encoded a protein of 1148 amino acids with a predicted molecular weight of 130 kDa (Fig. 4.3). The deduced amino acid sequence of duck iNOS (dkiNOS) was modelled (smart.embl-heidelberg.de) and displayed conserved regions for the binding of iNOS cofactors: heme, calmodulin, FMN, FAD and NADPH (Fig. 4.6). Table 4.1

shows that dkiNOS has a relatively high amino acid identity to iNOS protein sequences between different species (ClustalW algorithm). Furthermore, the dkiNOS protein was comparable (98%) to the published chiNOS protein (Lin *et al.,* 1996) and phylogenetic analyses showed that dkiNOS clusters near chiNOS, supporting the fact they are similar and may have a conserved function (Fig. 4.5). With this identification of the duck iNOS gene we then analysed the basal levels of iNOS expression in various tissues and compared these duck levels to the basal iNOS levels observed in chickens. Gene analysis by Q-RTPCR showed that iNOS expression (fold increase relative to muscle iNOS levels) in ducks was generally similar to the levels observed in chickens, however, in the lung it appears that the chicken may have higher basal levels of iNOS gene expression (Fig. 4.7).

4.2.3 iNOS is induced by mitogens in duck and chicken splenic leukocytes

Previous studies have shown that iNOS levels can be elevated following the addition of LPS or IFNγ in cell culture. To assess this newly identified duck iNOS gene, and compare its expression with that of chicken, splenocyte activation cultures were carried out. After 24 hours of culture, iNOS levels were elevated in chicken and duck cells stimulated with IFNγ and LPS (increased 80 and 50 fold, respectively). However, the synthetic dsRNA analog poly(I:C) only increased iNOS expression in duck splenic leukocytes when compared to chicken splenic leukocytes (Fig. 4.8). To test whether increased iNOS expression is associated with increased NO production, splenocytes were cultured with LPS for 48 hours and supernatants were tested using the colorimetric NO assay. At a range of timepoints LPS stimulated NO production in a fashion that correlated with iNOS gene induction (Fig. 4.9 A and B).

4.2.4 Chickens and ducks infected with H5N1 have increased iNOS expression

Since we observed increases in NO in H5N1 virus infected sera we wanted to assess the levels of iNOS, as a potential indicator of NO, in a range of tissues from infected animals. Therefore, chickens and ducks inoculated with H5N1 Vt453 influenza virus (Muscovy duck/Vietnam/453/2006) were sampled and iNOS levels measured at the peak of their relative infection (highest viral titres were at 24 h.p.i, for chickens and at

72 h.p.i, for ducks). The largest change in iNOS expression was observed in the liver of infected chickens (220 fold), with caecal tonsil, spleen and lung tissue also showing increased iNOS mRNA expression (30 fold, 17 fold and 15 fold, respectively). However, the heart and brain showed little change in iNOS mRNA expression (Fig. 4.10). In comparison to chickens, infected ducks were observed to have lower levels of iNOS in all organs except cardiac tissue. Ducks appeared to show an increased iNOS expression in heart tissue (5 fold), with the highest increase in aortic tissue (15 fold). Caecal tonsil tissue also showed some elevation in iNOS expression (5 fold), however, unlike chickens, lung, liver and spleen tissue showed only marginal increases compared to uninfected ducks.

4.2.5 Co-localisation of iNOS and H5N1 influenza virus antigen

As iNOS gene expression was upregulated in H5N1 virus infected tissues, we assessed the co-localisation of iNOS expression with regard to virus. Antibody staining for H5N1 antigen was observed in a wide range of chicken organs. In contrast, H5N1 antigen was restricted to fewer sites in infected ducks. In the chicken H5N1 antigen was predominantly found in respiratory and intestinal tissue, spleen, liver as well as heart. However, H5N1 antigen was mainly restricted to the respiratory tract, intestinal tissue and heart of infected ducks (Fig. 4.11). Antibody staining for iNOS was observed to be present in tissues which were high in H5N1 antigen and was more prevalent in chickens than in ducks (Fig. 4.12). Uninfected chickens and ducks had little iNOS staining present. During infection with H5N1, chicken lung tissue showed iNOS staining in the respiratory epithelium and the surrounding submucosa. In contrast, duck lung tissue showed iNOS staining in the hyaline cartilage, with less staining present in the submucosa (Fig. 4.12 A and B). In both chicken and duck caecal tonsil tissue, iNOS was prevalent in the lamina propria and in lymphocyte aggregates (Fig. 4.12 C and D). Nevertheless, tissue from infected chicken liver showed iNOS staining in the lumen of sinusoidal areas, whilst duck liver had little iNOS staining present (Fig. 4.12 E and F). Chicken heart tissue stained positive for iNOS in the myocardium and in endothelial cells surrounding blood vessels. Duck heart tissue had few iNOS positively stained cells present in the myocardium (Fig. 4.12 G and H). Uninfected tissues stained with either rabbit serum directed against

H5N1 influenza virus nucleoprotein or with a rabbit polyclonal antibody directed against iNOS (Abcam ab3523), showed no staining in either chickens or ducks. Control antibody staining sections were run following identical protocols but using an irrelevant primary antibody control and showed no staining.

4.3 Discussion

Continued sporadic outbreaks of H5N1 influenza virus infection highlights the need for alternative strategies to deal with infection. Moreover, without a greater understanding of host-pathogen interactions, the management of H5N1 will continue to be difficult. NO is produced primarily as an effector molecule as part of the hosts defence response (Bogdan *et al.,* 2000). However, it has been postulated that the outcomes of this defence response may be either beneficial or detrimental to the host depending on the level of NO produced (Akaike *et al.,* 1996; Arstall *et al.,* 1999; Arstall *et al.,* 1999). Therefore, understanding the role that NO and iNOS play during H5N1 infection in chickens and ducks may provide insights into the underlying mechanisms and differences observed in disease severity (Burggraaf *et al*., 2011 ; Isoda *et al*., 2006; Mase *et al*., 2005; Swayne *et al*., 1988 ; Wasilenko *et al*., 2009). To investigate this we identified the presence of the iNOS gene in ducks and confirmed that dkiNOS acts in a similar fashion to its chicken counterpart (Lin *et al.,* 1996). Furthermore, we describe the changes in iNOS levels in H5N1 virus infected chicken tissues and compared these to iNOS levels in infected duck tissue. Tissue specific increases in iNOS production in the chicken appear to be associated with a higher degree of disease severity during H5N1 virus infection in chickens when compared to ducks. However, although we have observed this association for the Vt453 H5N1 strain, since there is some degree in pathogenicity between the various isolates of H5N1 virus, there may also be varying degrees in the association of iNOS with pathogenicity.

As iNOS is typically induced following influenza infection, its role in promoting NO production and the sensitivity of NO regulation may be important in avian species. NO is known to be a key molecule in the immune response against viral infections

(Karupiah *et al.,* 1993; Kreil *et al.,* 1996; Kreil *et al.,* 1996). Studies of human and mouse iNOS and the associated NO production, have reported that these molecules are often active in concert with IFNγ to protect against viral infection (Karpala *et al.,* ; Moran *et al.,* 2005; Li *et al.,* 2009), however, their role in avian species is less defined. Moreover, it has been shown that upregulation of IFNγ and the induction of other proinflammatory molecules increase the production of NO (Chan *et al.,* 2001; Li *et al.,* 2009). The demonstration that dkiNOS expression is increased in a similar fashion to chiNOS, when stimulated by inflammatory mitogens, such as LPS and IFNγ (Fig. 4.8 and Fig. 4.9), may suggest a comparable role in the innate immune response of avian species. As dkiNOS is structurally similar to other iNOS homologues (Fig. 4.6) and appears to be associated with NO production, it may be expected that the iNOS response in ducks is similarly conserved. Furthermore, given the growing evidence for the antiviral activity of iNOS in the chicken (Ahmed *et al.,* 2007; Ahmed *et al.,* 2007), particularly following influenza infection (Kacergius *et al.,* 2006; Wasilenko *et al.,* 2009), it may be further expected that dkiNOS has the ability to be upregulated during the course of H5N1 influenza virus infection.

It is still unclear what effect iNOS and other proinflammatory molecules have during influenza infection. IFNγ has been shown to upregulate iNOS expression (Chan *et al.,* 2001) and likewise we observed that the addition of IFNγ to duck cells increased both iNOS mRNA expression and NO production. Correspondingly, H5N1 influenza virus infection studies in mammals have shown that IFNγ levels are increased and similarly H5N1 virus infected chickens have both elevated levels of IFNγ and other cytokines (Karpala *et al.,* ; Suzuki *et al.,* 2009). H5N1 viruses are often associated with an increase in inflammatory molecules (Chan *et al.,* 2005), supporting a possible role for iNOS (Wasilenko *et al.,* 2009). With this in mind, we examined NO and iNOS expression during H5N1 infection in chickens and ducks. In H5N1 virus infected chickens we observed that iNOS expression was increased in the lung, spleen and caecal tonsil by as much as 30 fold. This widespread increase in iNOS may be due to the fact that in chickens H5N1 strains tend to have a broad tissue tropism (Mo *et al.,* 1997; Chan *et al.,* 2005; Chen *et al.,* 2008). Viral analysis showed high viral titres in chicken lung, caecal tonsil, liver and heart tissue. In chickens, the respiratory system

appears to be the predominant site of infection, however, virus quickly spreads to other organs, such as the heart and liver, with the infection becoming systemic (Kobayashi *et al.,* 1996). This may be crucial in the manifestation of excessive levels of NO during infection.

Intriguingly, the levels of iNOS expression were increased over 200-fold in the liver. Basal levels of iNOS gene expression are relatively low in chicken liver tissue compared to chicken lung, spleen and caecal tonsil (Fig. 4.7) and this may partly account for its high fold increase in this organ. Nevertheless, the high levels of iNOS observed in chicken liver tissue may also be associated with the observed production of acute phase proteins (Sandri *et al.,* 2008). An acute phase response is involved in many inflammatory infections (Miwata *et al.,* 1993; Miwata *et al.,* 1993; Cheung *et al.,* 2002; Chan *et al.,* 2005) whereby proteins, such as serum amyloid A (SAA), have been associated with NO production from macrophages (Sandri *et al.,* 2008). Furthermore, during studies investigating the acute phase response following H5N1 influenza virus infection, we have observed increased levels of SAA in chickens, whilst the levels of SAA in H5N1 virus infected ducks was comparably low (Chapter 3). This higher level of iNOS production in tissues infiltrated with virus raises the question of the role iNOS regulation has in the outcome of viral infections. In chickens, high iNOS production may have an impact on the severity of the pathology witnessed during H5N1 infection.

In contrast to the chicken, H5N1 virus infection in ducks is characterised by a predilection for viral replication in muscle and lung tissue (Bingham *et al.,* 2009). We observed the highest viral levels in the heart with less viral replication occurring in the lung, liver and caecal tonsil tissue. We identified that heart and aorta tissue in ducks had the greatest increases in iNOS expression, although IHC for iNOS in duck heart tissue was less prevalent than in infected chickens. Levels of iNOS expression were also increased in the caecal tonsil of ducks and IHC for iNOS expression showed its localisation in the proximity of H5N1 antigen in the lamina propria. This increase in iNOS could be associated with an upregulated inflammatory response during infection. It has been reported that H5N1 infection of duck heart muscle may cause

myocarditis and similarly infection of the brain may cause encephalitis (Bingham *et al.,* 2009). The resultant inflammatory responses in these organs may contribute to some of the observed morbidity in infected ducks. Levels of iNOS may potentially become increased at sites of inflammation, suggesting that in a similar fashion to the chicken, increases in duck iNOS may be associated influenza infection. This may be due to either the action of genes that iNOS stimulates, such as acute phase proteins and cytokines or the actual production of NO and its potential to contribute to apoptosis (Wildhirt *et al.,* 1995; Wildhirt *et al.,* 1995; MacMicking *et al.,* 1997a; Arstall *et al.,* 1999). Nevertheless, NO has the potential to generate free radicals which may damage cells (Akaike *et al.,* 1996) and therefore it is important that NO and iNOS be tightly regulated (MacMicking *et al.,* 1997a). Kacergius *et al*., (2006) indicated that during influenza infection increases in NO synthesis through iNOS gene induction contributed to lung damage. For these reasons, iNOS has been implicated in the pathology associated with some viral models (Karupiah *et al.,* 1993; Reiss *et al.,* 1998; Wasilenko *et al.,* 2009). The question still remains as to the relevance of the association of iNOS and NO in the observed pathology of H5N1 influenza virus infection. At present there is a clear association, however, the role iNOS plays in viral pathogenicity for each of the species is yet to be fully determined. Therefore, future work may be directed at determining whether the pathological outcomes from infection are a result of iNOS expression and the cause of disease severity. Techniques such as RNA interference (RNAi), to target iNOS induction and inhibit its function during H5N1 virus infection, may help in futher elucidating its role during infection.

Although both H5N1 virus infected chickens and ducks have increased circulating levels of NO in comparison to uninfected animals, the levels of iNOS gene expression and tissue expression observed by Q-RTPCR and IHC are markedly different between the two species. IHC staining for iNOS in chicken H5N1 infected liver tissue showed its presence predominantly in the sinusoidal areas (presumably endothelial cells), whilst in caecal tonsil and lung tissue iNOS was also observed in lymphoid aggregates and in endothelial cells around blood vessels. In comparison to infected chickens, IHC for duck iNOS appears restricted to the caecal toncil, with infrequent

staining in upper respiratory and heart tissue. Furthermore, no iNOS staining was found in infected duck liver tissue. This suggests that iNOS may contribute to higher NO production within H5N1 virus infected chicken organs than in infected duck organs. However, comparably higher NO levels in the chicken may be only a part of the problem for the associated increased severity of H5N1 infection. It is likely other immune genes such, as viral recognition receptors and inflammatory cytokines have a role in the varied pathogenicity following H5N1 influenza infection. For example, Barber *et al*., (2010), demonstrated that chickens lack the viral sensing receptor RIG-I which when expressed in chicken cells improves the response to H5N1 influenza virus infection (Barber *et al.,* 2010). Furthermore, studies by Okada *et al*., (2009) suggested that the response to H5N1 influenza in chickens could be improved following reduction of the inflammatory immune cascade (Okada *et al.,* 2009). Therefore, it would appear that further research may be required to fully elucidate the mechanisms behind the varied immune response between chickens and ducks.

In conclusion, it may be postulated that since H5N1 viruses are often associated with an increased pathogenicity in chickens, it may be that iNOS expression in chickens becomes dysregulated and this may impact on disease severity through the overproduction of free radicals and the resultant damage to cellular function. With this in mind, further investigation into the inflammatory response during H5N1 viral infection is needed. A better understanding of the inflammatory response following H5N1 influenza virus infection may help in developing new strategies and approaches for modulating a more positive outcome for these infections in chickens and ducks.

Table 4.1 Duck iNOS amino acid similarity between different species

Organism	Organism (common name)	Size (aa)	Identical (%)[a]	Conserved (%)[b]	Total (%)[c]
Gallus gallus	Chicken	1136	91.02	07.21	98.23
Bos taurus	Cow	1156	68.51	22.05	90.57
Mus musculus	Mouse	1144	65.46	24.73	90.20
Canine	Dog	1154	67.15	22.35	89.51
Oncorhynchus mykiss	Trout	1083	59.92	27.88	87.81
Rattus novegicus	Rat	1147	65.82	23.53	89.36
Homo Sapien	Human	1147	65.56	23.71	89.27

[a] aa identical to DkiNOS; [b] aa similar to DkiNOS; [c] aa identical and similar to DkiNOS

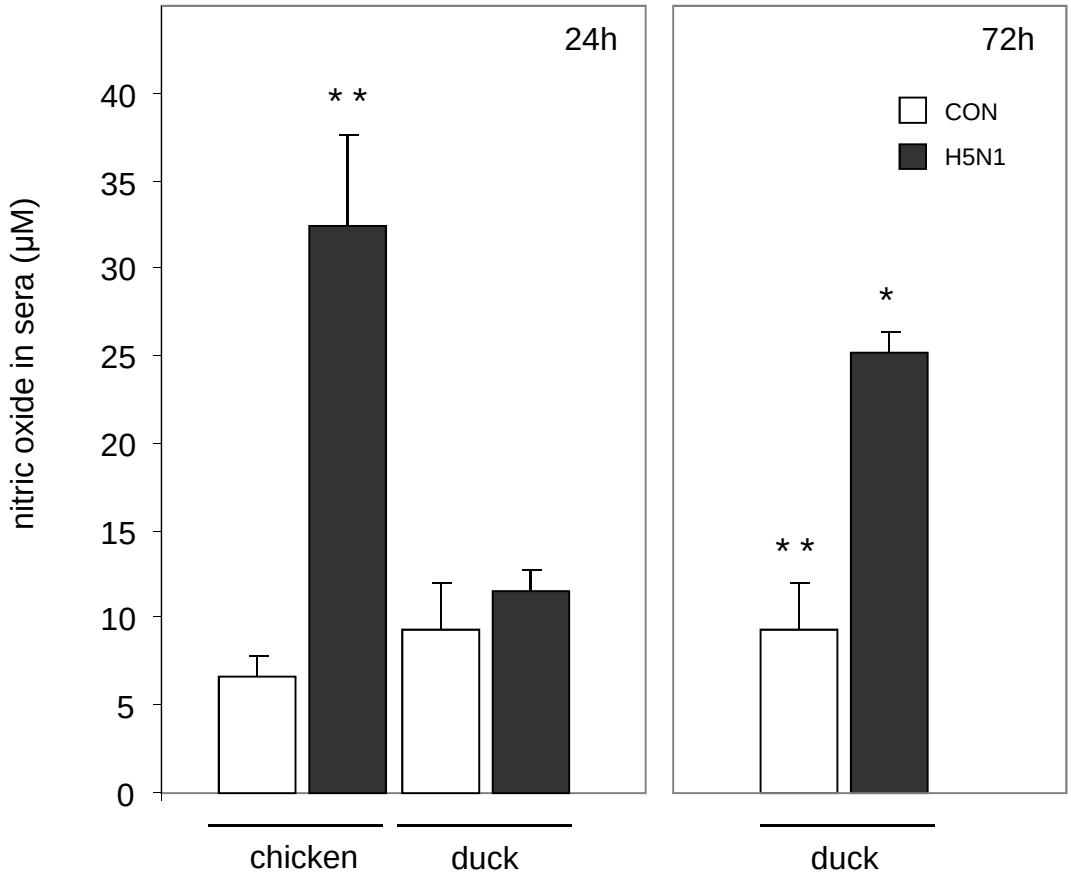

Figure 4.1. H5N1 influenza infection increases serum NO levels in both chickens and ducks. Serum collected from chickens and ducks infected with the H5N1 strain Muscovy/duck/Vietnam/453, as assayed for NO production during the peak of influenza infection (chickens at 24 h.p.i, ducks at 24 and 72 h.p.i.). Values are the mean of 2 experiments with 4 birds in each group, error bars display SE. A single asterisk indicates statistically significant differences of means with $p<0.05$, double asterisk indicates statistically significant differences of means with $p<0.01$.

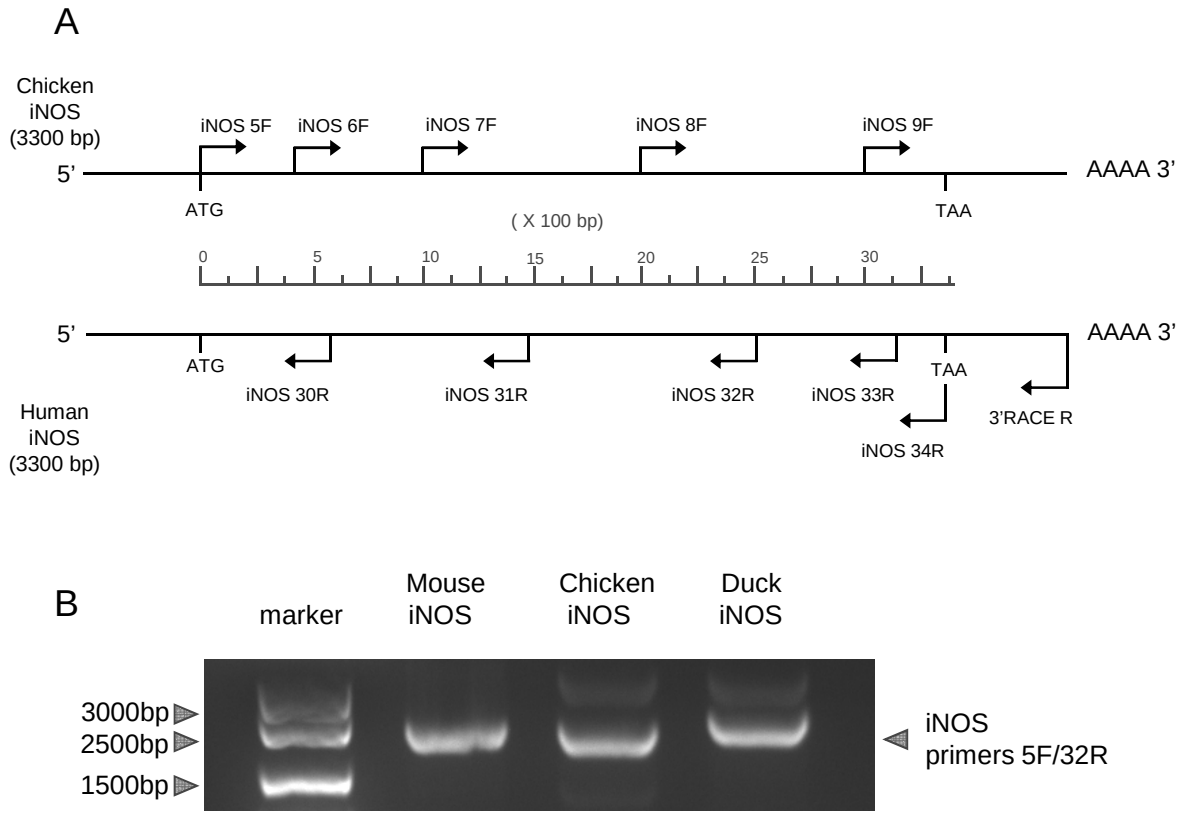

Figure 4.2. PCR product generated with iNOS specific primers or RLM RACE kit. (A) Diagram showing predicted binding sites for iNOS primers **(B)** Duck splenocytes were activated with LPS for 24 hours and PCR amplification with degenerative primers directed at duck iNOS was performed and the product run on a 1% agarose gel. A partial (isoform II NOS) iNOS cDNA product was obtained using 5F/32R which was approximately 2500 bp and showed 89% sequence identity with chicken iNOS cDNA. RLM 3-prime RACE (Invitrogen) was then used to capture the complete iNOS sequence which was 3447 bp and encoded a protein of 1148 amino acids with a predicted molecular weight of 130 kDa.

```
   1  atgctgtgcc catggcagtt tgcattcaaa cctcatgctg ctaagaaccg gtcctccaaa
        M  L  C   P  W  Q   F  A  F  K   P  H  A   A  K  N   R  S  S  K

  61  gtgacggata tcaataataa cgtggagaaa gatatgaaga tcaatggctt agtgaaagat
        V  T  D   I  N  N   N  V  E  K   D  M  K   I  N  G   L  V  K  D

 121  gatgccaaat tgcacgatct aatcaagaag cagaaagaga agctgcctat cgtaacttca
        D  A  K   L  H  D   L  I  K  K   Q  K  E   K  L  P   I  V  T  S

 181  gcagagaaac ccccagaaaa tggtatcaaa gcttcaaacc aaatatcaag atgtccaaga
        A  E  K   P  P  E   N  G  I  K   A  S  N   Q  I  S   R  C  P  R

 241  catgtaaaag taagaaactt ggaaaatgga tccagctttc ttgacacact acacctgaca
        H  V  K   V  R  N   L  E  N  G   S  S  F   L  D  T   L  H  L  T

 301  gcaaaggagg ttatcaattg ccgcaccaaa gcatgccaag gggcacttat gaccccaaag
        A  K  E   V  I  N   C  R  T  K   A  C  Q   G  A  L   M  T  P  K

 361  ggcttggtga ggggcactag agatgggcca gttcctcagg cagagctttt acctcaggca
        G  L  V   R  G  T   R  D  G  P   V  P  Q   A  E  L   L  P  Q  A

 421  atagactttg tcaagcagta ctacagttca tttaaagagt caaaaataga agaacacctg
        I  D  F   V  K  Q   Y  Y  S  S   F  K  E   S  K  I   E  E  H  L

 481  gcccgactag aaacagtgac caaagagata gaaacaacag gaacctacca tctgacacag
        A  R  L   E  T  V   T  K  E  I   E  T  T   G  T  Y   H  L  T  Q

 541  gatgaactga tctttgctgc caaacaggcc tggaggaatg ctccgagatg tattgggaga
        D  E  L   I  F  A   A  K  Q  A   W  R  N   A  P  R   C  I  G  R

 601  atccagtggt ccaatctaca ggtattcgat gcacgtgact gtaaaacagc caaggaaatg
        I  Q  W   S  N  L   Q  V  F  D   A  R  D   C  K  T   A  K  E  M

 661  tttgagcata tctgtcgtca tgttcagtat gcaacaaaca atggaaacat aaggtcagcc
        F  E  H   I  C  R   H  V  Q  Y   A  T  N   N  G  N   I  R  S  A

 721  atcactatct tcccacagag gactgacggg aagcatgatt tccgtgtttg gaacagccag
        I  T  I   F  P  Q   R  T  D  G   K  H  D   F  R  V   W  N  S  Q

 781  ctcatccgat atgctggata tcaaatgcca gatgggtctg tcctaggagg ccctgcaagt
        L  I  R   Y  A  G   Y  Q  M  P   D  G  S   V  L  G   G  P  A  S

 841  gtggagttca caaagttgtg cattgatctt gggtggaagc cgaaatatgg ccgctttgat
        V  E  F   T  K  L   C  I  D  L   G  W  K   P  K  Y   G  R  F  D

 901  gtagttccac tcattctcca agcaaacggc caagatccag aaatatttga attcccgcca
        V  V  P   L  I  L   Q  A  N  G   Q  D  P   E  I  F   E  F  P  P

 961  aaaattatcc ttgaagtgcc aatggagcat ccaaagtatg aatggtttaa ggagttagat
        K  I  I   L  E  V   P  M  E  H   P  K  Y   E  W  F   K  E  L  D

1021  ctgaaatggt acgcgctgcc tgctgttgcc aacatgctcc ttgaggtggg aggtctggaa
        L  K  W   Y  A  L   P  A  V  A   N  M  L   L  E  V   G  G  L  E

1081  tttactgcgt gtcctttcaa tggctggtac atggggacag agataggagt gcgagacttc
        F  T  A   C  P  F   N  G  W  Y   M  G  T   E  I  G   V  R  D  F

1141  tgtgatgtgc agcggtacaa tatcctgaag gaggtaggaa gaagaatggg actggaaaca
        C  D  V   Q  R  Y   N  I  L  K   E  V  G   R  R  M   G  L  E  T

1201  aacaaacttt catcactatg gaaagaccga gctgttgtag agataaatgt ggctgtgctt
        N  K  L   S  S  L   W  K  D  R   A  V  V   E  I  N   V  A  V  L

1261  tatagcttcc aaaaacaaaa cgttactatc atggatcacc actcagctgc tgaatccttc
        Y  S  F   Q  K  Q   N  V  T  I   M  D  H   H  S  A   A  E  S  F

1321  atgaaatata tgcagaatga gtaccgtgtg cgaggaggct gccccgctga ctgggtgtgg
        M  K  Y   M  Q  N   E  Y  R  V   R  G  G   C  P  A   D  W  V  W
```

```
1381  attgtacctc ctatgtcagg gagcataact cccgtgttcc accaggagat gttgaactat
        I  V  P   P  M  S   G  S  I  T   P  V  F   H  Q  E   M  L  N  Y

1441  gtcctcactc ccttctttta ctaccaggtg gatgcatgga aaacacacat ctggcatgat
        V  L  T   P  F  F   Y  Y  Q  V   D  A  W   K  T  H   I  W  H  D

1501  gagtctcaga ggccaatgaa aagagaaata aaattgagtg tcttggcaaa ggctgtactc
        E  S  Q   R  P  M   K  R  E  I   K  L  S   V  L  A   K  A  V  L

1561  tttgcatctt cactcatgca aaaagcaatg gcaacaaggt ccaaggtcac tgtgatctat
        F  A  S   S  L  M   Q  K  A  M   A  T  R   S  K  V   T  V  I  Y

1621  gcaacagaga ctgggaaatc tgaaacacta gccaacaatc tgtgcagctt gttcagctgt
        A  T  E   T  G  K   S  E  T  L   A  N  N   L  C  S   L  F  S  C

1681  gccttcaaca ctaagattct gtgcatggat gaatacaaca tcagtgacct ggaaaaagaa
        A  F  N   T  K  I   L  C  M  D   E  Y  N   I  S  D   L  E  K  E

1741  acgcttcttt tagtggttac tagcactttt ggaaatggag attctccaaa taatggaaag
        T  L  L   L  V  V   T  S  T  F   G  N  G   D  S  P   N  N  G  K

1801  acattgaaga actccttgct caccctgaaa ttgctggaaa agaaaattag atatgccgtg
        T  L  K   N  S  L   L  T  L  K   L  L  E   K  K  I   R  Y  A  V

1861  tttggtttgg gatctaccat gtatcctgaa ttctgtgcct ttgctcatgc cattgaccaa
        F  G  L   G  S  T   M  Y  P  E   F  C  A   F  A  H   A  I  D  Q

1921  aaactggccc aactaggggc ttcacagctc attccaatag gtgaaggaga tgaactcaat
        K  L  A   Q  L  G   A  S  Q  L   I  P  I   G  E  G   D  E  L  N

1981  gggcaagaag aagcctttcg cacatgggca gtcagtgcat tcaagactgc ctgtgacatt
        G  Q  E   E  A  F   R  T  W  A   V  S  A   F  K  T   A  C  D  I

2041  tttaacatcc gtgggaaaaa cagtattcag ttgcctaaga tgtatacctc agatgaaatc
        F  N  I   R  G  K   N  S  I  Q   L  P  K   M  Y  T   S  D  E  I

2101  tggaatccta agaaatacag gatactgcat gagtctcaaa ccatggactt ggctaaagca
        W  N  P   K  K  Y   R  I  L  H   E  S  Q   T  M  D   L  A  K  A

2161  cttacaaaca ttcatggaaa ggatgtaatt cccatgaagc tgaaattcag acagaatctt
        L  T  N   I  H  G   K  D  V  I   P  M  K   L  K  F   R  Q  N  L

2221  cagagtttaa aatccagtcg cgttaccatt ctagttaagc tttcctgtga gactaatcag
        Q  S  L   K  S  S   R  V  T  I   L  V  K   L  S  C   E  T  N  Q

2281  gaagtgcgct acctgcccgg agaacatatt gggattttcc caggcaacca gccagaactg
        E  V  R   Y  L  P   G  E  H  I   G  I  F   P  G  N   Q  P  E  L

2341  gtccatggcc tcatttcacg tgttaaggat gcccctccag ctgatcagac tgtcagactt
        V  H  G   L  I  S   R  V  K  D   A  P  P   A  D  Q   T  V  R  L

2401  gaaacctgca ctgaaggtgg ctactggaca agtgacaaaa agattccagc ctgcacactc
        E  T  C   T  E  G   G  Y  W  T   S  D  K   K  I  P   A  C  T  L

2461  tcccaagctt tgacatattt gcttgatatc actactccac cctcccaaca actgctaaaa
        S  Q  A   L  T  Y   L  L  D  I   T  T  P   P  S  Q   Q  L  L  K

2521  aagatttccc agctggtaac agcggaagga gacaaacaga gactggaagt tctatgtcat
        K  I  S   Q  L  V   T  A  E  G   D  K  Q   R  L  E   V  L  C  H

2581  aacacagaag aatacaataa atggaagttt tacaacagcc caaacatcct ggaggtcctg
        N  T  E   E  Y  N   K  W  K  F   Y  N  S   P  N  I   L  E  V  L

2641  gaagagtttc cttctgctga agtctcaaca gctttcttgt tgactcagct gccatttctg
        E  E  F   P  S  A   E  V  S  T   A  F  L   L  T  Q   L  P  F  L
```

```
2701  aaacccaggt actgttctgt cagttcttcc tgtgacatga cacccagaga gattcatctg
        K  P  R   Y  C  S   V  S  S  S   C  D  M   T  P  R   E  I  H  L

2761  acagttgcag tagtcaatta caggacaaga gatggacaag ggcctttgca tcatggagtt
        T  V  A   V  V  N   Y  R  T  R   D  G  Q   G  P  L   H  H  G  V

2821  tgcagcacat ggctgaataa aataagtctc aatgaagtag tgccatgctt tgtgcgcagt
        C  S  T   W  L  N   K  I  S  L   N  E  V   V  P  C   F  V  R  S

2881  ggtaacggat tccagctccc aaaggagcca accaagccct gcattctgat cggcccagga
        G  N  G   F  Q  L   P  K  E  P   T  K  P   C  I  L   I  G  P  G

2941  acaggaattg ctccattcag aagtttctgg cagcaacgcc tctatgactt ggaaaagaga
        T  G  I   A  P  F   R  S  F  W   Q  Q  R   L  Y  D   L  E  K  R

3001  gggatcaaag gtggtgacat gacattattg tttggctgcc ggcagccaga catggatcat
        G  I  K   G  G  D   M  T  L  L   F  G  C   R  Q  P   D  M  D  H

3061  atctacagag aagaaacaga agaaatgaag aggaaaggag ttctgaaaga agtttttaca
        I  Y  R   E  E  T   E  E  M  K   R  K  G   V  L  K   E  V  F  T

3121  gcttactcca ggcaacctgg ccaagctaaa gtctatgttc aagacattct tcaaaacaag
        A  Y  S   R  Q  P   G  Q  A  K   V  Y  V   Q  D  I   L  Q  N  K

3181  ttggaagcca aagtgtgcaa tctcttgcat aaagaggatg gacatctgta tgtctgtgga
        L  E  A   K  V  C   N  L  L  H   K  E  D   G  H  L   Y  V  C  G

3241  gatgtacgca tggccaggga tgttgctcag actttgaaag ggatacttgt aaaaaatctg
        D  V  R   M  A  R   D  V  A  Q   T  L  K   G  I  L   V  K  N  L

3301  aacctcacag agcagcaggc agaagagtat ttcttcgagc taaagagcca aaaacgttac
        N  L  T   E  Q  Q   A  E  E  Y   F  F  E   L  K  S   Q  K  R  Y

3361  catgaagata tatttggggc tgtgttccca catgaagtca aaagagatgt aaaagctttg
        H  E  D   I  F  G   A  V  F  P   H  E  V   K  R  D   V  K  A  L

3421  caacccatca gtccaagcac aaattaa
        Q  P  I   S  P  S   T  N  -
```

Figure 4.3. Nucleotide and predicted amino acid sequence of DkiNOS. Full length DkiNOS (3447 bp) was obtained from LPS activated splenic leukocytes using a combination of degenerative primers and RLM 3-prime RACE (Invitrogen). The nucleotide sequence was subsequently deposited to GeneBank (accession No. FJ966247) and is shown here with the predicted amino acid translation.

```
DKINOS          MLCPWQFAFKPHAAKNRSSKVTDINNNVEKD--MKINGLVKDDAKLHDLIKKQKEKLPIV 58
CHINOS          MLCPWQFAFKPHAVKNQSSEEKDINNNVEKD--VKVHSFVKDDAKLHSLSKKQMKMSPII 58
HUINOS          MACPWKFLFRVKSYQGDLKEEKDINNNVEKTPGAIPSPTTQDDPKSH---KHQNGFPQFL 57
                * ***:* *: :: :.  .: .******         .:**.* *   *:*     ::

DKINOS          T-SAEKPPENGIKASNQISRCPRHVKVRNLENGSSFLDTLHLTAKEVINCRTKACQGALM 117
CHINOS          T-SAEKHPQNGIKASNQISRCPRHVKVRNMENGSSLLDTLHLTAKEVINCRTRACQGALM 117
HUINOS          TGTAQNVPESLDKLHVTPSTRPQHVRIKNWGNGEIFHDTLHHKATSDISCKSKLCMGSIM 117
                * :*:: *:.  *     *  *:**:::*  **. : **** .*.. *.*::: * *::*

DKINOS          TPKGLVRGTRDGPVPQAELLPQAIDFVKQYYSSFKESKIEEHLARLETVTKEIETTGTYH 177
CHINOS          TPKGLVRSTRDGPVPPAELLPQAVDFVKQYYSSFKELKIEEHLARLETVTKEIETTGTYH 177
HUINOS          NSKSLTRGPRDKPTPVEELLPQAIEFINQYYGSFKEAKIEEHLARLEAVTKEIETTGTYQ 177
                ..*.*.*..** *.*  ******::*::***.**** **********:**********:

DKINOS          LTQDELIFAAKQAWRNAPRCIGRIQWSNLQVFDARDCKTAKEMFEHICRHVQYATNNGNI 237
CHINOS          LTKDELIFAAKQAWRNAPRCIGRIQWSNLQVFDARDCKTAKEMFEYICRHIQYATNNGNI 237
HUINOS          LTLDELIFATKMAWRNAPRCIGRIQWSNLQVFDARSCSTASEMFQHICRHILYATNSGNI 237
                ** ******:* **********************.*.**.***::****: ****.***

DKINOS          RSAITIFPQRTDGKHDFRVWNSQLIRYAGYQMPDGSVLGGPASVEFTKLCIDLGWKPKYG 297
CHINOS          RSAITIFPQRTDGKHDFRVWNSQLIRYAGYQMPDGSVIGDPASVEFTKLCIELGWKPKYG 297
HUINOS          RSAITVFPQRNDGKHDFRIWNSQLIRYAGYQMPDGTIRGDPATLEFTQLCIDLGWKPRYG 297
                *****:****.*******:*****************:: *.**::***:***:*****:**

DKINOS          RFDVVPLILQANGQDPEIFEFPPKIILEVPMEHPKYEWFKELDLKWYALPAVANMLLEVG 357
CHINOS          RFDVVPLILQANGQDPEIFEYPPEIILEVPMEHPKYEWFKELDLKWYALPAVANMLLEVG 357
HUINOS          RFDVLPLVLQAHGQDPEVFEIPPDLVLEVTMEHPKYEWFQELGLKWYALPAVANMLLEVG 357
                ****:**:***:*****:** **.::***.*********:**.*****************

DKINOS          GLEFTACPFNGWYMGTEIGVRDFCDVQRYNILKEVGRRMGLETNKLSSLWKDRAVVEINV 417
CHINOS          GLEFTACPFNGWYMGTEIGVRDFCDVQRYNILKEVGRRMGLESNKLASLWKDRAVVEINV 417
HUINOS          GLEFPACPFNGWYMGTEIGVRDLCDTQRYNILEEVGRRMGLGTHTLASLWKDRAVTEINA 417
                ****.*****************:**.******:******** ::.*:*********.***.

DKINOS          AVLYSFQKQNVTIMDHHSAAESFMKYMQNEYRVRGGCPADWVWIVPPMSGSITPVFHQEM 477
CHINOS          AVLHSFQKQNVTIMDHHSAAESFMKYMQNEYRVRGGCPADWVWIVPPMSGSITPVFHQEM 477
HUINOS          AVLHSFQKQNVTIMDHHTASESFMKHMQNEYRARGGCPADWIWLVPPVSGSITPVFHQEM 477
                ***:**************:*:*****:******.********:*:***:************

DKINOS          LNYVLTPFFYYQVDAWKTHIWHDESQRPMKREIKLSVLAKAVLFASSLMQKAMATRSKVT 537
CHINOS          LNYVLTPFFYYQVDAWKTHIWHDETRRPKKREIKLSILAKAVLLASLLLQKTMAARPKVT 537
HUINOS          LNYVLSPFYYYQIEPWKTHIWQDEKLRPRRREIRFTVLVKAVFFASVLMRKVMASRVRAT 537
                *****:**:***::.******:**. ** :***::::*.***::** *::*.**:* :.*

DKINOS          VIYATETGKSETLANNLCSLFSCAFNTKILCMDEYNISDLEKETLLLVVTSTFGNGDSPN 597
CHINOS          VIYATETGKSETLANSLCSLFSCAFNTKILCMDEYNISDLEKETLLLVVTSTFGNGDSPN 597
HUINOS          VLFATETGKSEALARDLAALFTYAFNTKVVCMEQYKANTLEEEQLLLVVTSTFGNGDCPS 597
                *:*********:**..*.:**: *****::**::*: . **:* *************.*.

DKINOS          NGKTLKNSLLTLKLLEKKIRYAVFGLGSTMYPEFCAFAHAIDQKLAQLGASQLIPIGEGD 657
CHINOS          NGKTLKNSLLTLKLLRKNIRYAVFGLGSTMYPEFCAFAHAIDQKLSQLGALQLTPVGEGD 657
HUINOS          NGQTLKKSLFMMKELGHTFRYAVFGLGSSMYPQFCAFAHDIDPKLSHLGASQLAPTGEGD 657
                **:***:**: :* * :.:*********:***:****** ** **::*** ** * ****

DKINOS          ELNGQEEAFRTWAVSAFKTACDIFNIRGKNSIQLPKMYTSDEIWNPKKYRILHESQTMDL 717
CHINOS          ELNGQEEAFRTWAVTAFKTACDIFDIRGKNSIQLPEIYTSDDSWNPKKHRIVYDSQTMDL 717
HUINOS          ELSGQEDAFRSWAVQTFRAACETFDVRSKHCIQIPKRYTSNATWEPEQYKLTQSPEPLDL 717
                **.***:***:*** :*::**: *::*.*:.**:*: ***:  *:*::::: ..:.:**

DKINOS          AKALTNIHGKDVIPMKLKFRQNLQSLKSSRVTILVKLSCETNQEVRYLPGEHIGIFPGNQ 777
CHINOS          TKALSDIHGKNVIPMKLKFRQNLQSLKSSRVTILVKLSCETNQEVHYLPGEHIGISPGNQ 777
HUINOS          NKALSSIHAKNVFAMRLKSLQNLQSEKSSRTTLLVQLTFEGSRGPSYLPGEHLGIFPGNQ 777
                 ***:.**.*:*:.*:**  ***** ****.*:**:*: * .:   ******:** ****
```

```
DKINOS          PELVHGLISRVKDAPPADQTVRLETCTEGG-YWTSDKKIPACTLSQALTYLLDITTPPSQ 836
CHINOS          PELVHGLIARVKDAPPADQTIRLETCTEGG-YWASEKKIPACTLSQALTYLLDITTPPTQ 836
HUINOS          TALVQGILERVVDCSSPDQTVCLEVLDESGSYWVKDKRLPPCSLRQALTYFLDITTPPTQ 837
                . **:*:: ** *....***: **.  *.* **..:*::*.*:* *****:*******:*

DKINOS          QLLKKISQLVTAEGDKQRLEVLCHNTEEYNKWKFYNSPNILEVLEEFPSAEVSTAFLLTQ 896
CHINOS          QLLKKLSQLVTAEGDKQRLEVLCHSTEEYNKWKFYNRPNILEVLEEFPSAEVSTAFLLTQ 896
HUINOS          LQLHKLARFATEETHRQRLEALCQ-PSEYNDWKFSNNPTFLEVLEEFPSLRVPAAFLLSQ 896
                  *:*:::::.* * .:****.**: ..***.*** * *.:********* .*.:****:*

DKINOS          LPFLKPRYCSVSSSCDMTPREIHLTVAVVNYRTRDGQGPLHHGVCSTWLNKISLNEVVPC 956
CHINOS          LPLLKPRYYSVSSSCDMTPREIHLTVAVVNYRTRDGQGPLHHGVCSTWLNKIALNETVPC 956
HUINOS          LPILKPRYYSISSSQDHTPSEVHLTVAVVTYRTRDGQGPLHHGVCSTWINNLKPEDPVPC 956
                **:***** *:*** * ** *:*******.******************:*::  :: ***

DKINOS          FVRSGNGFQLPKEPTKPCILIGPGTGIAPFRSFWQQRLYDLEKRGIKGGDMTLLFGCRQP 1016
CHINOS          FVRSADGFRLPKEPAKPCILIGPGTGIAPFRSFWQQRLYDLEKKGIKGGDMILLFGCRHP 1016
HUINOS          FVRSVSGFQLPEDPSQPCILIGPGTGIAPFRSFWQQRLHDSQRRGLKGGRMTLVFGCRHP 1016
                **** .**:**::*::*********************:* :::*:*** * *:****:*

DKINOS          DMDHIYREETEEMKRKGVLKEVFTAYSRQPGQAKVYVQDILQNKLEAKVCNLLHKEDGHL 1076
CHINOS          DMDHIYKEEVEEMKRKGVLKEVFTAYSRQPGQPKVYVQDILQNELETKVCNILHKEEGHL 1076
HUINOS          EEDHLYQEEMQEMVRKGVLFQVHTGYSRLPGKPKVYVQDILQKELADEVFSVLHGEQGHI 1076
                : **:*:** :** ***** :*.*.*** **:.*********::*  :* .:** *:**:

DKINOS          YVCGDVRMARDVAQTLKGILVKNLNLTEQQAEEYFFELKSQKRYHEDIFGAVFPHEVKRD 1136
CHINOS          YVCGDVRMARDVAQTLKRMLVKKLNHTEQQAEEYFFQLKSQKRYHEDIFGAVFPHEVKRI 1136
HUINOS          YVCGDVRMARDVATTLKKLVAAKLNLSEEQVEDYFFQLKSQKRYHEDIFGAVFSYGVKKG 1136
                ************* *** ::. :** :*:*.*:***:****************.: **:

DKINOS          VKALQPISPSTN 1148
CHINOS          ------------
HUINOS          NALEEPKGTRL- 1147
```

Figure 4.4. Duck iNOS amino acid sequence alignment. The open reading frame of the duck iNOS (DKINOS) sequence was analyzed through the Clustal W program and the predicted amino acid translation is shown in comparison to that of human (HUINOS) and chicken (CHINOS) iNOS. An asterisk (*) indicates identical amino acid residues while a colon (**:**) indicates a strongly conserved amino acid substitution and a dot (**.**) represents a weakly conserved amino acid substitution. Dashed sections (-) represent gaps introduced to optimize the alignment and numbers represent aa number. The nucleotide sequence was subsequently deposited to GeneBank (accession No. FJ966247).

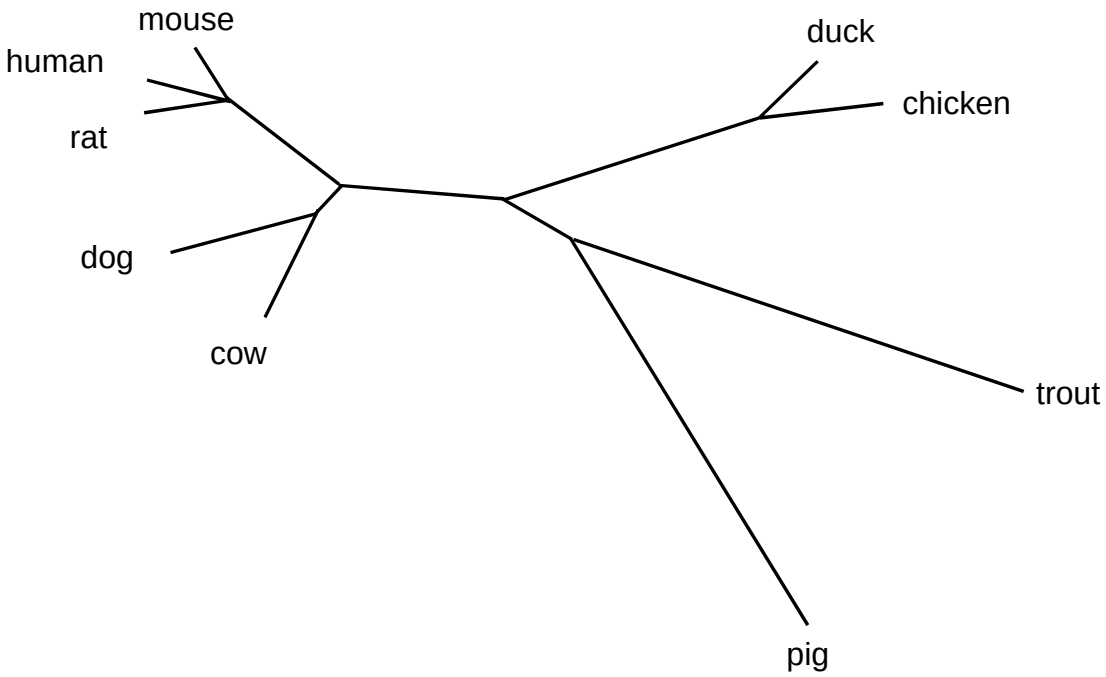

Figure 4.5. Unrooted phylogenetic tree of the iNOS gene from a range of species. An unrooted tree was constructed based on 1000 bootstrapped amino acid alignments of various iNOS members using the neighbour joining method. GeneBank accession numbers: chicken (*Gallus gallus*) NM_20496, cow (*Bos taurus*) DQ_676956, mouse (*Mus musculus*) NM_010927, dog (*Canine*) NM_001003186, rat (*Rattus novegicus*) NM_012611, human (Homo sapien) NM_000625, trout (*Oncorhynchus mykiss*) AJ_300555, pig (*Sus suscrofa*) NM_001143690.

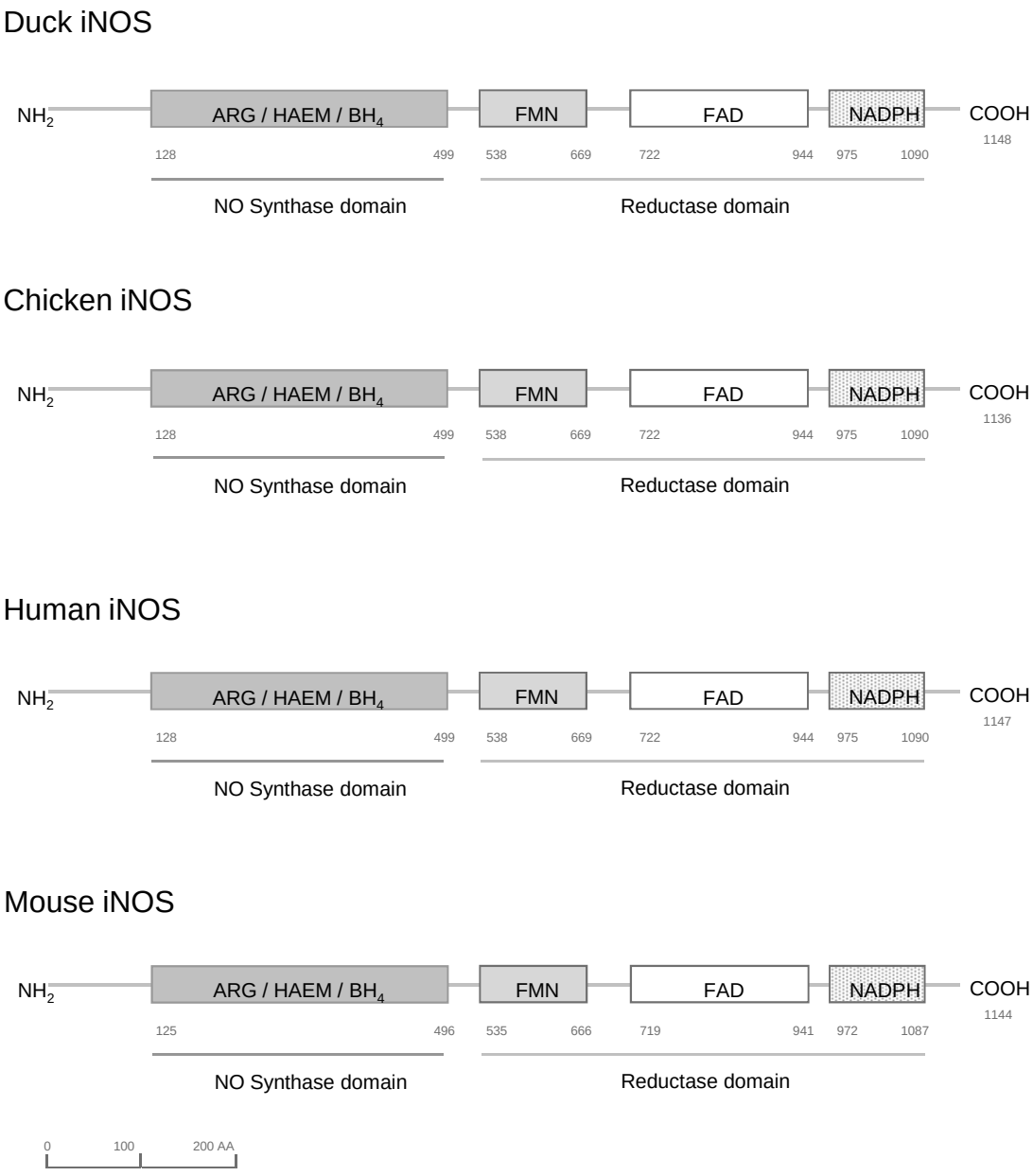

Figure 4.6. Comparative predicted domain organisation for DkiNOS and selected iNOS domains from a range of species. SMART analysis software was used to predict the domain structure of selected iNOS sequences (genebank accession numbers: chicken (*Gallus gallus*) NM_20496, mouse (*Mus musculus*) NM_010927, human (Homo sapien) NM_000625 and duck (*Anas Anas*) FJ_966247. Modelling showed DkiNOS contained both the NO synthase domain and the reductase domain conserved in other species.

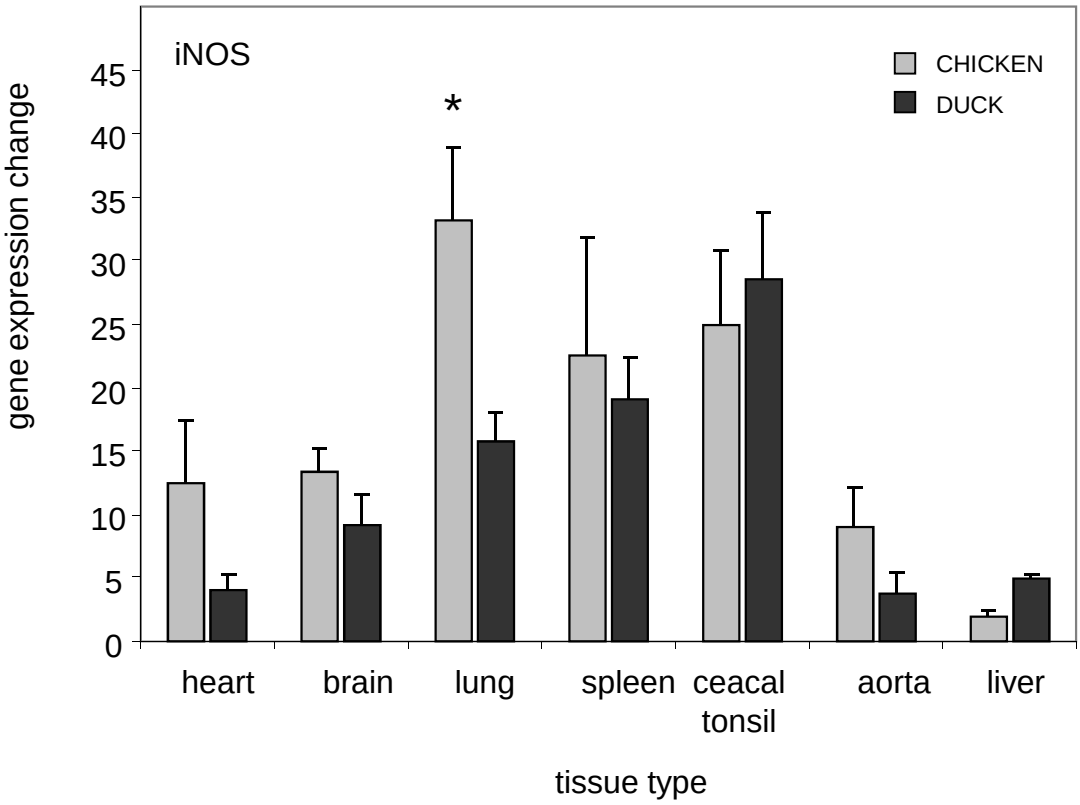

Figure 4.7. Comparison of iNOS levels in un-stimulated chicken and duck tissues measured by Q-RTPCR. iNOS mRNA expression was measured by Q-RTPCR in chicken and duck organs. GAPDH was used as a housekeeping gene to standardize results. Expression change is shown as the basal fold change increase of iNOS relative to muscle tissue. Experiments were performed in triplicate with the data representative of 3 independent experiments, error bars display SE. An asterisk indicates statistically significant differences of means with $p<0.05$.

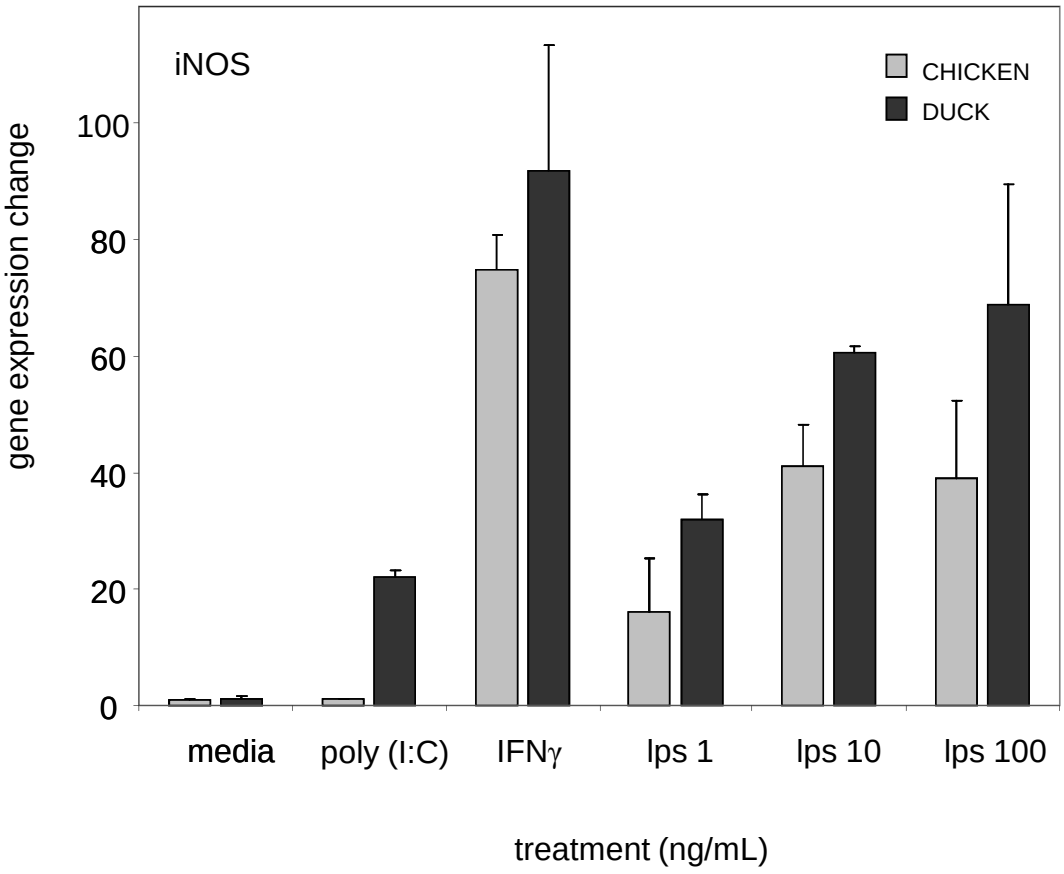

Figure 4.8. iNOS expression levels in activated chicken and duck splenocytes. Chicken and duck splenocytes were cultured for 20 hours with a range of concentrations of LPS (1, 10, and 100μg/mL) or a single concentration (10 μg/mL) of poly(I:C) or recombinant chicken IFNγ protein. RNA was collected from the cells and iNOS gene levels determined by Q-RTPCR. Obtained iNOS mRNA values were normalized to GAPDH and expression levels are shown as mean fold expression change relative to unstimulated controls. Experiments were performed in triplicate and data are representative of 3 independent experiments, error bars shoe SE.

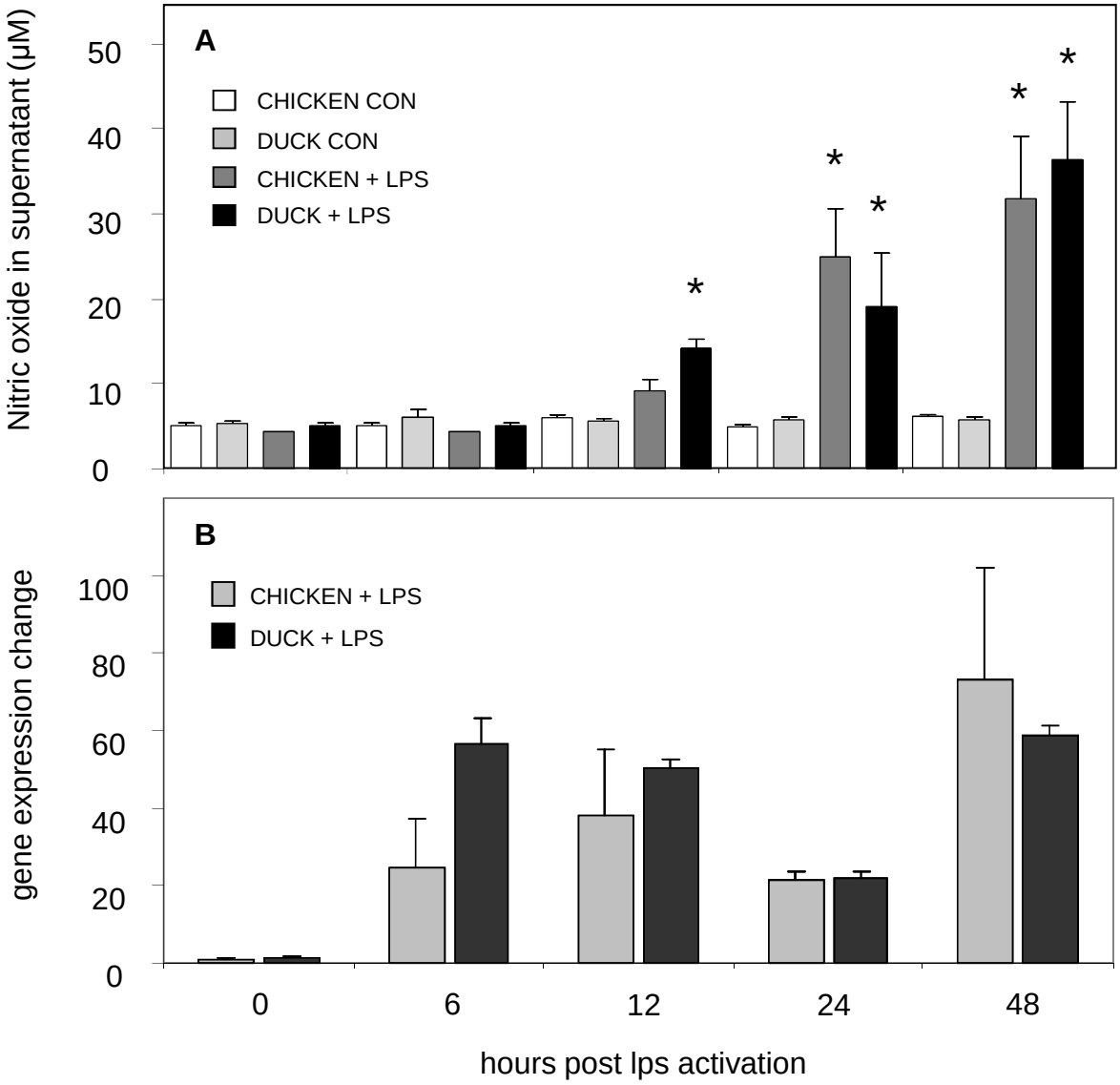

Figure 4.9. NO and iNOS levels increase in a similar fashion following LPS stimulation of chicken and duck splenocytes. Chicken and duck splenocytes were cultured over a time-course of 6 - 48 hours with a concentration of LPS (10 μg/mL). **(A)** NO levels, measured by a colorimetric assay, were increased in a similar fashion in chicken and duck cell culture supernatants. **(B)** iNOS gene expression levels, measured by Q-RTPCR, were increased in chicken and duck splenocytes. Levels of iNOS gene expression were determined in conjunction with the house keeping gene GAPDH and expression levels are shown as mean fold expression change relative to un-stimulated splenocytes. Experiments were performed in triplicate and data are representative of 3 independent experiments, error bars display SE. An asterisk indicates statistically significant differences of means with $p<0.05$.

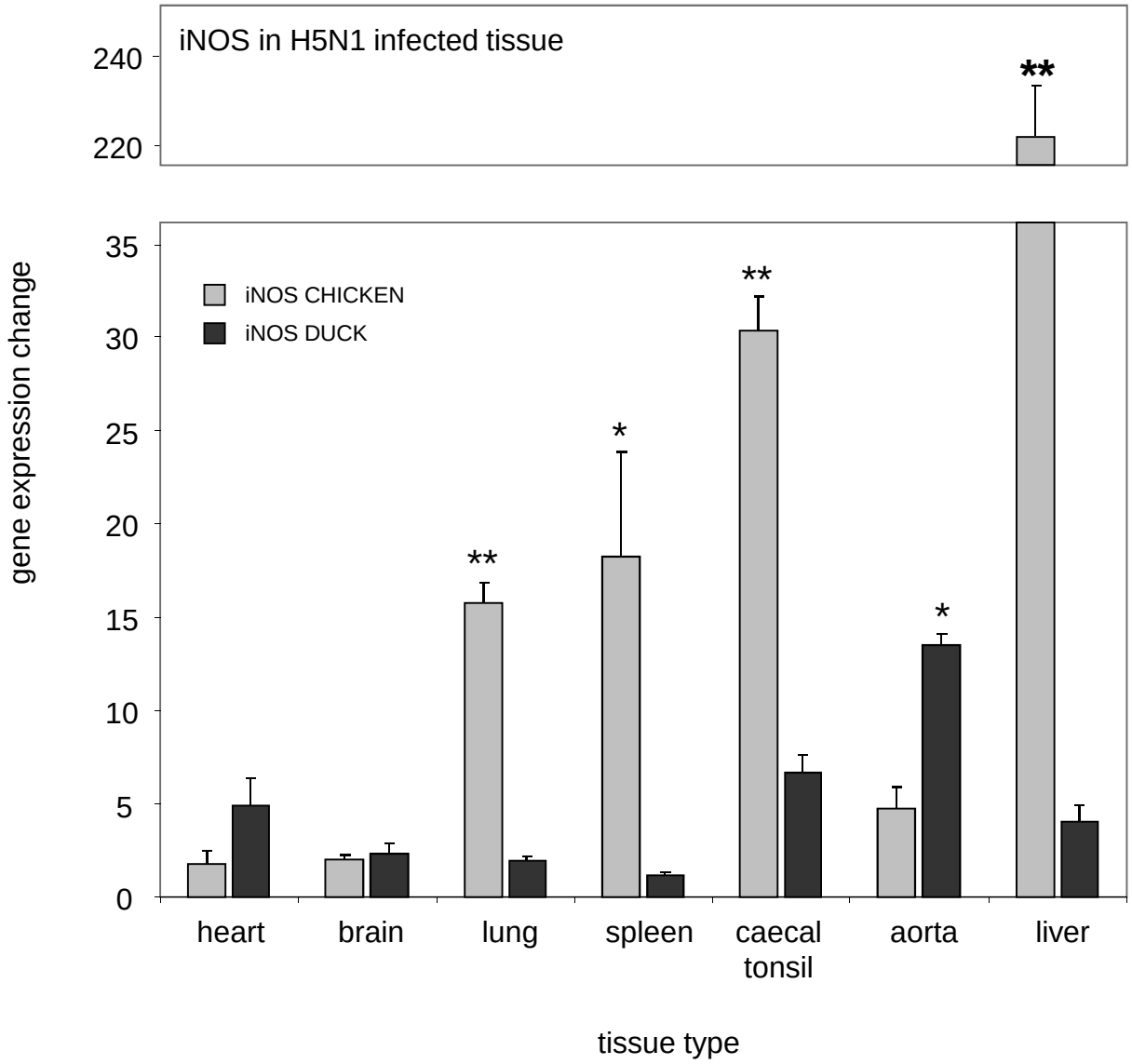

Figure 4.10 H5N1 influenza infection increases iNOS expression at higher levels in chickens compared to ducks. Q-RTPCR was carried out on various tissues from chickens and ducks infected with H5N1 and compared to uninfected controls. Chicken samples were taken at 24 h.p.i, duck samples were taken at 72 hp.i. Data represents the mean fold expression of either duck or chicken iNOS relative to each uninfected tissue type with GAPDH used as the housekeeping gene. Displayed values are the mean of 2 experiments with 4 birds in each group, error bars show SE. Single asterisk indicates statistically significant differences of means with $p<0.05$, double asterisk indicates statistically significant differences of means with $p<0.01$.

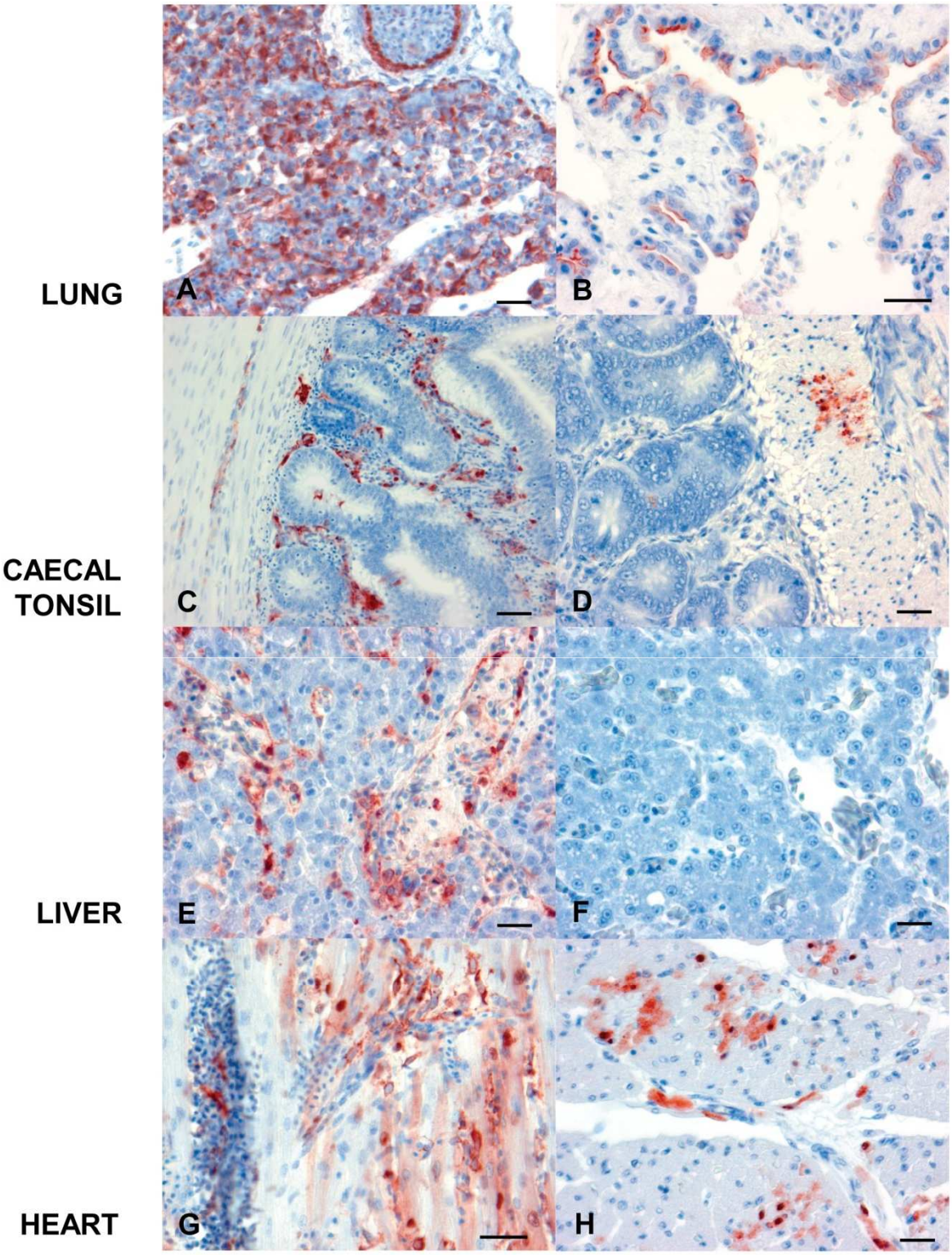
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Figure 4.11. H5N1 infection appears more widespread in chicken tissues in comparison to duck tissues. IHC for H5N1 antigen, left-hand panels shows staining in chicken tissues, right-hand panels shows staining in duck tissues **(A)** Chicken lung 24 (h.p.i), with IHC stain showing H5N1 viral antigen as red/brown colour. **(B)** Duck lung 72 h.p.i., H5N1 antigen was less prevalent in duck lung tissue than in chicken. H5N1 antigen was detected in single cells scattered within the lung parenchyma and in the hyaline cartilage. **(C)** Chicken caecal tonsil 24 h.p.i., with H5N1 in caecal lymphoid follicles and submucosa. **(D)** Duck caecal tonsil 72 h.p.i., with similar H5N1 antigen staining. **(E)** Chicken liver tissue 24 h.p.i., with severe H5N1 antigen staining. **(F)** Duck liver 72 h.p.i., showed no signs of viral antigen. **(G)** Chicken heart tissue 24 h.p.i. showed H5N1 staining in the myocardium and typically near blood vessels. **(H)** Duck heart tissue 72 h.p.i., with IHC H5N1 antigen staining the myocardium. All scale bars = 50 μm.

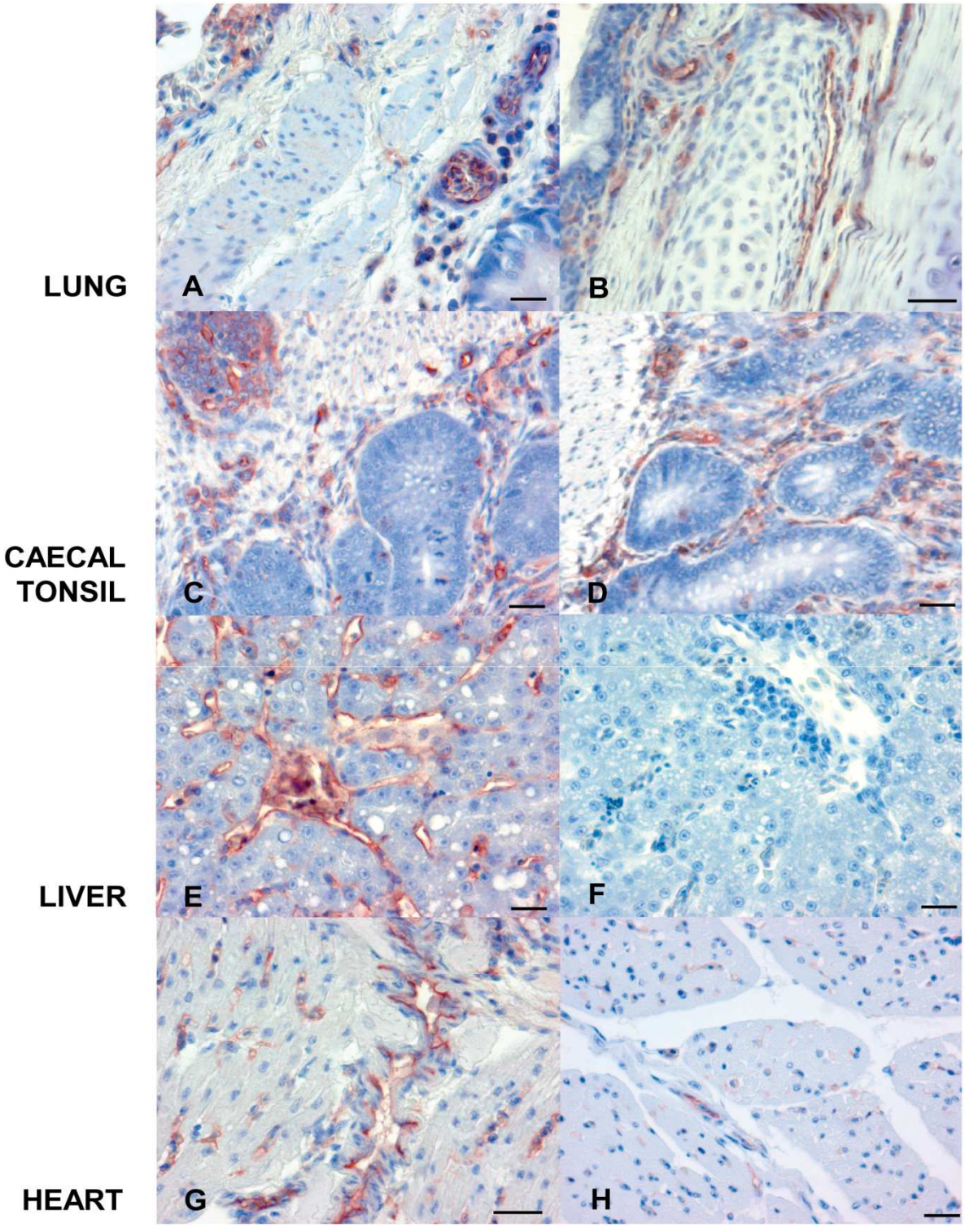
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Figure 4.12. During H5N1 influenza infection iNOS appears more highly expressed in chicken tissues when compared to ducks. IHC tissue section staining for iNOS, with left-hand panels showing chicken tissues and right-hand panels showing duck tissues. **(A)** Chicken lung 24 h.p.i., IHC stain (red/brown) for iNOS mainly in the submucosa. **(B)** Duck lung 72 h.p.i., IHC iNOS staining in the submucosa. **(C)** Chicken caecal tonsil 24 h.p.i., with iNOS in lymphoid follicles, the submucosa and lymphoid aggregates. **(D)** Duck caecal tonsil 72 h.p.i., IHC staining of iNOS mainly in the submucosa. **(E)** Chicken liver tissue 24 h.p.i., iNOS present in the sinusoidal endothelium. **(F)** Duck liver 72 h.p.i., little or no iNOS detected. **(G)** Chicken heart 24 h.p.i., iNOS in the myocardium and proximity to blood vessels (presumably endothelial cells). **(H)** Duck heart 72 h.p.i., with low levels of IHC staining for iNOS. All scale bars = 50 μm.

Chapter 5

Madindoline-A and Galiellalactone impact on the IL6 response in chickens.

5.1 Introduction

A range of viral and bacterial infections activate inflammation in the chicken. It may be argued that the pathology associated with increased inflammation often depends on the immune action that the host produces in response to infection (Glisson, 1998; Svanborg *et al.,* 1999). Considering the association of inflammatory molecules with severe influenza infection, observed in Chapters 3 and 4 of this thesis, there is a need to better understand how inflammation and inflammatory molecules, like IL6 and the IL6 pathway, may contribute to disease severity during infection. Infections in poultry by *Escherichia coli* cause a variety of disease syndromes (Pourbakhsh *et al.,* 1997), including respiratory tract and yolk sac infection, scepticemia, omphalitis and cellulitis (MacOwan *et al.,* 1984; Morley *et al.,* 1984; Peighambari *et al.,* 1995). Many of these symptoms are normally attributed to the direct effects of activation by LPS, a component of the bacterial cell wall (Westphal, 1975) and are probably mediated by cytokines such as IL6 (De Boever *et al.,* 2009). Although the systemic release of inflammatory mediators may cause many symptoms and signs of infection (Chappell *et al.,* 2009), local cytokine production can also lead to the recruitment of inflammatory cells and lymphocytes that participate directly in the clearance of bacteria from sites of infection (Withanage *et al.,* 2005). In this way, inflammation is a two-edged sword, crucial for antimicrobial defence but also responsible for disease associated tissue destruction (Svanborg *et al.,* 1999). Therefore, LPS activation of the chicken inflammatory response may be a good model for testing inhibitors of the inflammatory pathway. By reducing the levels of inflammation during diseases such as H5N1 influenza the host may be able to mount a more effective immune response.

One such cytokine which may be involved in contributing to an excessive inflammatory response is IL6 (Hodge *et al.,* 2005). IL6 is a multifunctional cytokine with immunoregulatory and proinflammatory roles (Stankiewicz *et al.,* 2002; Enjuanes *et al.,* 2008). Following severe infection, such as that of H5N1 influenza virus, high levels of IL6 are found in serum and various tissues (Dinarello, 1989). Gram negative bacterial sepsis also induces high levels of IL6 and initial studies have manipulated the levels of IL6 to examine the correlation of its expression with this disease state (Starnes *et al.,* 1990; Dalrymple *et al.,* 1996). However, there is some conjecture as to whether the presence of IL6 is deleterious or beneficial during bacterial infection or following LPS activation (Heremans *et al.,* 1992). High serum levels of IL6 have also been observed in several other infectious diseases including malaria (Kern *et al.,* 1989), leishmania, dengue fever and highly pathogenic avian influenza (Karpala et al., ; Cheung et al., 2002) and are associated with increased severity during the disease period.

Furthermore, individuals with high circulating levels of IL6 often have increased organ damage in concert with an associated increase in IL6 stimulated genes (Kern *et al.,* 1989; Gao *et al.,* 2010). This may be due to the role IL6 plays in promoting acute phase reactants, such as SAA, AGP and CRP, which have the ability to contribute to chronic inflammation and cellular toxicity (Villa *et al.,* 1996; Olsson *et al.,* 1999; Shagdarsuren *et al.,* 2005). Following severe infection acute phase reactants, such as SAA, AGP and CRP may contribute to tissue damage in a myriad of ways, either through chemotaxis, reactive amyloidosis, or complement activation (Olsson *et al.,* 1999; Griselli *et al.,* 1999). Similarly, IL6 is associated with increased levels of free radicals following infection, such as iNOS, a gene know to upregulate NO production (Evans, 1995; Arstall *et al.,* 1999). As previously discussed, NO can have a cytotoxic effect and can cause damage through the build up of peroxynitrite (Hibbs *et al.,* 1988; Palmer *et al.,* 1992; Saleh *et al.,* 1998). These increased levels of IL6, SAA and iNOS may be responsible for promoting excessive inflammation and lead to celleular damage and therefore a better regulation of IL6 may be able to improve the immune response in chickens.

In mammals, modulation of IL6 has been demonstrated through the inhibition of the IL6/gp130 receptor complex and IL6 signalling pathway by various synthesised compounds (Hayashi *et al.,* 2002a; Hellsten *et al.,* 2008). Galiellalactone, a fungal metabolite, has been shown to inhibit the IL6 signalling pathway by interfering with Stat3 signalling, whilst the indole alkaloid, Madindoline-A binds competitively to gp130 to prevent homodimerization of the receptor complex (Hayashi *et al.,* 2002a; Hellsten *et al.,* 2008). In mice tumour models, Mad-A/Gal administrations showed a beneficial reduction of IL6 signalling and are potential candidates for helping improve IL6 driven inflammation in diseases such as cancer, alzheimers and arthritis (Hayashi *et al.,* 2002a; Hellsten *et al.,* 2008). Therefore, it was postulated that Mad-A/Gal administration may inhibit IL6 signalling in other species, such as the chicken. Therapeutics such as Mad-A/Gal, which are easy to deliver and can successfully reduce IL6 driven inflammation may also have benefits for the treatment of other prominent infections which are associated with increased inflammation, like avian influenza.

To better understand the role of IL6 during inflammatory interactions and to help develop strategies aimed at reducing the severity of H5N1 influenza virus infection, we investigated the modulation of IL6 in the chicken. Since Mad-A/Gal are as yet untested in chickens, we sought to conduct preliminary experiments using LPS as a model for increased inflammatory cytokine production. We aimed to investigate the effects of Mad-A/Gal on IL6 signalling and IL6 stimulated genes in the chicken. We observed that as in other species, chicken splenocytes treated with Mad-A/Gal showed attenuated IL6 responses following *Escherichia coli* LPS activation. Furthermore, in chickens administered an LPS model of sceptic shock, treatment with Mad-A/Gal decreased IL6 induction and reduced the levels of IL6 stimulated genes, such as the acute phase reactant, SAA and the free radical synthezising gene iNOS. Understanding how these inflammatory pathways can be better modulated may help in the development of tools to enhance the immune response and protect from disease.

5.2 Results

5.2.1 Chicken splenocytes activated with LPS have increased levels of IL6 and IL6 stimulated genes

Infections involving *Escherichia coli* have been shown to stimulate IL6 production in a range of cells (Kaiser *et al.,* 2000). To investigate the role of IL6 and the time-course of IL6 production, chicken splenocytes were treated with LPS, a major component of gram negative bacterial cell wall for a 24 hour period. LPS induced the production of IL6 gene expression to increase 100 fold compared to untreated cells (Fig. 5.1). Furthermore, the gene expression levels of SAA and iNOS were also raised following IL6 activation, up to 140 fold and 30 fold, respectively, after 12 hours of LPS treatment (Fig 5.1). Whilst IL6 appeared to be induced first, with a peak between 6 and 12 hours after LPS activation, SAA expression levels peaked later between 12 and 24 hours after LPS treatment.

5.2.2 A combination of Mad-A and Gal treatment reduces IL6, SAA and iNOS expression in chicken splenocytes activated with LPS

IL6 is known to help mediate the inflammatory response through a signalling complex consisting of the ligand binding IL6-Rα chain and the signal transducing component, gp130. In mammals the IL6 signalling pathway can be inhibited through the use of Mad-A and Gal (Hayashi *et al.,* 2002a; Hellsten *et al.,* 2008). To assess the IL6 inhibitory properties of Mad-A/Gal, chicken splenocytes were activated with LPS and the levels of IL6, SAA and iNOS measured by Q-RTPCR after 24 hours. A combination of Mad-A/Gal together showed the greatest levels of IL6 inhibition compared to splenocytes without Mad-A or Gal (Fig 5.2). Whilst Mad-A alone reduced IL6 expression levels, the levels of SAA and iNOS were only modestly reduced. In contrast, the administration of Gal alone reduced SAA and iNOS expression levels but only partially reduced IL6 levels, suggesting a combination of Mad-A/Gal as the most effective inhibition strategy. Furthermore, to examine how a dose titration of Mad-A/Gal treatment would impact chicken cells and to test for possible toxicity, splenocytes were activated with LPS and administered increasing

concentrations of Mad-A/Gal (0.75, 1.5, 15 and 30 μg/mL). After 24 hours of treatment, splenocytes populations appeared healthy and RNA was isolated for Q-RTPCR analysis. The gene expression levels of IL6, SAA and iNOS were examined and found to be downregulated in a dose dependant fashion (Fig 5.3A). The highest concentration of Mad-A/Gal (15 μg/mL Mad-A and 15 μg/mL Gal) appeared to show the greatest level of reduction in IL6 and iNOS gene expression. The reductions in SAA levels were similar between 15 μg/mL and 30 μg/mL Mad-A/Gal administration. Furthermore, the levels of NO in the supernatant of LPS activated splenocytes were found to be reduced following Mad-A/Gal administration (Fig 5.3B).

5.2.3 Reduced levels of IL6 and IL6 stimulated genes by Mad-A and Gal following in vivo LPS administration

To investigate the role of the proinflammatory cytokine IL6 in response to LPS mediated sepsis, chickens were administered a 2mg/kg dose of LPS. Chicken sera were examined 24 hours post treatment for the levels of IL6 and AGP protein and compared to non LPS control chickens. Chickens administered LPS without Mad-A/Gal had elevated levels of IL6 and AGP, whilst in comparison, chickens administered with a combination of Mad-A/Gal (at 0.10, 0.30 and 0.75 μg/kg) showed reduced IL6 levels comparable to control chickens (Fig 5.4A). The levels of the acute phase marker, AGP were also reduced by Mad-A/Gal administration (Fig 5.4B).

5.2.4 Reduced gene expression levels of IL6, SAA and iNOS but not IFNγ by Mad-A and Gal following LPS administration in chickens

To help establish what tissues types are sensitive to Mad-A/Gal administration following I.P injection in combination with LPS, we investigated the gene expression of IL6 and the induction of downstream IL6 stimulated genes. Using Q-RTPCR the levels of IL6, SAA, iNOS and IFNγ were measured in liver, lung and spleen samples (Fig 5.5). The levels of IL6 gene expression were increased in LPS administered chickens without Mad-A/Gal in all tissues examined. However, in chickens treated with Mad-A/Gal the levels of IL6 were reduced to levels comparable with those

observed in control chickens and in a dose dependant fashion. Similarly, the levels of SAA were increased in LPS administered chickens but comparably decreased in chickens treated with Mad-A/Gal, particularly in liver tissue. The levels of the NO inducing gene iNOS, were only moderately increased (between 1 to 5 fold increases) in lung and liver tissue. Furthermore, Mad-A/Gal treatment had limited impact in reducing iNOS expression levels, however, the non-IL6 stimulated gene, IFNγ, showed increased expression levels in LPS administered chickens with or without Mad-A/Gal treatment.

5.2.5 IL6 inhibition is associated with decreased CD4, CD8 and IgM cell types following LPS administration in chickens

Given the role IL6 plays in stimulating immune cells and its potential proliferative effect on B cells (Tosato *et al.,* 1988), we investigated the impact that IL6 modulation might have on lymphocyte populations following LPS administration in chickens. The results show that CD4+ and CD8+ lymphocyte populations are moderately reduced in PBMCs from both LPS treated chickens and LPS with Mad-A/Gal treated chickens in comparison to non LPS treated control chickens. A key feature of IL6 is to initiate IgM production (Goldstein *et al.,* 1993). The results show that following LPS administration, IgM populations increase in chicken PBMCs when compared to control chickens. In contrast, the population of IgM positive cells from chickens with LPS and Mad-A/Gal treatment is reduced to similar relative cell numbers as seen in control chickens (Fig 5.6).

5.3 Discussion

Following severe infection, such as that by H5N1 influenza virus, high levels of IL6 can be found in sera and a range of tissues (Cheung *et al.,* 2002; Makhija *et al.,* 2005). Given the difficulties of conducting HPAIV studies, we sought to use LPS as a model for increased inflammatory cytokine production, in order to test IL6 modulation.. The endotoxin (LPS) is a major component of the outer membrane of Gram-negative bacteria and a critical factor in the pathogenesis of Gram-negative sepsis. LPS induces

high levels of IL6 and an associated increase in IL6 stimulated genes, like acute phase reactants (Beutler *et al.,* 2003). This elevation of IL6 and accumulation of IL6 stimulated molecules is considered to be important in contributing to the severity of infection (Svanborg *et al.,* 1999). However, without a better knowledge of the mechanisms behind these chicken inflammatory pathways, the management of inflammatory diseases may continue to be difficult. Therefore, we aimed to investigate how the manipulation of IL6 signalling would impact on the inflammatory response during an LPS model of infection in the chicken.

IL6 signalling through the IL6/gp130 receptor complex is usually well regulated and part of a complex inflammatory feedback loop. One example of this is the role of IL6 in signalling IL17. Studies by Ogura *et al.*, (2008) demonstrate that IL6 not only functions upstream of IL17A but also acts as a downstream target of IL17A. Furthermore, IL17A together with IL6 triggers a positive-feedback loop of IL6 expression through the activation of NFκB and STAT3 (Ogura *et al.,* 2008). In mammals, Mad-A and Gal have been shown to inhibit the activities of IL6 through suppressing dimerization of the IL6 / IL6receptor / gp130 complex and through interfering with the IL6 STAT3 signalling pathway (Hayashi *et al.,* 2002a; Hellsten *et al.,* 2008). Moreover, in mice tumour models, Mad-A/Gal administration showed improved treatment of disease caused by excessive IL6 production and prevention of other refractory diseases (Hayashi *et al.,* 1996; Weidler *et al.,* 2000; Hellsten *et al.,* 2008). Given the need to better understand the role IL6 plays in contributing to excessive inflammation and since IL6 signalling, and to a degree IL6 production, was suppressed in mice by Mad-A/Gal treatment, we investigated whether Mad-A and Gal were able to inhibit IL6 signalling in chickens. Upon activation with LPS, chicken splenocytes responded rapidly by firstly upregulating IL6 mRNA expression, followed by the upregulation of SAA and iNOS mRNA expression. In comparison, when LPS activated splenocytes were administered Mad-A/Gal the levels of IL6, SAA and iNOS mRNA expression were significantly reduced. Therefore, our results confirm that chicken IL6, like mammalian IL6, is activated by LPS from *Escherichia coli* and following Mad-A/Gal administration the ability of IL6 to induce downstream inflammatory genes appears reduced, as the expression of the IL6 stimulated gene

SAA and AGP is decreased. Intriguingly, it appears that Mad-A/Gal inhibition of IL6 signalling may also impact on other downstream inflammatory molecules, such as the NO inducing gene iNOS. Finally, the reduction in both IL6 sera levels and IL6 gene expression levels across a range of tissues in Mad-A/Gal treated chickens following LPS administration may suggest that inhibition of IL6 signalling can impact on the production of IL6, possibly through a positive feedback mechanism (Sansone *et al.,* 2007).

As increased levels of IL6 have been associated with various infections and can result in the induction of acute phase genes and other inflammatory cytokines (Kern *et al.,* 1989; Kaiser *et al.,* 2000; Cheung *et al.,* 2002), we aimed to modulate the level of IL6 in chickens. Following intra-peritoneal injection of *Escherichia coli* LPS, as a model of IL6 induction, chickens were administered three different concentrations of Mad-A/Gal (0.10, 0.30 and 0.75 μg/kg) and markers of inflammation investigated. We observed that chickens treated with all three concentrations of Mad-A/Gal had reduced levels of IL6 protein in their sera compared to chickens without IL6 inhibitors. Moreover, the sera levels of the acute phase reactant AGP were moderately reduced in chickens treated with Mad-A/Gal in comparison to chickens without Mad-A/Gal treatment, although AGP levels were higher than those observed in control chickens. This suggests that Mad-A/Gal administration can decrease systemic levels of IL6 protein and may also contribute to the reduction of downstream acute phase genes. As the IL6 signalling pathway is not the only mechanism leading to acute phase gene induction (Conti *et al.,* 1995) it is not surprising that AGP levels are raised in comparison to those observed in non LPS treated chickens. One reason for this may be that LPS activates inflammatory molecules, such as IL1, which Mad-A/Gal appear unable to inhibit and in turn, IL1 may signal acute phase gene expression (Won *et al.,* 1991; Blanque *et al.,* 1999). Given that systemic IL6 levels in Mad-A/Gal treated chickens were comparable with non LPS administered control chickens, we sought to identify the whether the gene expression levels of IL6 were increased in a range of tissues.

To confirm that Mad-A/Gal treatment could impact on IL6 signalling in a range of chicken organs and to investigate the gene expression levels of inflammatory molecules associated with increased IL6 production, we measured the mRNA levels of IL6, SAA and iNOS. The levels of IFNγ were also measured to confirm LPS administration was inducing an inflammatory cytokine response. The IFNγ response to LPS administration has been well characterised in the chicken and in concert with IL6, is highly upregulated (Leshchinsky *et al.,* 2003; Sijben *et al.,* 2003; Okamura *et al.,* 2004; Tassiulas *et al.,* 2007). Liver tissue showed the highest levels of IL6 and SAA expression in LPS treated chickens without Mad-A/Gal administration. In contrast, Mad-A/Gal treated chickens showed comparably lower levels of IL6 and SAA. SAA levels were reduced primarily in liver tissue however, the extrahepatic gene expression of SAA appeared to be less inhibited by Mad-A/Gal administration. These results suggest that Mad-A/Gal administration can provide effective inhibition of IL6 throughout a range of chicken tissues and may also reduce the downstream IL6 gene expression cascade observed during severe bacterial infections. Intriguingly, the expression levels of the NO inducing gene iNOS, were only significantly raised in lung tissue when compared to non LPS treated chickens. Furthermore, although iNOS levels appeared to be reduced in the liver and lung of Mad-A/Gal administered chickens, the role of iNOS during *in vivo* LPS administration in chickens was less prevalent than that observed during *in vitro* LPS treatment of chicken splenic leukocytes. As many studies have shown the relationship between IFNγ production and iNOS induction, and since LPS is suggested to significantly increase IFNγ expression it is intriguing that iNOS levels are not further increased in chicken tissues (Reis *et al.,* 2001; Jarosinski *et al.,* 2005). As expected, the modulation of IL6 signalling by Mad-A/Gal appeared to have little or no effect on IFNγ mRNA expression levels in LPS treated chickens. In other species LPS administration has been observed to upregulate IFN levels (Blanchard *et al.,* 1986), and similarly, chickens with or without Mad-A/Gal, had increased IFNγ mRNA expression levels in a range of tissues.

In conjunction with increased cytokine production, LPS induced infections have also been associated with increased immune cell markers and antibody production in

chickens. It has been suggested previously that the administration of *Escherichia coli* LPS can increase the proliferation of B cells (Gupta *et al.,* 1988; Marcelletti, 1996). Chart *et al*., (1992) demonstrated that following treatment with LPS from *Salmonella*, IgM levels were significantly raised in chickens, whilst it has also been observed that IL6 binding to B cells can trigger a multistep cascade which results in IgM production (Goldstein *et al.,* 1993). However, there appears to be no definitive evidence of LPS being directly responsible for increased B cell proliferation. To test whether Mad-A/Gal inhibition of IL6 signalling and the associated reduction in IL6 expression levels influenced key immune cell populations, we investigated the proportions of CD4, CD8, IgM and MHCII expressing cells. Following LPS challenge, we observed little difference in the populations of $CD4^+$ and $CD8^+$ cells in chickens treated with or without Mad-A/Gal. However, we did observe an increase in the proportion of B cells in the blood, marked by an increased percentage of IgM positive cells. Since there is only anecdotal evidence of IL6 acting to increase B cell numbers (Levy, 2007), it may be more plausible that Mad-A/Gal treatment is having an effect on the regulation of other genes which do induce B cell proliferation. Galielallactone, for instance, can inhibit AP-1, TNF and NFkB in a transient fashion (Weidler *et al.,* 2000). This may also account for some of the reduction of IgM expressing cells witnessed in Mad-A/Gal treated chickens following LPS challenge.

In this study we have illustrated that Mad-A/Gal compounds can act in chickens to improve an LPS induced systemic inflammatory response. The administration of Mad-A/Gal reduced both IL6 signalling levels and also dampened the effect IL6 has on downstream inflammatory molecules, such as SAA and NO which have been previously associated with increased disease severity. As such, Mad-A/Gal show potential as therapeutics to target inflammatory diseases and infections that cause sustained increases in IL6 and IL6 stimulated genes.

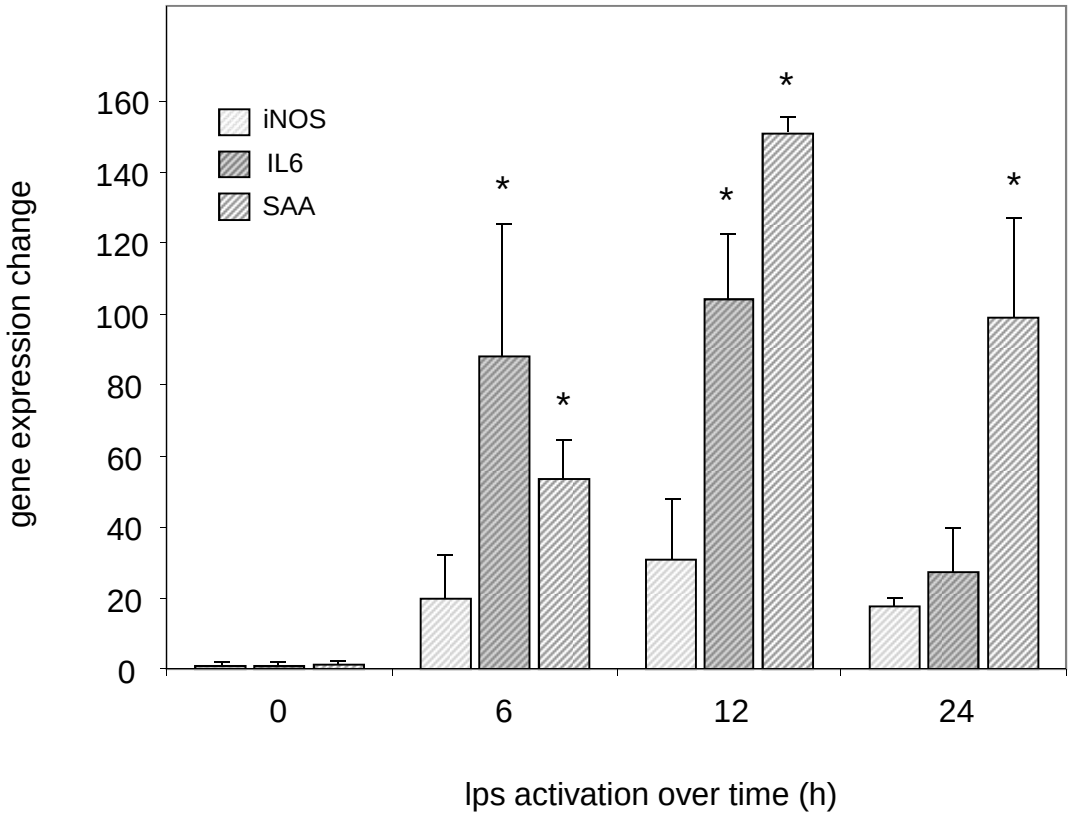


Figure 5.1. Chicken splenocytes activated with LPS have increased levels of IL6 and IL6 stimulated genes. Chicken splenocytes activated with LPS (10μg/mL) were investigated for the expression levels of IL6, SAA and iNOS. RNA was synthesized from the cells after 6, 12 and 24 hours and Q-RTPCR was carried out for IL6, SAA and iNOS gene expression levels determined relative to the house keeping gene GAPDH. Experiments were performed in triplicate with the data representative of 3 independent experiments, error bars display SE. An asterisk indicates statistically significant differences of means with $p<0.05$.

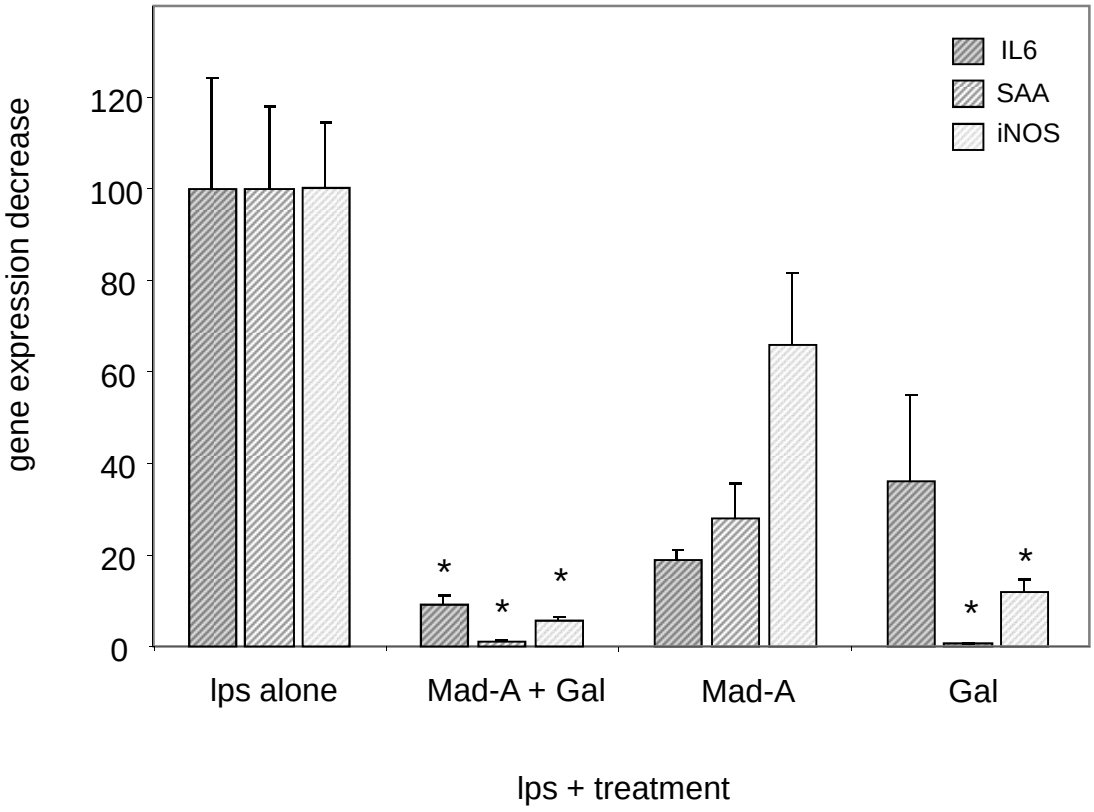
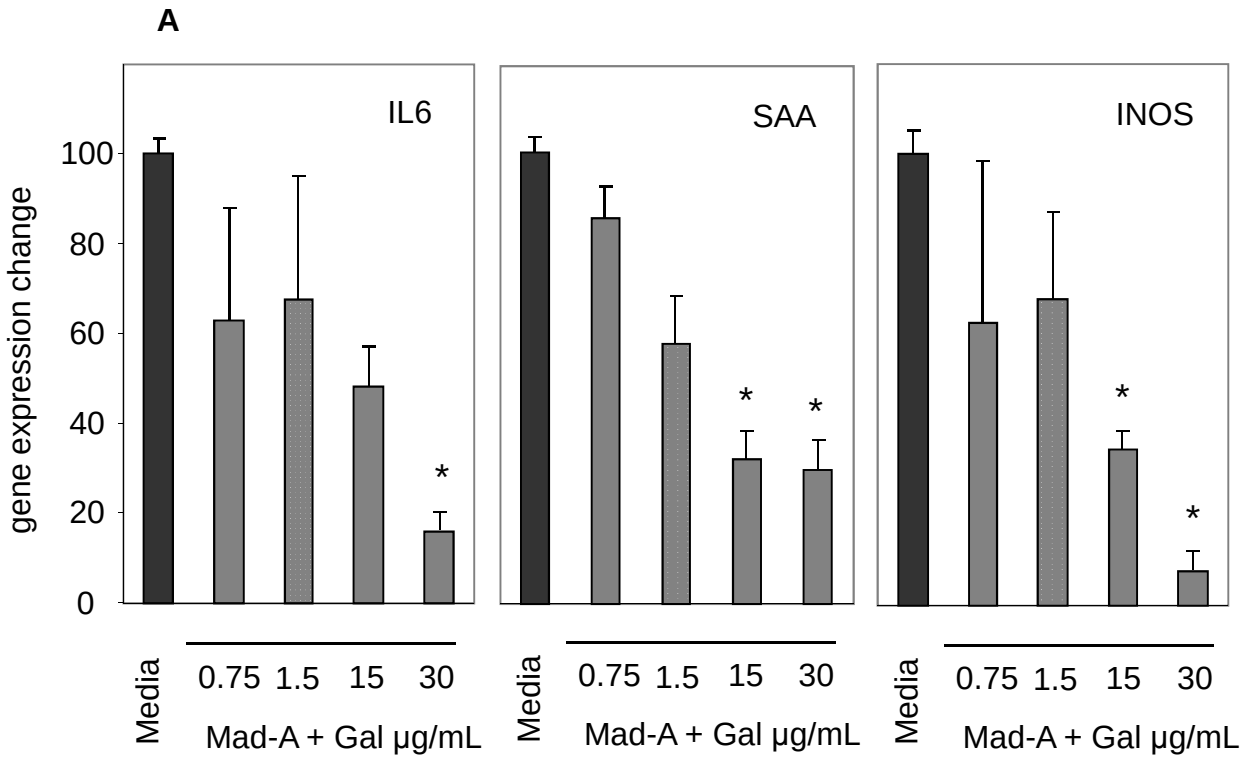


Figure 5.2. Suppression of IL6 and IL6 stimulated genes by a combination of Mad-A and Gal or each individually. Chicken splenocytes activated with LPS (10μg/mL) were treated with (15 ng/mL) of either Mad-A, Gal, or Mad-A and Gal (7.5 ng/mL each) together. RNA was synthesized from the cells after 24 hours and Q-RTPCR was carried out for IL6, SAA and iNOS. Gene expression levels were determined in conjunction with the house keeping gene GAPDH. Experiments were performed in triplicate with the data representative of 3 independent experiments, error bars display SE. An asterisk indicates statistically significant differences of means with $p<0.05$.


A
IL6
SAA
INOS
gene expression change
100
80
60
40
20
0
*
Media
0.75 1.5 15 30
Mad-A + Gal µg/mL

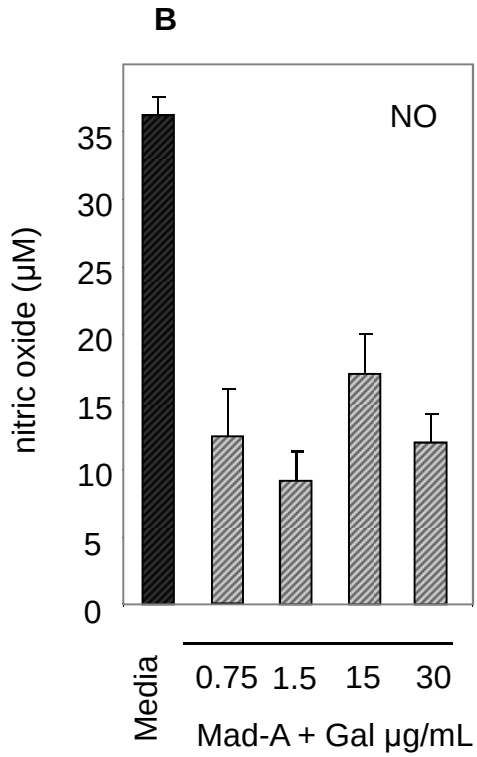

B
NO
nitric oxide (µM)
35
30
25
20
15
10
5
0
Media
0.75 1.5 15 30
Mad-A + Gal µg/mL

Figure 5.3. Suppression of IL6 and SAA in a dose dependent manner by Mad-A and Gal treatment. (A) Chicken splenocytes activated with LPS (20μg/mL) were treated with (0.75, 1.5, 15 and 30 ng/mL) of Mad-A and Gal. RNA was synthesized from the cells after 24 hours of treatment and Q-RTPCR was carried out for IL6, SAA and iNOS gene expression levels. Fold change decrease was calculated from the LPS alone activated splenicytes determined in conjunction with the house keeping gene GAPDH. **(B)** Supernatant from splenocytes cells cultured with LPS and Mad-A/Gal were sampled by colorimetric assay for NO production after 24 hours. Experiments were performed in triplicate with the data representative of 3 independent experiments, error bars display SE. An asterisk indicates statistically significant differences of means with $p<0.05$.

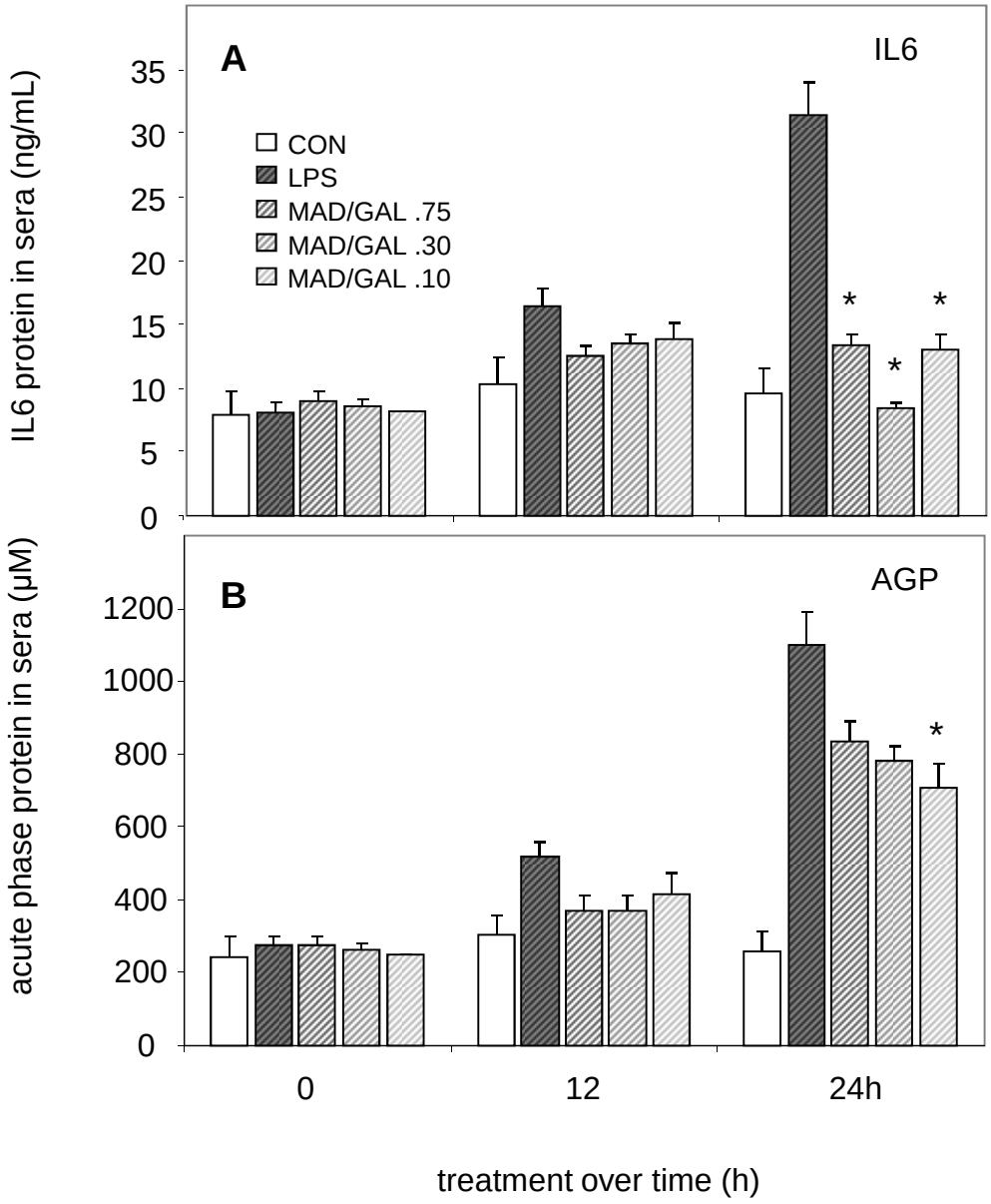


Figure 5.4. Following LPS administration levels of IL6 (A) and AGP (B) are increased in chickens but reduced by Mad-A/Gal treatment. (A) Levels of IL6 protein measured in the sera of LPS administered chickens using an IL6 dependant 7TD1 cell line. Examining IL6 at 0, 12 and 24 h.p. treatment indicated that chickens with Mad-A/Gal administration have reduced IL6 expression. **(B)** Levels of the acute phase protein alpha-1-acid glycoprotein (AGP) were measured by an SRID radial immunodiffusion assay. The levels of AGP were reduced by Mad-A/Gal treatment compared to untreated chickens. Error bars display SE, (n=4) and an asterisk indicates statistically significant differences of means with $p<0.05$.

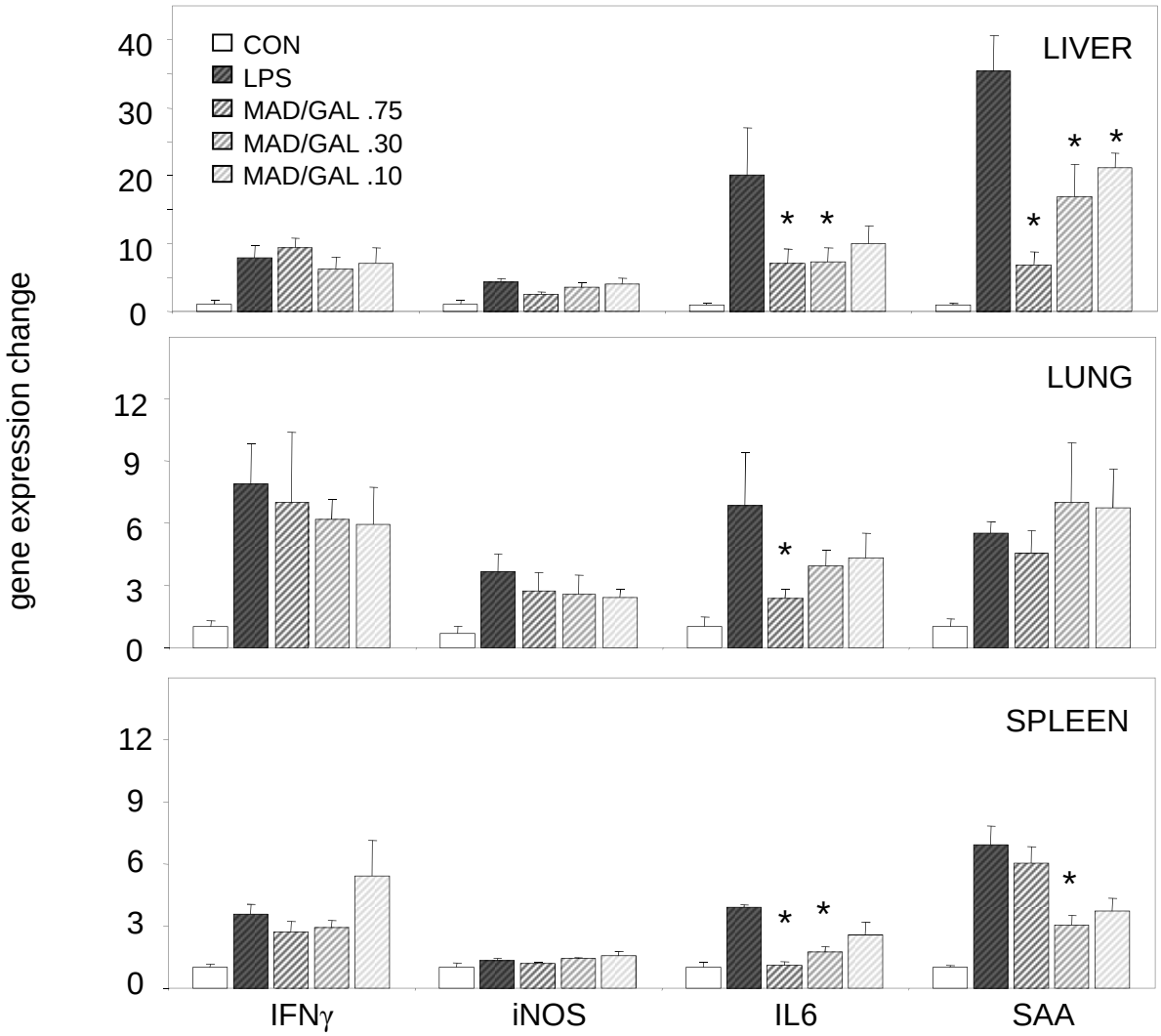


Figure 5.5. Chickens administered with LPS have reduced levels of IL6 and IL6 stimulated genes following treatment with Mad-A/Gal. Chicken administered with LPS (2mg/kg) and treated with or without Mad-A/Gal, were examined for their gene expression levels of inflammatory molecules by Q-RTPCR. Liver, lung and spleen tissues were harvested and RNA isolated 24 h after treatment. Q-RTPCR was carried out for IFNγ, IL6, SAA and iNOS. Gene expression levels were determined in conjunction with the house keeping gene GAPDH. Data represents the mean fold expression relative to unstimulated controls with 4 birds in each group, error bars display SE. An asterisk indicates statistically significant differences of means with $p<0.05$.

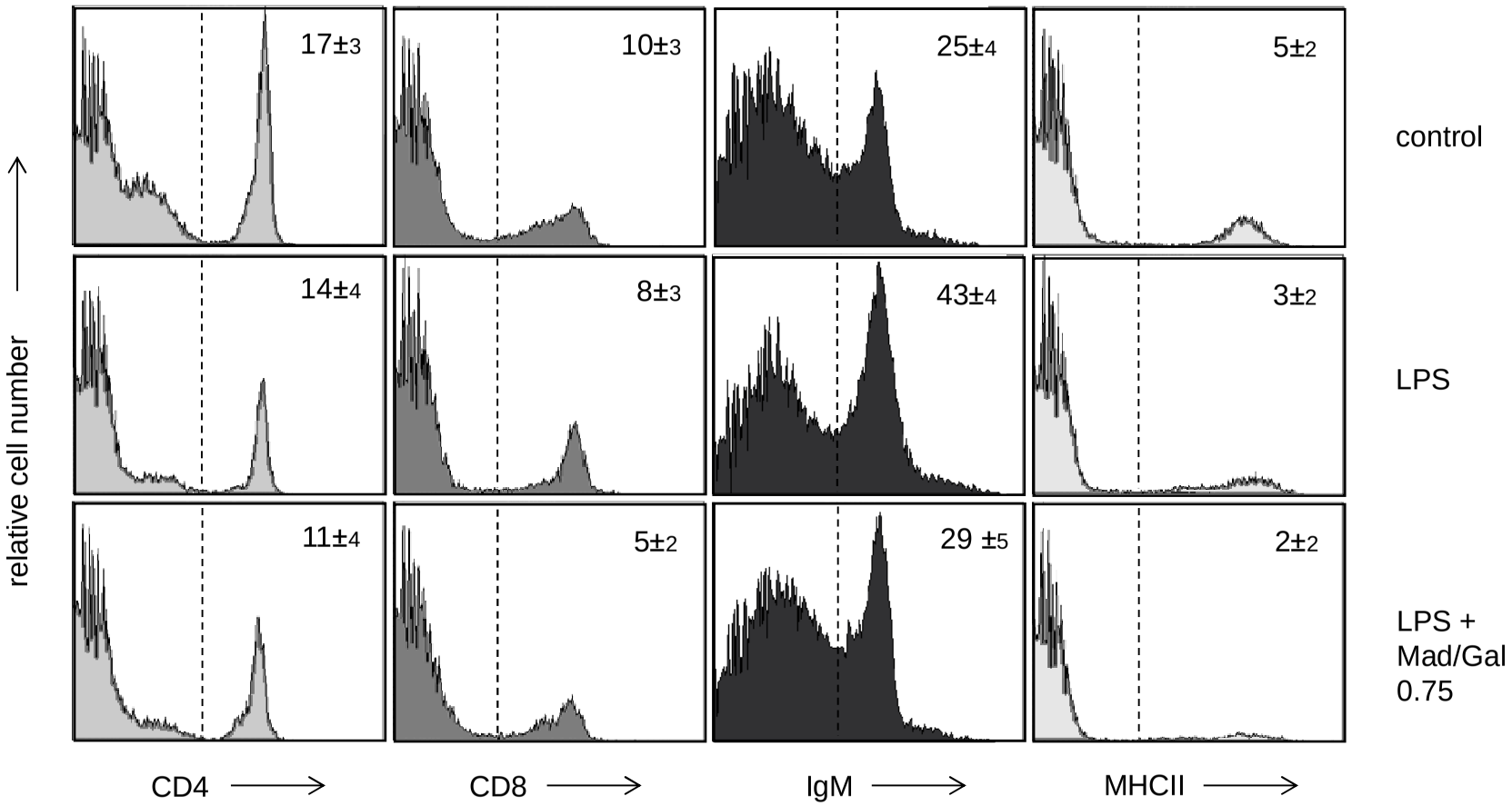

17±3
10±3
25±4
5±2
14±4
8±3
43±4
3±2
11±4
5±2
29 ±5
2±2
relative cell number
CD4
CD8
IgM
MHCII
control
LPS
LPS +
Mad/Gal
0.75

Figure 5.6. Cell surface phenotype populations of chicken PBMC, CD4, CD8 and IgM are reduced in Mad-A/Gal treated chickens following *in vitro* LPS administration. Flow cytometric histograms show the staining of CD4+, CD8+, IgM and MHCII from PBMCs isolated from chickens activated with LPS or PBS control, with or without Mad-A/Gal. The percentage of positive cells for each antibody is indicated in the panel. Viable lymphocyte cells were gated for the analysis, whilst non LPS, non Mad-A/Gal treated PBMCs served as negative controls Values are expressed as the mean ± SE (n=3).

Chapter 6

Manipulating the IL6 signalling pathway potentially impacts on H5N1 avian influenza virus replication.

6.1 Introduction

Avian H5N1 influenza viruses continue to spread throughout poultry and migratory birds in many countries despite current prevention and control strategies. Infections by these H5N1 influenza viruses are highly pathogenic in human patients with a mortality rate near 60% (Subbarao *et al.,* 1998). Deficiencies in antiviral administration and the induction of a severe cytokine response has been attributed to the often unsatisfactory outcome of treatment for H5N1 influenza virus infections (Peiris *et al.,* 2004). The role of cytokines in the pathology associated with H5N1 virus infections is somewhat controversial. However, deciphering the impact of cytokines during infection is a difficult process; it is not clear if hypercytokinemia induces pathology or alternatively, whether pathology induces hypercytokinemia (de Jong *et al.,* 2006b; Us, 2008). Similarly, it is difficult to ascertain if H5N1 influenza infection is responsible for cytokine induction or whether elevated cytokine levels promote increased levels of infection. Previous chapters have dealt with investigations into H5N1 virus induced hypercytokinemia, which appears to erupt when there is no resolution to the viral infection (Cheung *et al.,* 2002; Chan *et al.,* 2005). Furthermore, studies of H5N1 influenza virus infection in ducks, a natural reservoir for avian influenza viruses (Kim *et al.,* 2009), demonstrated lower levels of proinflammatory cytokines when compared to the same H5N1 influenza virus infection in chickens. Ducks are often resilient to H5N1 influenza viruses (Kishida *et al.,* 2005; Liu *et al.,* 2005), supporting the hypothesis that high cytokine levels may be associated with an increase in disease severity and therefore, a reduction of this hyper-active immune response may be beneficial.

A pertinent finding of Chapter 4, was the observation of a clear difference in the levels of IL6 between ducks and chickens during H5N1 influenza virus infection. Whilst a range of cytokines are upregulated during H5N1 influenza virus infection, IL6 in particular is known to be capable of inducing both the acute phase response and regulating a proinflammatory cytokine feedback-loop (Olsson *et al.,* 1999; Ogura *et al.,* 2008). Moreover, studies of human H5N1 influenza virus infection are beginning to suggest that individuals with high circulating levels of IL6 often have increased organ damage and an associated increase in IL6 stimulated genes (Gao *et al.,* 2010). Gao *et al.,* (2010) suggest one possible explanation for the increased cytokine levels is due to circulating immune cells being a target of H5N1 influenza virus and therefore these cells may also play a role in viral dissemination. If this is the case, it may be possible to use anti cytokine compounds as therapeutics to target influenza infections, providing a new strategy to reduce the severity of disease.

One of the major problems in combating influenza infection is the high genetic variability of the virus, resulting in the rapid formation of variants that escape the acquired immunity against previous influenza virus strains, or have resistance to antiviral therapies. The clinically approved anti influenza therapies currently available are targeted against essential viral protein functions. Adamantane compounds have a specific inhibitory effect by blocking the ion-channel activity of the viral M2 protein, whilst zanamivir and oseltamivir block the activity of the NA protein, required for viral release (Cox *et al.,* 1999; Gubareva *et al.,* 2000; McKimm-Breschkin, 2005). However, adamantine sensitive influenza strains quickly develop resistance (Zhou *et al.,* 2011) and due to various mutations, influenza strains in patients are now escaping the selective pressure of neuraminidase inhibitors (Gubareva *et al.,* 2000; Gubareva *et al.,* 2002; Hurt *et al.,* 2011). Therefore, ongoing studies are attempting to find new therapies which reduce viral replication through mechanisms which do not target viral function or structure. Instead, there is an increasing interest in targeting specific host cellular functions, which would make adaptation for influenza viruses more difficult and may therefore reduce replication independent of the type, strain and antigenic properties of the pathogen.

Given that H5N1 influenza viruses manipulate the expression of a variety of immune genes shortly after infection, it is likely that the activity of many other intracellular signalling mediators may also be altered (Vlahos *et al.,* 2011; Lee *et al.,* 2011). Signalling processes can be initiated by the cell as a defence against a pathogen, but may also be hijacked by influenza viruses to support replication (Brass *et al.,* 2009; Watanabe *et al.,* 2010). Cell culture RNAi based screens by Brass *et al*., (2009) have identified a range of host factors involved in transport, trafficking, endosocytosis, translation initiation and cytokine induction, which are all exploited by the influenza virus to help increase viral replication. With this in mind, early investigations of H5N1 influenza virus infected mice administered inflammatory inhibitors in combination with antivirals (neuraminidase inhibitor) have shown increased survival. Similarly, multiple investigations by Lee *et al.,* (2008) and Zheng *et al.,* (2008) have suggested that suppression of cyclooxygenase-2 (COX-2), a gene involved in the inflammatory process, may help suppress viral replication (Zheng *et al.,* 2008; Lee *et al.,* 2011).

The contributing role of cytokines in the increased severity of H5N1 influenza infection is difficult to deduce. Elevated levels of IL6 may increase the pathology associated with H5N1 virus infection, thus, we sought to investigate whether inhibition of IL6 signalling improves the immune response in chickens. Since the Mad-A/Gal compounds had demonstrated significant inhibition of IL6 signalling and IL6 stimulated genes in chickens, they were used to modulate the influenza generated IL6 response. The present study clearly demonstrates that Mad-A/Gal significantly suppresses IL6 signalling, reducing the inflammatory cytokine cascade typically observed in chickens following infection with the Muscovy duck/Vietnam/453 H5N1 virus isolate. Furthermore the levels of viral replication were also found to be reduced following IL6 inhibition. Given the lack of efficient antivirals against H5N1 virus and the narrow time frame during which certain anti viral drugs are effective, a combination of antiviral with anti-inflammatory treatments may better impact on reducing the pathogenesis caused by H5N1 influenza virus.

6.2 Results

6.2.1 Mad-A/Gal treatment reduces circulating levels of IL6 and other inflammatory molecules in H5N1 virus infected chickens

Typically, H5N1 influenza virus infections are associated with increased IL6 levels in the sera of patients during the peak of disease pathogenesis. To investigate the impact IL6 has on the inflammatory cascade during H5N1 influenza virus infection, chicken sera was analysed following treatment with the IL6 inhibitors Mad-A and Gal. The levels of IL6, AGP and NO were examined 24 h.p.i. Chickens infected with H5N1 virus showed a 12 fold increase in the levels of IL6 in their sera (in comparison to uninfected chickens, shown in Fig. 6.1). In contrast, H5N1 virus infected chickens treated with Mad-A/Gal showed levels of IL6 7 fold more than uninfected chickens, significantly less IL6 than in the sera of chickens without Mad-A/Gal treatment ($p<0.05$ or $**p<0.01$). Furthermore, the levels of the acute phase protein AGP (Fig. 6.2) and the levels of the free radical, NO (Fig. 6.3) were also reduced in Mad-A/Gal treated chickens compared to H5N1 virus alone infected chickens. The levels of IL6, AGP and NO in the sera of chickens 18 h.p.i, appeared similar between H5N1 virus infected with or without Mad-A/Gal and uninfected animals.

6.2.2 Mad-A/Gal treatment reduces cytokine gene expression levels in a range of tissues following H5N1 influenza virus infection

High cytokine levels may play a role in the increased pathology observed in organs following H5N1 influenza infection. Since we observed a decrease in the circulating levels of IL6, AGP and NO following Mad-A/Gal treatment, we assessed whether IL6 expression was reduced in specific tissues. The gene expression levels of IL6 and the IL6 stimulated genes, SAA and iNOS were examined by Q-RTPCR in the lung, liver, spleen and heart. Chickens infected with H5N1 influenza without Mad-A/Gal treatment showed rapid increases in IFNγ, iNOS, IL6 and SAA 24 h.p.i, compared with uninfected levels (Fig. 6.4). IL6 had the largest increases in mRNA expression (between 250 to 450 fold increases), whilst iNOS appeared increased to the highest degree in liver tissue (250 fold increase) and SAA was observed to be increased

primarily in liver and lung tissue (220 and 120 fold respectively). In comparison, chickens infected with H5N1 virus but treated with Mad-A/Gal showed IL6 levels that were significantly lower ($p<0.05$ or $^{**}p<0.01$) reduced by almost half in lung and spleen tissue (220 and 120 fold respectively). Mad-A/Gal administration also appeared to reduce the levels of the IL6 stimulated gene, SAA in a range of tissues. Similarly, iNOS levels were reduced in H5N1 virus infected chickens with Mad-A/Gal administration (Fig. 6.4). The levels of IFNγ were decreased only in liver tissue of Mad-A/Gal treated chickens, whilst in other tissues, IL6 inhibition appeared to have little or no effect on IFNγ gene expression levels. However, the levels of cytokines in H5N1 virus infected chickens with Mad-A/Gal treatment were still significantly higher than uninfected animals.

6.2.3 Chickens treated with Mad-A/Gal have lower levels of H5N1 virus compared to untreated infected chickens

To compare the viral replication efficiency between H5N1 infected chickens without IL6 inhibition and infected chickens which had been administered Mad-A/Gal, we harvested organs for viral analysis. Within 24 h.p.i, chicken lung, liver and heart tissues were found to have high viral titres of H5N1 influenza virus, averaging between 4.9 and 5.5 $\log_{10}$ tissue culture infectious doses ($TCID_{50}$). The spleen of chickens also contained large amounts of virus, in excess of 4.5 $\log_{10}$ $TCID_{50}$ (Fig. 6.5). In contrast, H5N1 virus infected chickens administered with Mad-A/Gal showed lower levels of viral replication in lung, liver and heart tissue. Liver and heart tissue showed the greatest reduction in viral titre levels, with more than 1 $\log_{10}$ $TCID_{50}$ reduction compared to chickens without Mad-A/Gal, whilst lung tissue titres were also reduced. The spleen tissue of H5N1 virus infected chickens with Mad-A/Gal showed similar levels of virus compared to infected chickens without IL6 inhibition.

6.2.4 Immunohistochemistry (IHC) shows reduced spread of H5N1 virus antigen in chickens treated with Mad-A/Gal

Since H5N1 viral antigen has been shown to be widespread in chicken organs during the peak of influenza infection (Fig. 3.3), we sought to investigate the distribution of

H5N1 virus in chickens with Mad-A/Gal reduced levels of IL6 compared to chickens without Mad-A/Gal treatment. IHC antibody staining for H5N1 antigen was observed in a wide range of organs in chickens without Mad-A/Gal treatment and appeared widely disseminated (chicken 60). In lung, spleen, liver and heart tissue, IHC staining for H5N1 antigen was observed throughout the entire tissue section with multiple foci of intense H5N1 virus staining, often surrounding sites of necrotic tissue and blood vessels. In contrast, H5N1 virus was restricted to less frequent, concentrated foci in heart and liver tissue and typically less widespread in Mad-A/Gal treated chickens (chicken 47). However, in lung and spleen tissue H5N1 antigen distribution appeared as equally disseminated as in non Mad-A/Gal treated chicken sections (Fig. 6.6).

6.2.5 Survival mean-death-times for H5N1 influenza virus infected chickens are similar for Mad-A/Gal treated and untreated chickens

Given the reduction of inflammatory cytokines and viral titre levels observed in H5N1 virus infected chickens treated with Mad-A/Gal, we investigated whether Mad-A/Gal administration had an impact on the mean survival times of influenza infected chickens. Initially, between 24 to 25 hours post H5N1 influenza virus infection, chickens treated with Mad-A/Gal showed better rates of survival compared to non IL6 inhibited chickens. However, by 28 to 30 h.p.i, chickens with or without Mad-A/Gal developed severe signs of infection and succumbed to H5N1 virus infection at a similar rate (Fig. 6.7). Therefore, there appeared to be no improvement in mean survival times for Mad-A/Gal treated chickens compared to untreated chickens.

6.3 Discussion

The recent concern over H5N1 influenza virus outbreaks has reinforced the urgent need for new pharmacological strategies that not only alleviate virus replication but also address the organ damage caused by inflammatory cytokines. The mechanisms behind how this severe immune response is initiated are still somewhat unclear (Chan *et al.,* 2005). Previous investigations have suggested an associated increase of inflammatory cytokines with increased disease severity but have been unable to

confirm that cytokines are responsible. The present study has shown that the reduction of IL6 signalling substantially reduces inflammatory markers in a range of tissues following infection with H5N1 influenza virus. Intriguingly, the suppression of IL6 and IL6 stimulated genes also enhances clearance of H5N1 virus. Therefore, modulation of IL6 activity may contribute to new therapeutic approaches aimed at improving the cytokine dysfunction observed during H5N1 influenza virus infection.

Exactly how crucial hypercytokinemia is in contributing to the increased severity witnessed during HPAIV infection is a subject of debate. It has been previously discussed, that studies by Salomon *et al*., (2007) found preventing components of the cytokine storm from erupting failed to protect H5N1 infected mice or improve survival. Similarly, studies using two genetically similar H7N1 strains, with varying pathogenicity (either HP or LP) in chickens, suggested that cytokines may play a limited role in determining pathogenicity (Rebel *et al.,* 2011). In contrast, trials using combination of an antiviral and immunomodulators such as cyclooxygenase-2 (COX-2) and ETR-P1 improved H5N1 influenza infection significantly and decreased inflammatory markers by almost half (Zheng *et al.,* 2008; Okada *et al.,* 2009). Similarly, Snelgrove *et al.,* (2006) and Vlahos *et al.,* (2011) have provided evidence for significant improvement from cytokine induced inflammation following H3N2 and H1N1 virus infection in mice deficient in Nox2, an inflammatory cell ROS (reactive oxygen species). This data suggests that reduced inflammation may help improve the antiviral response. Some of the confliction between these studies may be due to differences in either the host response to infection or the isolate of influenza virus investigated. Two key differences between H5N1 influenza infection in mice and chickens appears to be both the length of infection and the dissemination of virus. Following challenge with the same highly pathogenic strains of H5N1 influenza, mice appear to succumb to a viral infection which primarily affects the respiratory system, whilst in chickens virus rapidly spreads and becomes systemic (Rao *et al.,* 2008). Moreover, mice suffer a longer infection period than chickens, with many of the 2003-2004 H5N1 isolates causing death after 9 to 12 days of infection in mice, while in comparison chickens may survive only 2 to 3 days (Lu *et al.,* 1999; Rao *et al.,*

2008; Yen *et al.,* 2009). Therefore, modulation of the immune system following H5N1 influenza may have different impacts in mice when compared to chickens.

Our investigations into the increased severity of H5N1 influenza infection in chickens suggested IL6 may play a role, since it was highly induced following H5N1virus infection in chickens but not in ducks. IL6 induction is associated with increased levels of inflammatory markers, signalling in concert with IL1 to induce acute phase reactants (Ramadori *et al.,* 1988). IL6 may also increase other inflammatory cytokines, such as IL1, through the suppression of its receptor antagonist (IL1Ra), inhibiting the complex feedback loop which regulates inflammatory cytokines (Jordan *et al.,* 1995). Following IL6 suppression by Mad-A/Gal, we observed reduced levels of both circulating acute phase reactants and gene expression levels of SAA in a range of tissues. Furthermore, the levels of NO in sera and levels of iNOS gene expression in a range of organs were reduced in H5N1 infected chickens following Mad-A/Gal treatment. As sustained increases in acute phase reactants, such as SAA, CRP and AGP have been implicated in causing cellular damage (Olsson *et al.,* 1999; Shagdarsuren *et al.,* 2005) these molecules may be important in contributing to the excessive levels of inflammation following H5N1 influenza infection (Falsey *et al.,* 2001; Lannergard *et al.,* 2003). Given the deleterious effect these genes can have, it was hoped that a more managed acute phase response would be beneficial in combating influenza infection. Moreover, other molecules aimed at viral clearance, such as NO may also contribute to disease severity when expressed at high levels, as discussed in Chapter 4 (Perrone *et al.,* 2008; Burggraaf *et al.,* 2011). As a consequence of a severe inflammatory response, NO may increase the cytotoxic activity of macrophages and can cause epithelial cell damage through the build up of peroxynitrite (Hibbs *et al.,* 1988; Palmer *et al.,* 1992; Saleh *et al.,* 1998). Therefore, preventing NO accumulation may also improve inflammation during H5N1 influenza infection. However, complete ablation may also be deleterious given the sensitive immune action these genes are involved in.

To determine whether reduced levels of IL6, SAA and iNOS could help alleviate the severity of H5N1 influenza infection we examined the levels of H5N1 viral

replication in chickens. Mad-A/Gal treatment reduced H5N1 viral replication in a range of chicken tissues, compared to H5N1 infected control tissues. Similarly, the distribution of H5N1 viral staining, determined by IHC, appeared reduced in the heart, liver and spleen of chickens treated with Mad-A/Gal. This suggests that increased cytokine levels are partly responsible for the severe pathogenicity observed during H5N1 influenza virus infection. However, viral replication levels were nevertheless more than 3.5 $\log_{10}$ $TCID_{50}$ in all tissues examined and this correlated with no improved survival from chickens with Mad-A/Gal administration. One reason for the lack of improvement in survival and the relatively small reduction in viral levels could be that Mad-A/Gal treatment did not reduce inflammation to a sufficient degree. Although IL6 was reduced, it was still significantly increased above uninfected levels and this in concert with the accumulative increases of IFNγ, iNOS and SAA may be damaging the host antiviral response. Therefore, a further reduction in IL6 expression and IL6 signalling, through the use of a higher dose of Mad-A/Gal, may be required to lower the overall inflammatory cytokine production below a deleterious threshold. This may allow for an immune response which is less preoccupied with inflammation, hopefully providing sufficient survival time until the more targeted adaptive immune action is initiated. Alternatively, IL6 inhibition may be compensated for by other cytokines with overlapping signalling mechanisms. Cytokines that are expressed at a significantly high level following viral infection, such as IL10, IL12 and IL15 can activate STAT3, the signalling pathway used by IL6 and IL1 (Heinrich *et al.,* 1998a; Akira, 2000; Ghoshal *et al.,* 2001). Therefore, it may be beneficial to explore immunomodulation of multiple inflammatory molecules to help improve the immune response following infection.

In conclusion, current strategies for improving H5N1 influenza infection are focussed primarily at alleviating mechanisms of viral infection and replication. However, less attention has been given to examining the mechanisms that modulate the host responses, which lead to inflammation and an associated increase in pathology. The modulation of IL6 has an impact on H5N1 influenza infection in chickens, lowering inflammatory markers and reducing viral levels. However, administration of Mad-A/Gal at 0.8mg/kg did not completely inhibit IL6 levels or reduce inflammatory genes

at a sufficient enough level to improve survival in chickens following H5N1 influenza infection. Further studies, investigating improved inhibition of IL6 in concert with manipulation of other crucial inflammatory molecules, such IL1 and IFNγ may help alleviate some of the redundancy that the inflammatory response has. This information provides a platform to further investigate the impact that cytokine modulation has on the immune response, with the aim to develop new strategies and approaches for improving the outcome following H5N1 influenza infection.

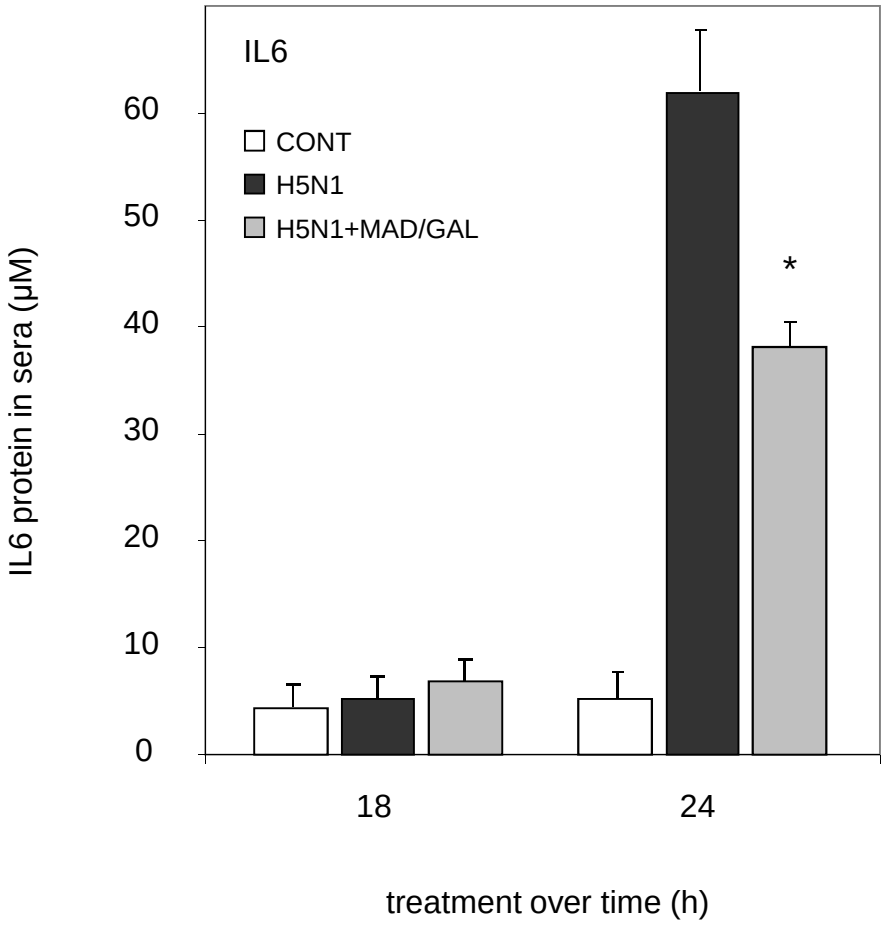


Figure 6.1. Following H5N1 influenza infection, the level of IL6 is increased in chickens but reduced by Mad-A/Gal treatment. The levels of IL6 protein were measured in the sera of chickens using an IL6 dependant 7TD1 cell line. Examining IL6 at 12 and 24 h.p.i, indicated that chickens with Mad-A/Gal treatment have reduced levels of IL6. Data shows average levels of IL6 from 6 birds in H5N1 infected groups and 4 birds from control group, error bars display SE. An asterisk indicates statistically significant differences of means with $p<0.05$.

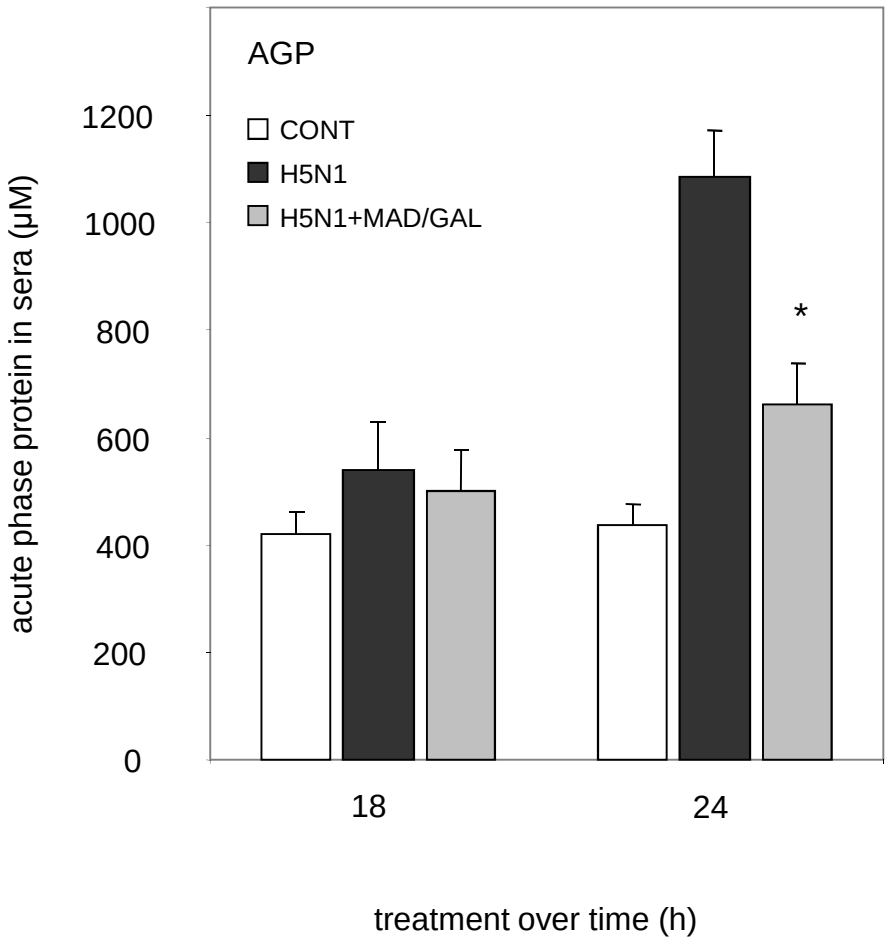


Figure 6.2. Following H5N1 influenza infection, the levels of AGP are increased in chickens but reduced by Mad-A/Gal treatment. Levels of the acute phase protein alpha-1-acid glycoprotein (AGP) were measured by an SRID radial immunodiffusion assay at 18 and 24 h.p.i.. The levels of AGP were reduced by Mad-A/Gal treatment compared to untreated chickens. Data shows average levels of AGP from 6 birds in H5N1 infected groups and 4 birds from control group, error bars display SE. An asterisk indicates statistically significant differences of means with $p<0.05$.

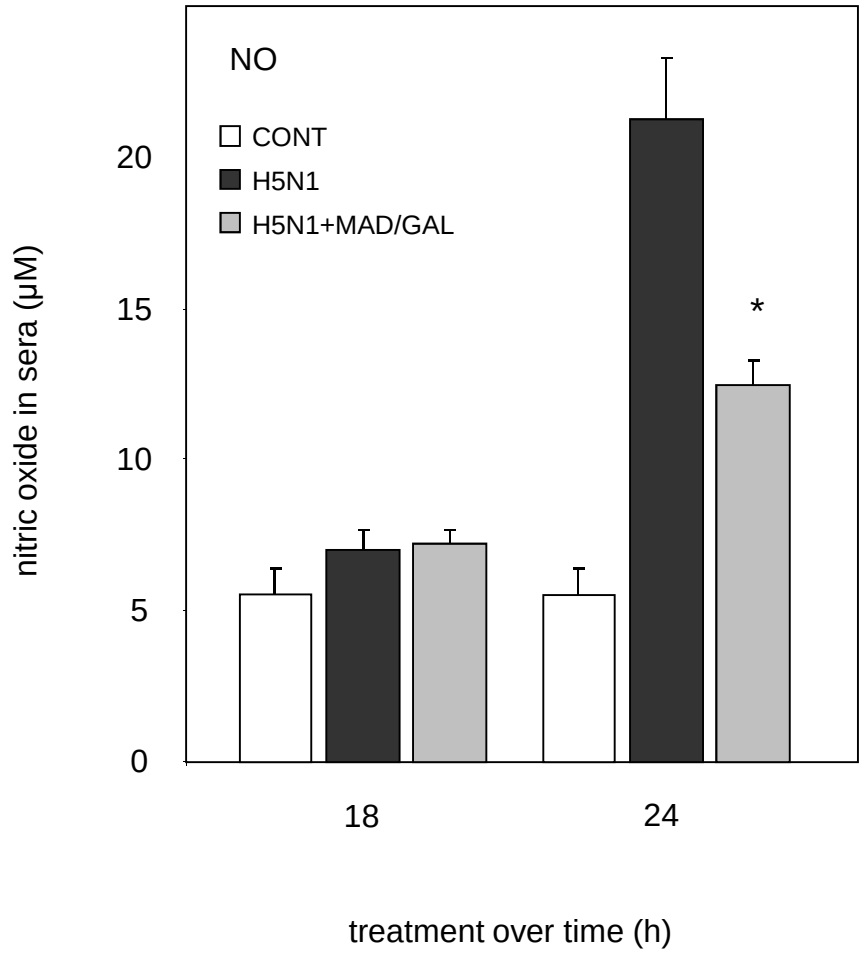


Figure 6.3. Following H5N1 influenza infection, the levels of NO are increased in chickens but reduced by Mad-A/Gal treatment. Chickens sera was assayed for the production of NO at 18 and 24 h.p.i. Chickens administered Mad-A/Gal showed reduced levels of NO in their sera. Data represents the mean NO production of 6 bird in the H5N1 infected groups and 4 birds in the control group, error bars show SE. An asterisk indicates statistically significant differences of means with $p<0.05$.

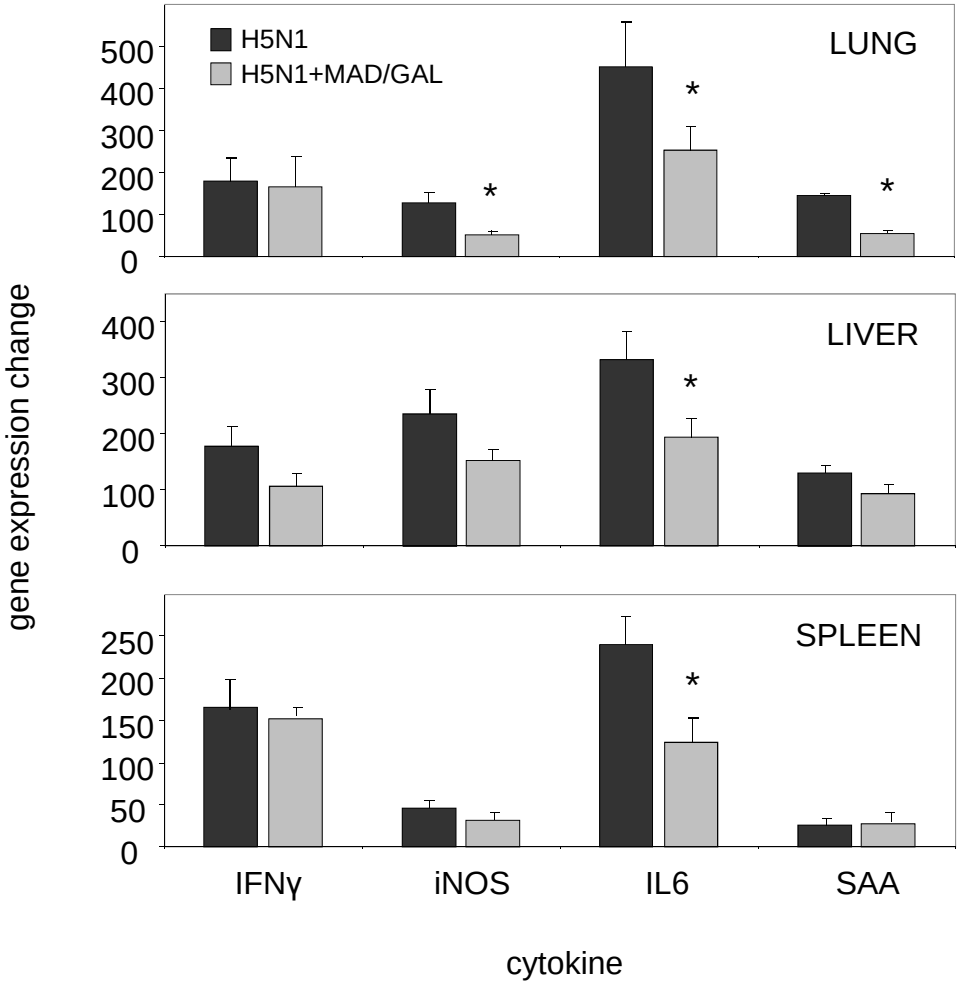


Figure 6.4. Chickens infected with H5N1 influenza virus have increased cytokine levels compared to Mad-A/Gal treated chickens. Chicken infected with H5N1 influenza and treated with or without Mad-A/Gal, were examined for their gene expression levels of IFNγ, iNOS, IL6 and SAA by Q-RTPCR. Liver, lung and spleen tissues were harvested and RNA isolated between 24-30 h.p.i. Gene expression levels were determined in conjunction with the house keeping gene GAPDH. Data represents the mean fold expression relative to uninfected controls with 6 birds in each H5N1 infected group and 4 birds in the uninfected control group, error bars show SE. An asterisk indicates statistically significant differences of means with $p<0.05$.

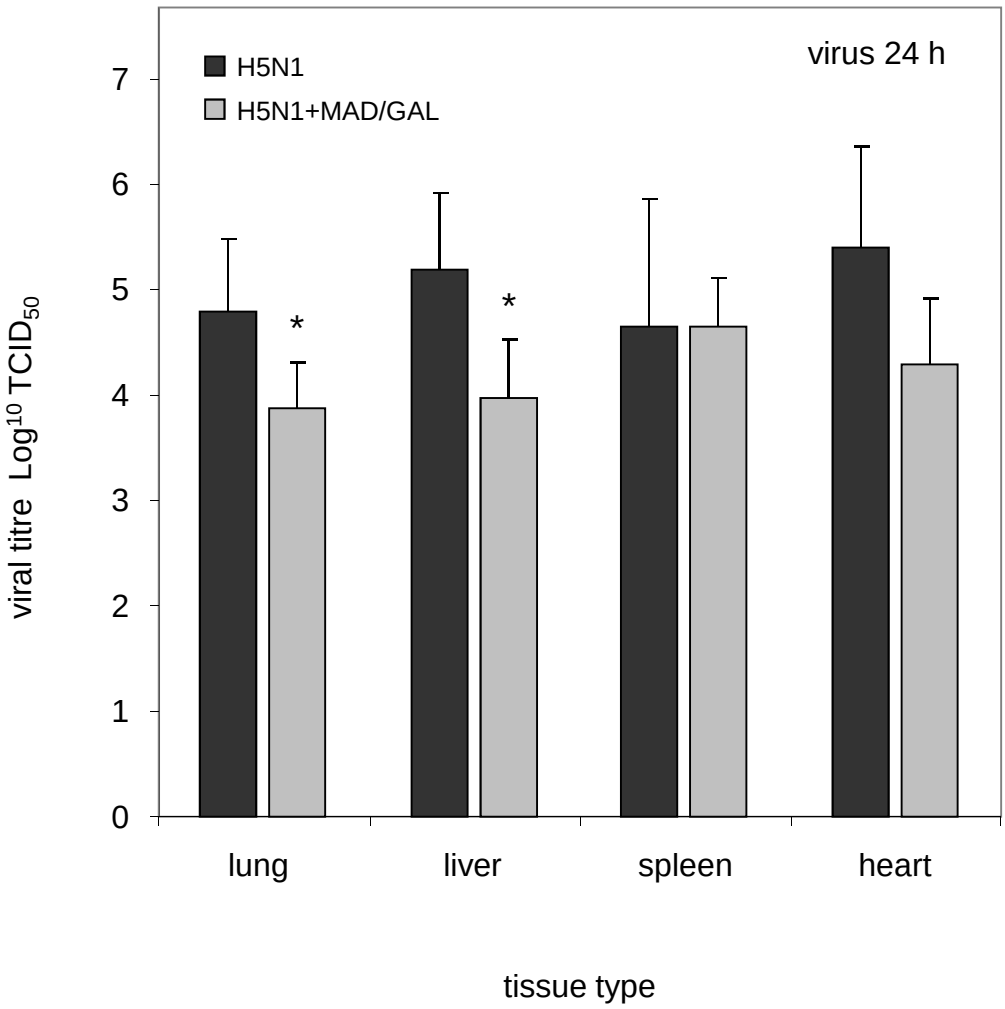


Figure 6.5. Chickens administered Mad-A/Gal have reduced levels of H5N1 viral replication. The graph shows that H5N1 viral titres appear reduced in chickens with Mad-A/Gal treatment. Titres of individual lung, liver, spleen and heart tissue was measured in Vero cells for CPE. The mean virus titres from six birds are shown (log^{10} $TCID_{50}$), error bars show SE. An asterisk indicates statistically significant differences of means with $p<0.05$.

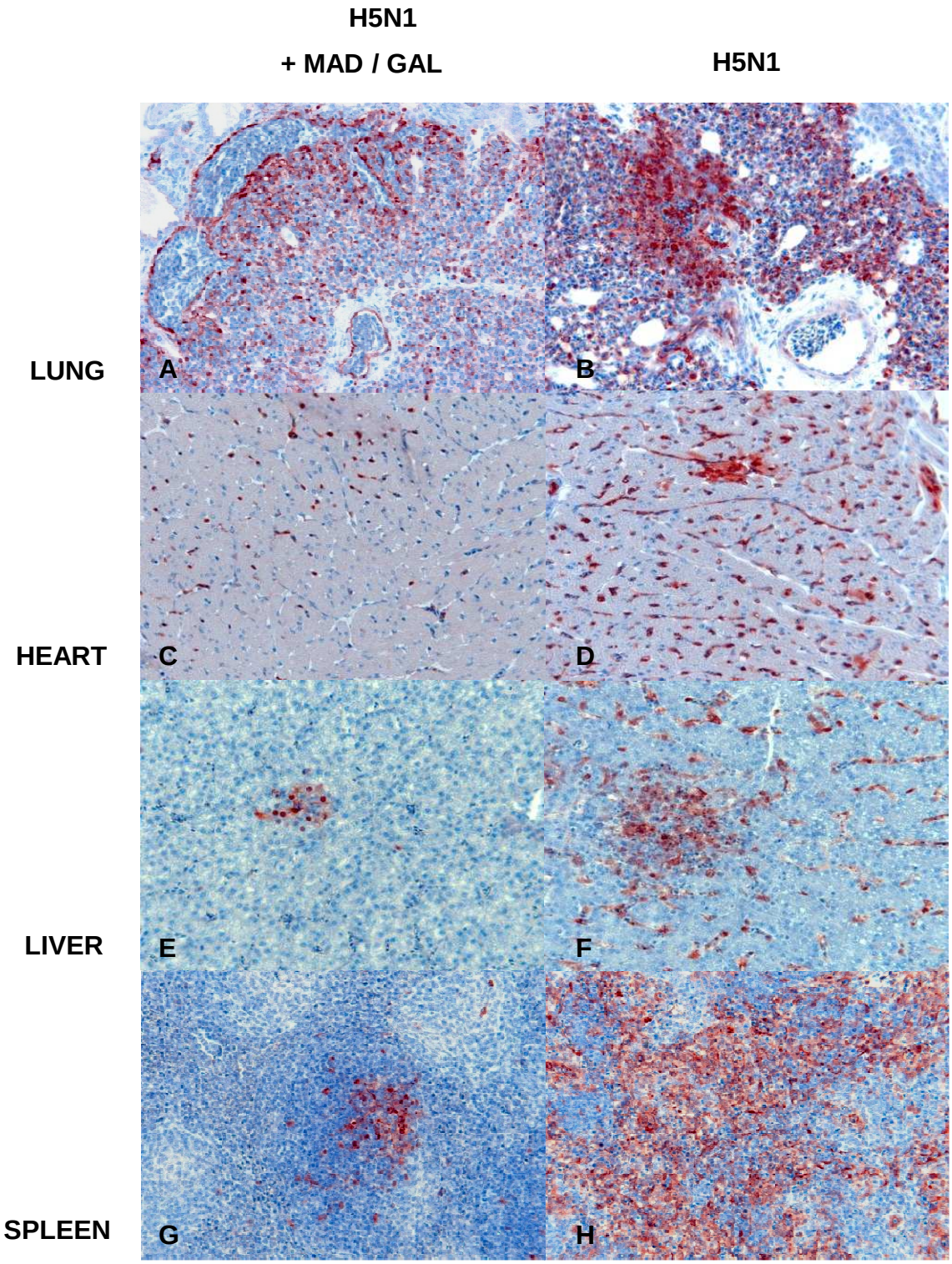

H5N1
+ MAD / GAL
H5N1
LUNG
HEART
LIVER
SPLEEN
A
B
C
D
E
F
G
H

Figure 6.6. Immunohistochemistry of chicken tissues shows reduced spread of H5N1 antigen following Mad-A/Gal treatment. Left hand panel shows H5N1 infected chicken (47) tissues administered with Mad-A/Gal (0.8 mg/kg). Right hand panel shows H5N1 infected chicken (60) tissues without Mad-A/Gal treatment. Both chickens 47 and 60 were euthanased approximately 24 h.p.i due to severe clinical signs of infection. **(A)** Chicken (47), with IHC stain showing H5N1 viral antigen as red/brown colour prevalent in the blood vessel endothelium and dense antigen staining in respiratory tissue of the lung **(B)** Chicken (60) with IHC stain showing a similar degree of dense H5N1 antigen in lung tissue. **(C)** Chicken (47) with IHC staining in capillaries but restricted to fewer foci. **(D)** Chicken (60) with IHC showing severe viral antigen staining in capillaries and myocardium. **(E)** Chicken (47) liver tissue, with IHC staining showing H5N1 antigen in small foci of sinusoidal endothelial cells and hepatocytes. **(F)** Chicken (60) liver tissue with IHC staining showing H5N1 antigen distributed widely throughout the sinusoidal cells and in necrotic lesions of hepatocytes. **(G)** Chicken (47) with IHC of spleen tissue, showing H5N1 antigen restricted to small foci in the red pulp generally in close proximity to blood vessels. **(H)** Chicken (60) with IHC staining showing dense H5N1 antigen in the red pulp, necrotic tissue and in proximity to blood vessels of the spleen. All scale bars = 50 μm.

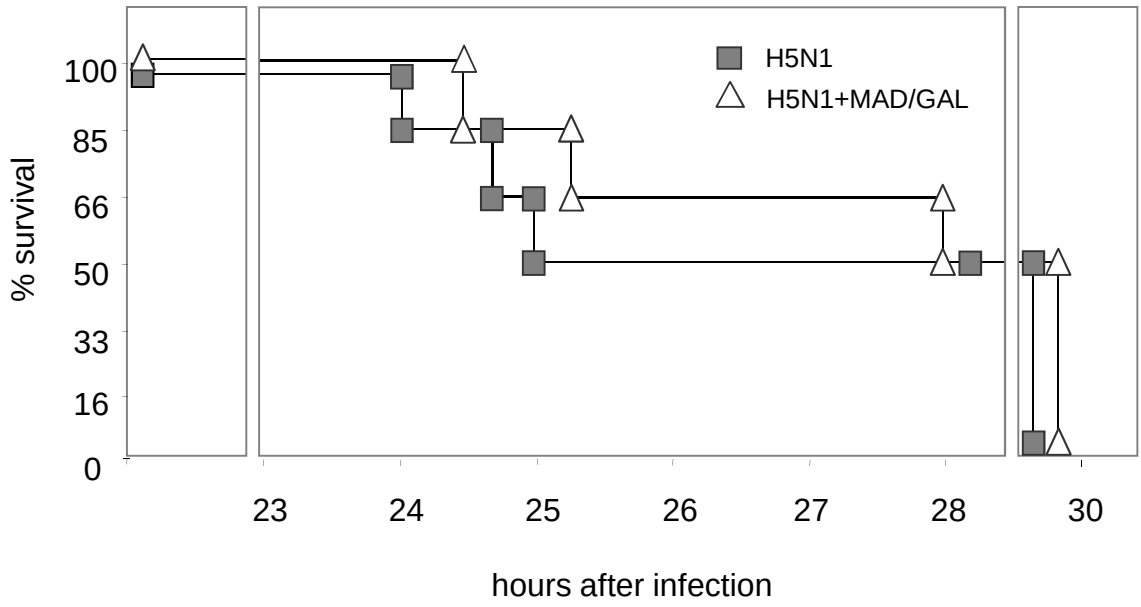


Figure 6.7. H5N1 influenza mortality in chickens is not improved by Mad-A/Gal treatment. Survival of chickens after oral-intranasal inoculation with H5N1 Vt453 influenza virus. Graph depicts the percentage survival of chickens without Mad-A/Gal treatment (squares) or chickens with Mad-A/Gal (triangles) inoculated with (H5N1) Vt453. Chickens needed to be euthanized or succumbed to viral infection between 24-30 h.p.i. Chickens treated with Mad-A/Gal had a mean survival time of 27 h and 50 m, whilst chickens without Mad-A/gal treatment had a mean survival time of 26 h and 45 m.

Chapter 7

General discussion

The emergence and spread of the highly pathogenic avian influenza virus (H5N1) in poultry and migratory birds has led to repeated cases of transmission to humans, raising concerns about a possible pandemic (Subbarao *et al.,* 2000; Lee *et al.,* 2010). Zoonotic H5N1 influenza infections continue to spread unabated and are likely to be endemic in many regions, particularly Asia. Given the large natural reservoir and the high mortality rate in humans following infection, around 60%, it is of critical importance to prevent and control influenza infection. While a novel H1N1 virus has recently spread worldwide and has become pandemic, it remains relatively mild in its severity (Michaelis *et al.,* 2009). Concern arises however, that this virus will become epizootic in swine, increasing the opportunities for pandemic H1N1 strains to re-assort with avian H5N1 strains of influenza, which have repeatedly been isolated from swine (Takano *et al.,* 2009). Although the risk of such an event may be low, its potential impact would be high due to the current difficulties in generating efficient H5N1 vaccines and inadequacies in treatment of influenza induced disease (Lee *et al.,* 2005a). Therefore, preventative approaches that utilize additional aspects of the host response, such as the innate immune responses, may offer new therapies to help fight influenza. However, little research has currently been conducted into host-pathogen responses following H5N1 influenza infection and consequently, control of H5N1 influenza outbreaks relies heavily on quarantine and slaughter of infected animals.

Host-pathogen interactions are crucial in the outcome of infection, setting the tone for subsequent immune action against pathogens through the interactions of receptors and their downstream regulation of immune genes (Kato *et al.,* 2006; Wang *et al.,* 2007; Ehrhardt *et al.,* 2009). To be highly virulent, a virus must be able to escape the host immune response and replicate rapidly. In addition, it has been recognized that extreme virulence is associated with an intense cytokine response which is generated by H5N1 influenza infection and leads to inflammation at sites of infection (Ichinohe *et al.,* 2009) and the death of large numbers of cells (Webster, 2001). Since

proinflammatory cytokines are markedly elevated during severe H5N1 influenza virus infection, it is hypothezised that hypercyokinemia or cytokine storm, may be crucial in contributing to increased disease severity (Cheung *et al.,* 2002). However, there is some conjecture as to how influential hypercytokinemia is in contributing to increased disease severity given the varied pathogenicity of H5N1 viruses in different species. Many migratory bird species, such as ducks, often appear asymptomatic to H5N1 influenza infection, however, little is known about the duck immune response to virus (Isoda *et al.,* 2006; Jeong *et al.,* 2009). Our research presented in chapter 3, shows that in contrast to chickens, which produce an intense cytokine response, ducks typically show little change in proinflammatory cytokine levels. These observations suggest that following H5N1 influenza infection there may be differing immune responses between species which are critical in determining the severity of disease.

Given the mechanisms for H5N1 disease pathogenesis are still somewhat unclear, research is needed to identify key elements involved in the immune response to influenza virus. Moreover, a better understanding of the inflammatory molecules which are associated with hypercytokinemia is needed, with the aim to provide information that may assist in developing new antiviral strategies and therapeutics for the chicken and potentially humans. In humans, H5N1 influenza infection is characterized by rapid viral replication and while dissemination outside the respiratory tract was not initially observed from outbreaks in 1997 (Peiris *et al.,* 2004), more recent H5N1 viruses appear capable to disseminate systemically to multiple organs contributing to unusual disease manifestations (de Jong *et al.,* 2005; de Jong *et al.,* 2006b). Lee *et al*., (2008), suggest that the available data of human H5N1 influenza infection has consistently found very few virus infected lung epithelial cells, despite widespread histopathological damage (To *et al.,* 2001; Peiris *et al.,* 2004). Perhaps with this in mind they hypothesize that the proinflammatory response may be rapid and occur prior to the release of progeny viruses and therefore, the effects of the proinflammatory cascade triggered by H5N1 virus may outpace the direct effects caused by the virus (Lee *et al.,* 2011). Furthermore, studies by Sarmento *et al.,* (2008) used microarrays to demonstrate that patterns of host gene expression are altered by differential H5N1 influenza infection as early as 6 hours post infection,

whilst viral growth was preceded by this distinct host response (Sarmento *et al.,* 2008).

As sequencing of influenza viruses has improved, it has been possible to create reverse genetic segments to investigate how important mutations in viral genes impact on viral replication. Hatta *et al*., (2001) have demonstrated that a glutamic acid-to-lysine substitution at residue 627 of the PB2 polymerase protein and an HA glycoprotein that can be readily cleaved is associated with the severe virulence of the H5N1 Hong Kong influenza virus. Similarly, investigations into the role of influenza genes such as PB1 and PB2 have demonstrated that they are important for altered viral replication following H5N1 influenza virus infection (Wasilenko *et al.,* 2008). Nevertheless, these proposed virulence mechanisms are not necessarily definitive and it appears that multigenic mechanisms contribute to the disease outcome. Moreover, elevated levels of serum cytokines and chemokines accompany these clinical manifestations (Peiris *et al.,* 2004) and may assist viral dissemination. In other diseases, increased levels of IL6 and inflammatory molecules, such as IFNγ, SAA and NO, are known to cause tissue damage when upregulated to excessive levels (Hibbs *et al.,* 1988; Palmer *et al.,* 1992; Cao *et al.,* 1998). Similarly, in human cases of H5N1 influenza infection, high cytokine levels have been associated with severe infection and may contribute to disease pathogenesis (de Jong *et al.,* 2005; de Jong *et al.,* 2006b). If the chicken immune response to influenza operates in a similar fashion to mammals this may lead to new approaches being developed in the chicken. Approaches may include the modulation of key inflammatory cytokines so as to prevent hypercytokinemia, allowing a more appropriate immune response against virus. However, the limited information regarding the role of key inflammatory genes, such as IL6, in contributing to hypercytokinemia has restricted these approaches to manage H5N1 influenza infection in the chicken. Therefore, our investigations provide new information that may assist to advance the inhibition of an inflammatory cytokine response which can become deleterious during severe influenza infection.

A key set of inflammatory genes appear to be upregulated during human H5N1 influenza infection (To *et al.,* 2001; Us, 2008; Cheng *et al.,* 2011). The relationship

between severe H5N1 infection and inflammatory cytokine production was first alluded to by Dybing *et al*., (2000) and later more fully recognized following observations of intense cytokine production in H5N1 infected human macrophages (Cheung *et al.,* 2002). In studies of H5N1 influenza viruses which have produced severe infections, inflammatory cytokines, particularly TNF, IL6, IL1β, IL18 and IFNs have all been shown to be elevated, either systemically or at focal sites of viral replication (Chan *et al.,* 2005; Szretter *et al.,* 2007). Furthermore, many of these inflammatory cytokines signal the downstream production of other inflammatory molecules. The interleukins IL1β and IL6 contribute to the excessive production of acute phase reactants which can cause cellular damage (Won *et al.,* 1991; Olsson *et al.,* 1999). We observed that high levels of IL6 were systemically expressed in chickens infected with both the Vt453 and Ind109 H5N1 strains and similarly the levels of SAA, an acute phase gene, were also increased. The interferons, like IFNγ play a role in increasing free radicals, such as reactive nitrogen intermediates (RNIs) and reactive oxygen species (ROS) (Hierholzer *et al.,* 1998a; Chan *et al.,* 2001). The changes in one such RNI linked enzyme, iNOS, in H5N1 influenza infected chicken tissues was observed and compared to iNOS and NO levels in infected ducks. These tissue specific increases in iNOS production in the chicken appear to be associated with a higher degree of disease severity in H5N1 infection in chickens. Furthermore, studies of mammalian H5N1 influenza infection have also described an increased production of iNOS and NO which is thought to be detrimental during infection (Wasilenko *et al.,* 2008; Aldridge *et al.,* 2009). Whether, the high levels of iNOS are directly caused by increased inflammatory cytokine production is currently unclear, however, the induction of iNOS by IFNγ has been well documented (Nathan *et al.,* 1994; Kacergius *et al.,* 2006; Wasilenko *et al.,* 2009). In chickens, this environment of high cytokine levels, acute phase genes and free radicals is associated with severe vascular leakage and lung edema during H5N1 infection and may promote viral dissemination (Perrone *et al.,* 2008; Tate *et al.,* 2010). This contrasts with typical H5N1 infections in ducks, whereby inflammatory cytokine levels are significantly lower but peak later in the infection period (Burggraaf *et al.,* 2011). This more closely regulated cytokine production may be more involved in directing an adaptive immune response which targets viral replication that is generally confined to fewer organs

(Bingham *et al.,* 2009; Burggraaf *et al.,* in submission). Therefore, examining the key components of the inflammatory response during H5N1 induced hypercytokinemia in chickens and ducks may help us understand how to survive the initial hyperinflammatory response and prolong survival so that an adaptive response which targets viral replication can commence.

In many aspects the chicken immune response to influenza infection appears similar to that observed in humans. However, a generally lower representation of orthologues of mammalian genes in the chicken genome appears to be an observation with regard to inflammatory cytokines (Kaiser *et al.,* 2005) and may have an outcome in the immune response against influenza. Tumor necrosis factor (TNF), a key molecule which regulates gene products involved in inflammation and tissue repair has not yet been identified in chickens (Tubak *et al.,* 2009). Given the elevated levels of TNF following mammalian H5N1 infection and the proposed role TNF plays in promoting an inflammatory cytokine cascade, such as through IL6 induction (Kozawa *et al.,* 1997; Tanabe *et al.,* 2010), the lack of TNF in the chicken is intriguing. Since our research demonstrates that IL6 is highly increased in chickens during H5N1 influenza infection, and given the lack of TNF induced IL6 production, investigations to determine through which mechanism IL6 is upregulated may be beneficial. It is likely chickens have a mechanism to compensate for the loss of TNF (Rautenschlein *et al.,* 1999) and appear capable of stimulating genes normally under the control of TNF. The TNF-like molecule (TL1A) may have an immuno-regulating role, however, it is not known what role this TNF-like gene plays during viral infection in chickens or whether it contributes to hypercytokinemia like during mammalian H5N1 influenza infection (Us, 2008).

Similarly, a number of pattern recognition receptors, which detect viral RNA, also appear to be condensed to fewer family members or in some cases, may be missing completely from the chicken genome. The chicken TLR family for example appears to use TLR7 to represent mammalian TLR7, 8 and 9 (Philbin *et al.,* 2005). As described by Philbin *et al*., (2005) and Jenkins *et al*., (2006), chicken TLR7 responds to agonists of mammalian TLR7 and importantly, these TLR7 agonists stimulate the

signalling of IL1β and IL6 and to a lesser degree induce an IFN response in chicken cell lines (Philbin *et al.,* 2005). This may have some implications for the antiviral role TLR7 plays, as differences in the TLR repertoire and TLR signalling may influence the patterns of host susceptibility to infections and the immune mechanisms responsible for the control of infectious challenge in different host species. We have observed that during H5N1 influenza infection, chicken TLR7 levels were significantly lower when compared to duck TLR7 levels. It is not known how this difference in TLR7 levels impacts on the innate immune response in chickens or whether lower TLR7 levels contribute to a reduced antiviral response when compared to ducks. Typically, TLR7 would act to stimulate type I IFN and IL6 responses (Glaum *et al.,* 2009), however the high levels of IFN and IL6 in chickens during H5N1 influenza infection, suggests other mechanisms are also associated in initiating an immune response against viral infection (Kato *et al.,* 2008; Yen *et al.,* 2009; Cheng *et al.,* 2011). Recent research into avian pattern recognition receptors shows that a host of viral recognition genes, such as melanoma differentiation associated gene 5 (Mda5) and the retinoic acid inducible gene I (RIG-I) may be important for an effective antiviral response. However, in the chicken, it appears that RIG-I is missing and as yet, a RIG-I like gene has not been identified (Barber *et al.,* 2010). Barber *et al*., (2010) suggest that when chicken cells express duck RIG-I, their immune response to H5N1 infection is improved. This would suggest that a lack of RIG-I hinders the chicken response during influenza infection. However, since mammals have functional RIG-I and yet are still susceptible to severe H5N1 infections, it may be that that the antiviral mechanisms of RIG-I alone are not sufficient to fully protect from HPAIV infection. Furthermore, there may be differences in the roles of other pattern recognition receptors between chickens and ducks. In chickens it is possible that Mda5 or other as yet unidentified receptors compensate for the absence of RIG-I by carrying out some of the functions attributed to RIG-I in mammals and ducks. Recent studies by Karpala *et al*., (2011) show that Mda5 is upregulated during H5N1 influenza infection (Karpala *et al.,* 2011). This complicated network of pattern recognition responses are likely pivotal in the induction of the inflammatory cytokine response following H5N1 influenza infection and a greater understanding of their role may help provide better regulation of many inflammatory molecules.

Whilst the entire chicken genome has been sequenced and many inflammatory genes characterised, there is a paucity of information regarding duck immune genes. Nevertheless, of the genes identified in the duck, many of the inflammatory cytokines upregulated during influenza infection share a close sequence similarity to those observed in chickens. The interleukin IL1β is 99% identical between chickens and ducks, whilst IFNγ is 80% identical. Moreover, there is functional similarity between many duck and chicken genes (Guo *et al.,* 1996; Wu *et al.,* 2007; Chen *et al.,* 2008). Our results demonstrate that chicken IFNγ can stimulate duck cells to produce iNOS at a similar gene level as witnessed in chicken cells and similarly it has been demonstrated that IFNγ is functionally similar in many avian species (Li *et al.,* 2007). The identification and cloning of duck iNOS also provides important information regarding inflammatory mechanisms in the duck, as its analysis suggested conservation of its role in the duck. Identification of duck iNOS also allowed for the investigation and comparison of the role of iNOS and NO during influenza infection in chickens and ducks. Mammalian studies of NO during influenza suggested iNOS expression and the downstream production of NO may be important in the outcome of disease (Aldridge *et al.,* 2009; Wasilenko *et al.,* 2009). Our studies revealed that high levels of NO and iNOS during H5N1 infection in chickens were associated with disease severity and may be due to the increased IFNγ levels observed in a range of tissues. IFNγ is crucial in promoting NO production and studies by Wasilenko *et al*., (2009), show that high levels of IFNγ and iNOS are associated with increased NO levels which may be involved in increased pathogenicity (Wasilenko *et al.,* 2008; Wasilenko *et al.,* 2009). Furthermore, it has been postulated that excessive levels of NO following influenza infection may cause severe cellular and organ damage (Akaike *et al.,* 1996). Akaike *et al*., (1998) and Karcegius *et al*., (2006) suggest that the contribution of iNOS as part of the mechanism for pathogenesis during influenza virus induced pneumonia is clear, whereby NO synthesis, through iNOS gene induction, contributes to lung damage through the build up of free radicals. However, it is likely that the inflammation present during influenza infection is multifaceted and caused by a number of inflammatory molecules. Therefore, therapeutics which impact on the modulation of the inflammatory response need to be either targeted against

early inflammatory genes which promote the cytokine cascade or against genes that have a broad ranging effect.

Many recent studies of mammalian and avian influenza infections have demonstrated that proinflammatory cytokines like IL6 are systemically elevated during infection. Increased IL6 levels impact on the regulation of immune molecules produced by epithelial and vascular endothelial cells during infection and have been implicated in the influenza induced pathogenesis of mammals (Kaiser *et al.,* 2001; Chan *et al.,* 2005). This increase in IL6 prior to death has been noted in humans and primates (To *et al.,* 2001; Chan *et al.,* 2005; Evseenko *et al.,* 2007) and our studies demonstrate that chickens, which have a similar symptom severity during infection, also have systemically increased levels of IL6. Furthermore studies of other zoonotic viruses which induce hypercytokinemia, such as SARS and Dengue, have shown that IL6 may contribute to disease severity and often lung injury (Hierholzer *et al.,* 1998b; Huang *et al.,* 2005; Pang *et al.,* 2007). As yet, how excessive IL6 levels contribute to disease severity is somewhat unclear. However, the disparity in cytokine production could potentially impact on the downstream regulation of IL6 stimulated genes. High levels of cytokines such as IL6 and IL1β promote the induction of acute phase reactants response, which consequently increase inflammation, which may become deleterious to the host (Mozes *et al.,* 1989; Miwata *et al.,* 1993; Griselli *et al.,* 1999). In chickens during H5N1 influenza infection, SAA was observed to be significantly elevated when compared to ducks. This suggests that a combination of increased IL6, IFNγ, SAA and NO promotes a deleterious inflammatory response in the chicken during H5N1 infection. This hyper inflammatory response appears to be unique to H5N1 influenza viruses, as chickens infected with a H5N3 strain of influenza showed significantly lower cytokine levels. Similarly, investigations into the inflammatory response in mammals infected with various H1N1 strains has demonstrated typically low inflammatory cytokine levels when compared to H5N1 infection (Lee *et al.,* 2010), suggesting that treatment of H5N1 virus strains may need therapies that target both the increased viral replication and high cytokine production.

The therapeutic use of inflammatory inhibitors requires consideration, since H5N1 virus strains appear to be associated with an intense cytokine response and have emerged to become resistant to antiviral treatment compared to seasonal influenza A virus strains. Moreover, therapeutics for the treatment of seriously ill influenza infected patients are lacking and neuraminidase inhibitors alone have not clinically proven effective for H5N1 influenza patients as they cannot address the lethal cytokine storm associated with infection (de Jong *et al.,* 2006b; Us, 2008; Cheng *et al.,* 2011). Studies of H5N1 influenza in mice treated in a delayed fashion (48 h.p.i), with the antiviral zanamivir, showed IL6 levels were reduced by 10 percent compared to infected control mice however survival was not improved (Zheng *et al.,* 2008). In contrast, when H5N1 infected mice were treated with a combination of an antiviral and immunomodulators survival was improved significantly and inflammation decreased (Zheng *et al.,* 2008). Similarly, the use of Mad-A and Gal to inhibit the signalling of IL6 during H5N1 infection in chickens suggested that viral replication could be reduced and inflammation improved through modulation of the inflammatory response. However, the levels of IL6 signalling did not appear to be reduced sufficiently, as inflammatory markers were still increased compared to uninfected chickens. Whether this was due to an insufficient Mad-A/Gal treatment dose or because of redundancy in the inflammatory response, whereby the inhibition of a single cytokine can be compensated for, is unknown and additional research is needed to confirm the benefits of inhibiting IL6 signalling. Nevertheless, these studies provide insight into the role possible therapeutics could play to target inflammation and improve the immune response during H5N1 infection.

7.1 Future directions

Currently, little exists in the way of therapeutic approaches to improve the disease caused by severe H5N1 influenza induced hypercytokinemia. In chapter 5 we demonstrate the potential of inhibiting the IL6 signalling pathway to reduce inflammation and decrease the downstream accumulation of damaging acute phase genes and free radicals. However, it may be that the cytokine response has some redundancy, whereby successful inhibition of a single cytokine is annulled through

the signalling of other cytokine genes and therefore, a multi-targeted approach may be necessary. Studies of anti-inflammatory therapies by Salomon *et al*., (2007) confirm that preventing a single component of the cytokine storm from erupting failed to protect H5N1 infected mice or improve survival. This data implies that it is necessary to focus on limiting virus replication as well as understanding the exacerbated cytokine response, which is driven through multiple inflammatory mechanisms. Recent studies by Perrone *et al*., (2010) support this multi targeted inhibition of inflammatory genes. In H5N1 influenza infected mice containing a triple mutation causing deficiencies in signalling receptors, TNF and IL1-RI, there was reduced morbidity and a significant delay in mortality following lethal viral challenge. This data suggests that the combined signalling from the TNF and IL1 receptors may promote increased lung inflammation in mice and may contribute to the severity of disease caused by H5N1 virus infection. However, to complicate matters further, Okada *et al.,* (2009) recently suggested that ETR-P1 administration alone rescued chickens from the lethal inflammatory response normally induced during H5N1 influenza infection. H5N1 induced ET-1 expression in the lungs of chickens was contributing to excessive inflammation through neutrophil activation. With these studies in mind it appears that additional research is needed to confirm whether complete inhibition of IL6 can improve the response against H5N1 influenza infection in chickens and should be considered in therapeutic strategies to target the virus. Furthermore, it may be that investigations aimed at reducing a combination of inflammatory factors, such as IL6, IL1β and NALP in concert with an antiviral have the most success in reducing H5N1 viral replication and improving survival times. In either case, it is hoped that reductions to the inflammatory cytokine cascade will result in providing an extended window for the adaptive immune response to become organized and target viral replication and ameliorate influenza infection.

One area in which progress has been steadily made, is the use of new technologies to help determine critical pathways during influenza infection. Among the various approaches used to explore host factor involvement in the influenza virus replication cycle, perhaps the most powerful is RNAi-based genome-wide screening, which has shed new light on the search for host factors involved in virus replication. Studies by

Karlas *et al.,* (2010), and Watanabe *et al.*, (2010), to name just two, have identified a range of host genes involved in transport, trafficking, endosocytosis, translation initiation and cytokine induction, which may be co-opted by the influenza virus to help increase viral replication (Watanabe *et al.,* 2010; Karlas *et al.,* 2010). These more detailed screens represent a starting point for exploration of important host factors and it is hoped that this may lead to the discovery of new drug targets for a broad range of influenza viruses.

7.2 Concluding remarks

The results of this study have important implications for the development of control strategies and therapeutics which aim to manipulate immune pathways to impact on the severity of viruses such as HPAI. The improved understanding of these pathways following infection is of paramount importance in order to progress these innovative viral control strategies as has been observed with other zoonotic diseases. The studies herein provide new insights into the varied pathogenicity associated with H5N1 influenza viruses in chickens and ducks and highlight the differing immune responses produced by the host during infection. Importantly, the specific inflammatory cytokine response that is produced during H5N1 influenza infection has helped identify downstream cascades that promote deleterious inflammation and vascular leakage. The initial identification of duck genes such as iNOS, led to the comparison of molecules which are associated with increased disease severity in chickens. Given the exacerbated response in chickens when compared to ducks, therapeutics which can help to alleviate the hypercytokinemia witnessed during H5N1 infection may be beneficial. Therefore, the IL6 inhibitors, Madindoline and Gallielalactone, were used in chickens to reduce the excessive inflammation normally associated with H5N1 influenza infection and observed to have an impact on viral replication. Moreover, the demonstrated benefit of anti-inflammatory therapy may now provide a mechanism, in concert with currently used antivirals, by which to treat H5N1 influenza infection and provide a platform for future work aimed at combating H5N1 influenza infections in susceptible species.

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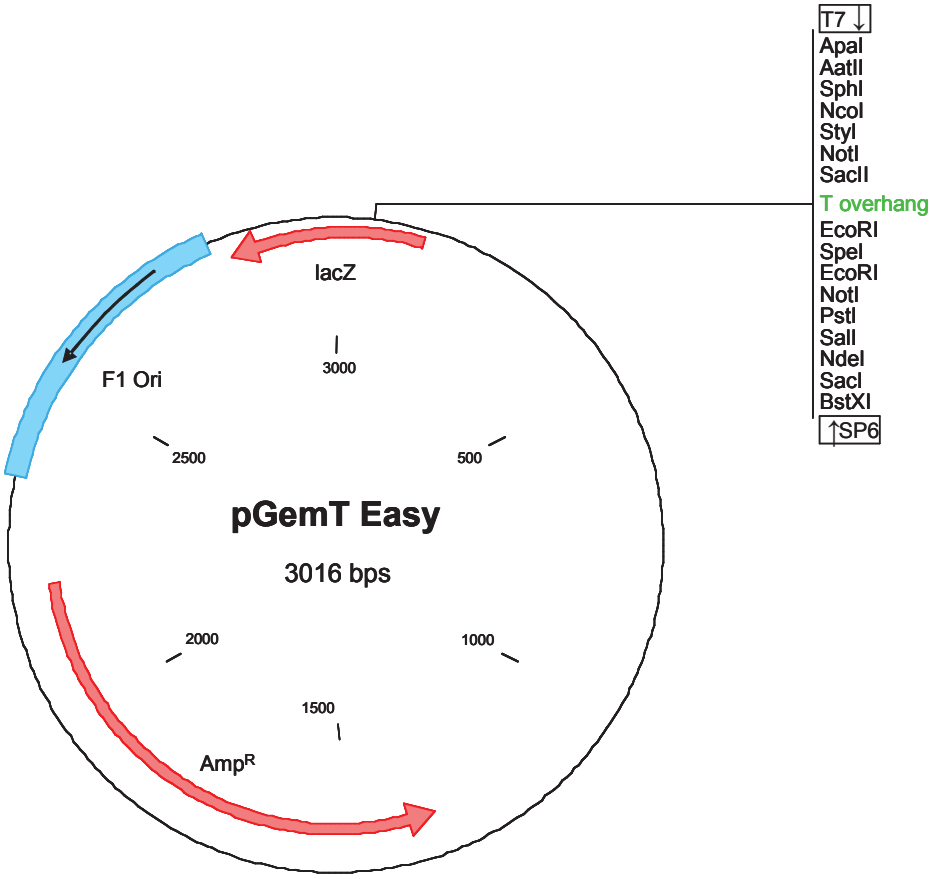
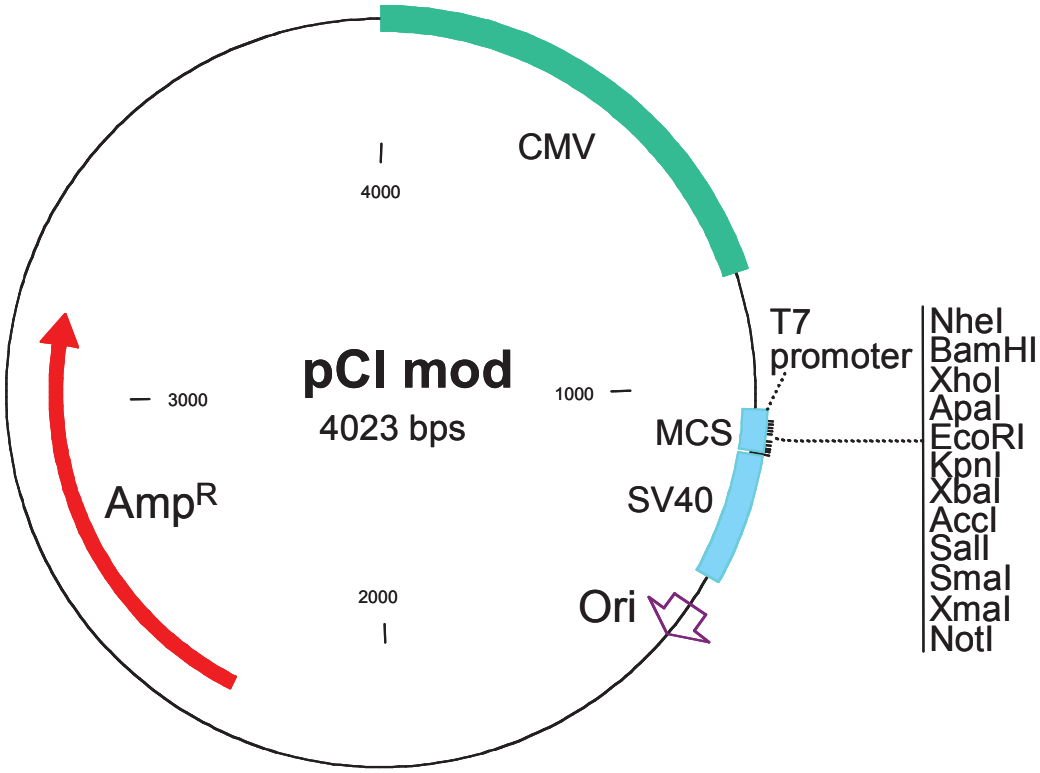
Seasonal A (H1N1) and Pandemic A (H1N1) 2009 Influenza Viruses in Guangdong, China, during 2008 and 2009. *Journal of Clinical Microbiology* **49**: 2651-5.

Appendices

8.1 Appendix 1. Vector maps



Appendix 1.1. Plasmid map of the pGemT Easy vector. The pGemT Easy plasmid (Promega, USA) contains single 3´-T overhangs at the insertion site to improve the efficiency of ligation. The pGemT Easy plasmid incorporates T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the alpha-peptide coding region of the enzyme beta-galactosidase (lacZ). Insertional inactivation of the alpha-peptide allows recombinant clones to be directly identified by colour screening on indicator plates. In addition pGemT Easy also contains β-lactamase gene for ampicillin resistance (AmpR), an f1 origin of replication (Ori).



Appendix 1.2. Plasmid map of the pCI-mod vector. The pCI-mod plasmid (Promega, USA) incorporates a β-lactamase gene for ampicillin resistance (AmpR), an f1 origin of replication (Ori), a human cytomegalovirus (CMV) immediate enhancer/promoter region for strong expression, a T7 RNA polymerase promoter, a multiple cloning site (MCS), and an SV40 late polyadenylation signal for efficient RNA processing.

8.2 Appendix 2. Solutions and reagents

AEC substrate

AEC chromagen powder

AEC buffer

200 µl distilled water

ATV

8.0 g NaCl

0.4 g KCl

1.0 g Glucose

0.35 g $NaHCO_3$

0.5 g Trypsin

0.2 g EDTA

0.06 g KH_2PO_4

0.09 g $Na_2HPO_4.12H_2O$

Make up to 1L MilliQ water. Stir the solution O/N at 4°C. Filter and aliquot (50 mL) and store at -20°C. Once thawed can be used for 1-2 weeks if kept at 4°C.

Carbonate buffer

15 mM Na_2CO_3 (Ajax Chemicals, Australia)

35 mM $NaHCO_3$ (Prolabo, France)

pH 9.6

Destaining solution

5% v/v methanol (BDH, Australia)

10% v/v acetic acid (Ajax Chemicals, Australia)

Dulbecco's Modified Eagle Medium (DMEM)

DMEM (Trace Biosciences, Australia) was diluted 1:5 in MilliQ water with the following additions:

20 mM HEPES (ICN, USA)

0.75 mg/mL $NaHCO_3$ (Prolabo, France)

1 mM L-glutamine (Sigma, Germany)

100 U/mL penicillin (CSL, Australia)

100 µg/mL streptomycin sulfate (CSL, Australia)

50 µM 2-mercaptoethanol (Sigma, Germany)

7TD1 media

DMEM with following additional additives

10% FCS

0.5 mM Arginine (Sigma, Germany)

0.24 mM Asparagine (Sigma, Germany)

FACS permeabilising solution

Perm/Wash™ buffer (Becton Dickinson, USA) diluted 1:10 in distilled water

FACS wash

2% FCS (Trace Biosciences, Australia)

0.01% NaN_3 (BDH, UK)

PBS

H_2SO_4 stop solution

0.5 M H_2SO_4 (BDH, Australia)

Inactivation of foetal calf serum (FCS)

FCS (Trace Biosciences, Australia) was inactivated at 56°C for 1 h and cooled to room temperature before use

Red cell lysis buffer

1 mL 170 mM Tris hydroxymethyl methane (BDH, Australia), pH 7.6

9 mL 0.83% NH_4Cl (BDH, Australia)

RNA Later

40 mL 0.5M EDTA

25 mL 1M Sodium Citrate

700 g Ammonium Sulfate

935 mL dH_2O

Stir on a hotplate stirrer on low heat until dissolved completely then allow to cool.

Adjust to pH 5.2 with H_2SO_4

Running buffer

25 mM Tris hydroxymethyl methane

192 mM glycine (APS, Australia)

170 mM sodium dodecyl sulphate (SDS; BDH, Australia)

pH 8.3

SDS-PAGE

For two 12% or 15% polyacrylamide gels

Solution components	Resolving gel		Stacking gel
	12%	15%	4%
MilliQ water (ml)	1.9	1.1	3
Acrylamide/Bis (Bio-rad, USA) (mL)	4.2	5	0.66
1 M Tris, pH 8.8 (mL)	3.8	3.8	
0.5M Tris, pH 6.8 (mL)			1.25
10% SDS (µL)	100	100	50
TEMED (Bio-rad, USA) (µL)	5	5	5
10% Ammonium Persulfate (APS) (Bio-rad) (µL)	100	100	50

Sodium casein

1% Casein sodium salt (Sigma, Germany) made up in PBS

TAE buffer (50x)

(Sambrook *et al.,* 1989)

2 M Tris hydroxymethyl methane

1 M acetic acid

0.05 M EDTA, pH 8.0 (Bio-rad,USA)

TBS buffer

10 mM Tris-Cl, pH 7.5

150 mM NaCl

TBS-Tween/Triton buffer

20 mM Tris-Cl, pH 7.5

500 mM NaCl

0.05% Tween-20 (Sigma, Germany)

0.2% TritonX-100 (Sigma, Germany)

TMB substrate solution

15 mL MilliQ H20

15 mL 200mM sodium acetate (BDH, Australia)

450 µL citric acid (BDH, Australia)

3.6 µL H_2O_2 (Sigma, Germany)

300 µL tetramethyl benzidine (TMB) (Boehringer Mannheim, Germany)

Trypticase™ Soy Broth (TSB)

(Becton Dickinson, USA)

1.7% Pancreatic digest of casein

0.3% Papaic digest of soybean meal

0.5% Sodium chloride

0.25% Dipotassium phosphate

0.25% Dextrose

TSB™ agar plates

1.5% Bacto Agar

TSB

Western transfer buffer

25 mM Tris hydroxymethyl methane

192 mM glycine

20% ethanol (v/v)

Western blocking buffer

3% bovine albumin (BSA) (Sigma, Germany)

10 mM Tris-Cl, pH 7.5

150 mM NaCl

0.1% Coomassie brilliant blue stain

0.1% Brilliant blue R250 (Sigma, Germany)

45% methanol

9% acetic acid

2x SDS-PAGE-loading buffer

125 mM Tris-Cl, pH 6.8

20% glycerol

4% SDS

200 mM Dithiothreitol (DTT; Progen, Australia)

1% Bromophenol Blue (Sigma, Germany)

10% Ammonium persulfate (APS)

1 g APS (Bio-Rad)

10 mL dH_2O

Filter sterilise

10x Loading Buffer

0.025 g Bromophenol blue (Sigma)

0.025 g Xylene cyanol

5 mL Glycerol

Made up to 10 mL with 10x TAE

50X TAE buffer

(Sambrook *et al.,* 1989)

2 M Tris base

1 M glacial acetic acid

0.05 M EDTA, pH 8.0

10x Running Buffer

30.5 g Tris base (Sigma)

143.65 g Glycine (AnalaR)

10 g SDS (BDH Lab. Supplies)

1M Tris pH 8.8

60.55 g Tris (Sigma)

400 mL dH_2O

Adjust pH to 8.8 with HCl

Make up to 500 mL

0.5M Tris pH 6.8

30.275 g Tris (Sigma)

400 mL dH_2O

Adjust pH to 6.8 with HCl

Make up to 500 mL

0.25% Dextrose

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PLoS one

Increased Inducible Nitric Oxide Synthase Expression in Organs Is Associated with a Higher Severity of H5N1 Influenza Virus Infection

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Abstract

Background: The mechanisms of disease severity caused by H5N1 influenza virus infection remain somewhat unclear. Studies have indicated that a high viral load and an associated hyper inflammatory immune response are influential during the onset of infection. This dysregulated inflammatory response with increased levels of free radicals, such as nitric oxide (NO), appears likely to contribute to disease severity. However, enzymes of the nitric oxide synthase (NOS) family such as the inducible form of NOS (iNOS) generate NO, which serves as a potent anti-viral molecule to combat infection in combination with acute phase proteins and cytokines. Nevertheless, excessive production of iNOS and subsequent high levels of NO during H5N1 infection may have negative effects, acting with other damaging oxidants to promote excessive inflammation or induce apoptosis.

Methodology/Principal Findings: There are dramatic differences in the severity of disease between chickens and ducks following H5N1 influenza infection. Chickens show a high level of mortality and associated pathology, whilst ducks show relatively minor symptoms. It is not clear how this varying pathogenicty comes about, although it has been suggested that an overactive inflammatory immune response to infection in the chicken, compared to the duck response, may be to blame for the disparity in observed pathology. In this study, we identify and investigate iNOS gene expression in ducks and chickens during H5N1 influenza infection. Infected chickens show a marked increase in iNOS expression in a wide range of organs. Contrastingly, infected duck tissues have lower levels of tissue related iNOS expression.

Conclusions/Significance: The differences in iNOS expression levels observed between chickens and ducks during H5N1 avian influenza infection may be important in the inflammatory response that contributes to the pathology. Understanding the regulation of iNOS expression and its role during H5N1 influenza infection may provide insights for the development of new therapeutic strategies in the treatment of avian influenza infection.

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Introduction

H5N1 influenza virus strains have been prevalent and highly pathogenic in gallinaceous birds, specifically chickens, causing acute systemic disease [1,2,3]. The severity of infection, however, varies dramatically between chickens and other avian species, such as ducks [1,4,5]. Infection of ducks is often asymptomatic, whereby H5N1 viruses, which are categorised as highly pathogenic in chickens, appear to display reduced clinical signs in the duck [6,7,8]. Furthermore, little is known about the immune response generated following H5N1 influenza infection in both chickens and ducks. Viral replication may be associated with an increased proinflammatory response [9,10,11], as chickens exhibit widespread viremia and an associated increase in cytokines, leading to inflammation. This suggests that H5N1 may be a potent inducer of proinflammatory cytokines, chemokines and free radicals in chickens [9,11,12,13]. Therefore, a key question that remains unanswered in the field is what drives the pathogenicity associated with H5N1 influenza infection?

The free radical NO is an important messenger molecule linked to an array of immune responses [14]. NO mediates macrophage cytotoxicity and plays an antiviral role [15,16,17]. NO is generated by the enzyme NOS, which catalyses the biosynthesis of NO in a range of tissues. Currently, a number of distinct NOS isoforms have been characterised in mammals: the neuronal isoform (nNOS), inducible isoform (iNOS) and endothelial isoform (eNOS) [18,19,20]. The two constitutive forms, nNOS and eNOS, are activated by, and dependant on, changes in intracellular calcium, [21,22] whereas iNOS is calcium independent [23]. It is this inducible NOS (iNOS) form which serves as a key molecule in combating viral infection, acting as a mediator of apoptosis and the acute phase protein response [24,25]. Excessive production of

iNOS, however, may have negative effects reacting with other damaging oxidants and promoting inflammation [26]. Recently the iNOS gene was cloned in the chicken [27], which has allowed investigations of iNOS activity and the role of this gene in a range of tissues following H5N1 influenza virus infection of chickens. However, at present there is a paucity of information surrounding the duck iNOS gene and its role in the immune response to influenza virus infection.

To explore the role of iNOS and NO production and contrast this following the immune response of chickens and ducks to H5N1 influenza, we identified and cloned iNOS in ducks. We showed that levels of NO were elevated in both infected chickens and ducks when compared to uninfected birds. Given the severity of pathology associated with H5N1 infection varies in the organs of chickens and ducks, we examined the pathways leading to NO production at the prominent sites of infection. Our results showed that infected chickens have an earlier and more prevalent spread of iNOS in lung, spleen, caecal tonsil and liver tissue as compared to ducks. The high expression of iNOS in chicken organs may account for the more severe disease associated with H5N1 infection in this species.

Materials and Methods

Animals

Five-week-old Pekin ducks were purchased from Luv-a-Duck (Nhill, Victoria, Australia) and six-week-old broiler Ross chickens were purchased from Bartters (Bannockburn, Victoria, Australia). Animals were individually identified with leg bands and assigned to treatment groups. The ducks and chickens were fed with commercial grower chicken pellets, *ad libidum*. Water troughs that were deep enough for the ducks to float and splash were placed in each room. Each room also had a partially enclosed dry retreat with wood shavings for the ducks and chickens to sit on. All animal work was conducted with the approval of the CSIRO - AAHL Animal Ethics Committee. All procedures were conducted according to the guidelines of the National Health and Medical Research Council as described in the Australian code of practice for the care and use of animals for scientific purposes [28].

Virus strains

Ducks and chickens were challenged with the Vietnamese H5N1 strain, A/Muscovy duck/Vietnam/453/2004. The virus was passed twice in chicken eggs to obtain a working stock. The working inoculum consisted of a 1:100 dilution of infected allantoic fluid. A total volume of 0.5 mL was inoculated through an oral-intranasal route with each duck and chicken dose containing approximately $10^{7.2}$ median egg infectious doses (EID_{50}) [29].

Virus titration

Lung, caecal tonsil, liver and heart tissues were dissociated by bead beating and 10% w/v homogenates in phosphate-buffered saline were prepared. Homogenates were titrated in flat-bottomed 96-well micro-titre plates which were seeded with a Vero cell suspension (10^6 cells per plate). Ten-fold dilutions of the samples were prepared and 0.1 mL of each dilution was added, as 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 CO_2 incubator and examined for the presence of cytopathic effect after 5 days. The lowest 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 per 0.1 mL ($TCID_{50}$/0.1 mL).

Isolation of lymphocytes and cell culture

Ducks and chickens were anesthetised by CO_2 asphyxiation and spleens harvested. Single cell suspensions of leukocytes were prepared from individual spleens by dispersal through a 70 μm strainer into Petri dishes containing DMEM. Suspensions were layered over lymphoprep (Nicomed Pharma AS, Oslo, Norway) and centrifuged at $1500g_{max}$ for 15 min. Mononuclear cells at the interface were collected, washed, resuspended, and cultured in DMEM supplemented with 10% fetal bovine serum (FBS).

Reagents

The synthetic dsRNA analog poly (I:C) (Invivogen) was prepared and stored as per manufacturer's instructions. Both lipopolysaccharides (LPS) from *E. coli* and chicken IFNγ were produced in our laboratory [30]. Nucleic acids were stored at −80°C, and cytokines were stored at 4°C.

RNA isolation and reverse transcription

RNA was harvested using Tri-reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Extracted RNA was subjected to DNase treatment using a DNase 1 (Sigma-Aldrich) and was then DNase-treated RNA was then reverse transcribed to complimentary DNA (cDNA) using a reverse transcription kit (Promega, Madison, WI).

Cloning, 3-prime RACE sequencing

Specific iNOS oligonucleotide primers were designed around the conserved regions in sequences aligned from the human, mouse and chicken iNOS genes [18,27,31,32]. A combination of primers (Table 1) were used to amplify the duck iNOS gene (GenBank accession No. FJ966247). Synthesised cDNA was made with gene specific primers in a standard PCR amplification performed using 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min, with a further 15 min extension at 72°C following the last cycle using an Applied Biosystems DNA Thermocycler 480 (Perkin Elmer, USA). To determine the complete iNOS sequence, a 3-prime RACE (Rapid Amplifiaction of cDNA Ends) kit (Invitrogen) was used according to the manufacturer's instructions. DNA products of interest were gel purified using a gel extraction kit (Qiagen, Valencia, CA) and then ligated into pGemT-Easy (Promega) for sequence analyses. The iNOS gene was sequenced by Micromon DNA sequencing facility, Monash University (Clayton). The method used dye-terminator reactions

Table 1. Primer sequences for RT-PCR and QRT-PCR used in this study.

Species	Primer name	Type	Sequence 5′-3′
duck	dkinosf1	RT-PCR FWD	ATGCTGTGCCCATGGCAGTTTGC
duck	dkinosr2	RT-PCR REV	TTAATTTGTGCTTGGACTGATGGG
chicken	chinosf3	RT-PCR FWD	ATGCTGTGCCCATGGCAGTTTGC
chicken	chinosr4	RT-PCR REV	GCCCGGACCAATGGGTTGCAAATC
duck/ chicken	chdkinos	QRT-PCR FWD	CCACCAGGAGATGTTGAATATGTC
duck/ chicken	chdkinos	QRT-PCR REP	TCCACCTGGTAGTAAAAG
duck/ chicken	chdkinos	QRT-PCR REV	CCAGATGTGTGTTTTCCATGCA

doi:10.1371/journal.pone.0014561.t001

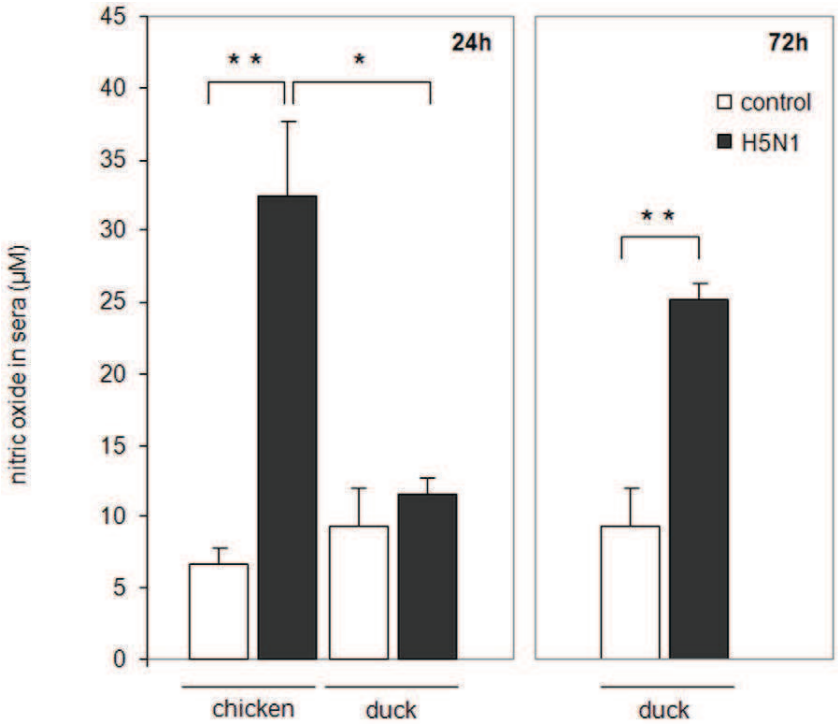

Figure 1. H5N1 influenza infection results in increased serum NO levels in both chickens and ducks. Serum collected from chickens and ducks infected with the H5N1 strain Muscovy/duck/Vietnam/453, as assayed for NO production during the peak of influenza infection (chickens at 24 hours, ducks at 24 and 72 hours post infection). Displayed values are the mean of 2 experiments with 4 birds in each group. A single asterisk indicates statistically significant differences of means with $p<0.05$, double asterisk indicates statistically significant differences of means with $p<0.01$.
doi:10.1371/journal.pone.0014561.g001

performed on an ABI integrated Thermal Cycler and then run on an ABI 377 sequencer (Applied Biosystems).

Nitrate production assay

NO production in sera was measured using a nitric oxide colorimetric assay kit (Roche cat No. 11756281001). Sera was irradiated and then filtered in a 10000 kd MWCO column (Satorious, Vivascience cat No. 13239-E). The assay was conducted as per manufacturer's instructions using a NO control with a standard curve plotted and samples were measured at a 550 nm wavelength.

Semiquantitative RT-PCR (QRT-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 manufacturer's instructions (Applied Biosystems, Foster City, CA). Primers and probes (Table 1) were designed using Primer express software and where possible were designed across intron:exon boundries. Probes were labeled with the reporter dye carboxyfluorescein (FAM) and the quencher tetramethyl-6-carboxyrhodamine (TAMRA). Breifly, triplicate (or more) samples were each measured in 25 μL reactions. PCR cycling was performed as follows: 95°C for 15 sec, 61°C for 30 sec and 68°C for 30 sec. 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 ΔΔCt (Applied Biosystems). Target gene Ct values were normalized to the endogenous control glyceraldehyde-3-phosphate-dehydrogenase, a house-keeping gene, to derive the ΔCt. This was compared with an untreated calibrator to derive the ΔΔCt and relative gene quantitation, or fold expression relative to the untreated control (calibrator), was derived using $2^{-\Delta\Delta Ct}$.

Immunohistochemistry (IHC)

Chicken and duck tissues were fixed in 4% formaldehyde in neutral buffered saline. After no more than 2 days of formalin fixation, tissues were processed into paraffin wax by routine histological methods. Sections of tissues were cut onto slides and stained using an immunoperoxidase test. Sections were quenched with 10% hydrogen peroxide for 10 min and digested with 5 to 7 mg/mL proteinase K for 6–7 min. Sections were then incubated for 1 h with either rabbit serum directed against H5N1 influenza virus nucleoprotein [29] or with a rabbit polyclonal antibody directed against iNOS (Abcam ab3523). A secondary (goat anti rabbit) horseradish peroxidase-conjugated antibody (DAKO Envision) was then used for 45 min. Control sections were run following identical protocols but using an irrelevant primary mouse antibody. Sections were stained with aminoethylcarbazol substrate chromogen (DAKO Envision) for 5 to 6 min, and counterstained with Mayer's haematoxylin.

Statistical analyses

To determine the significant differences between experimental groups, ANOVA or Mann-Whitney *U*-tests were performed using the fold change scores. All data are expressed as the mean±SEM and *p-values* were set at 0.05 ($p\leq0.05$) unless indicated otherwise.

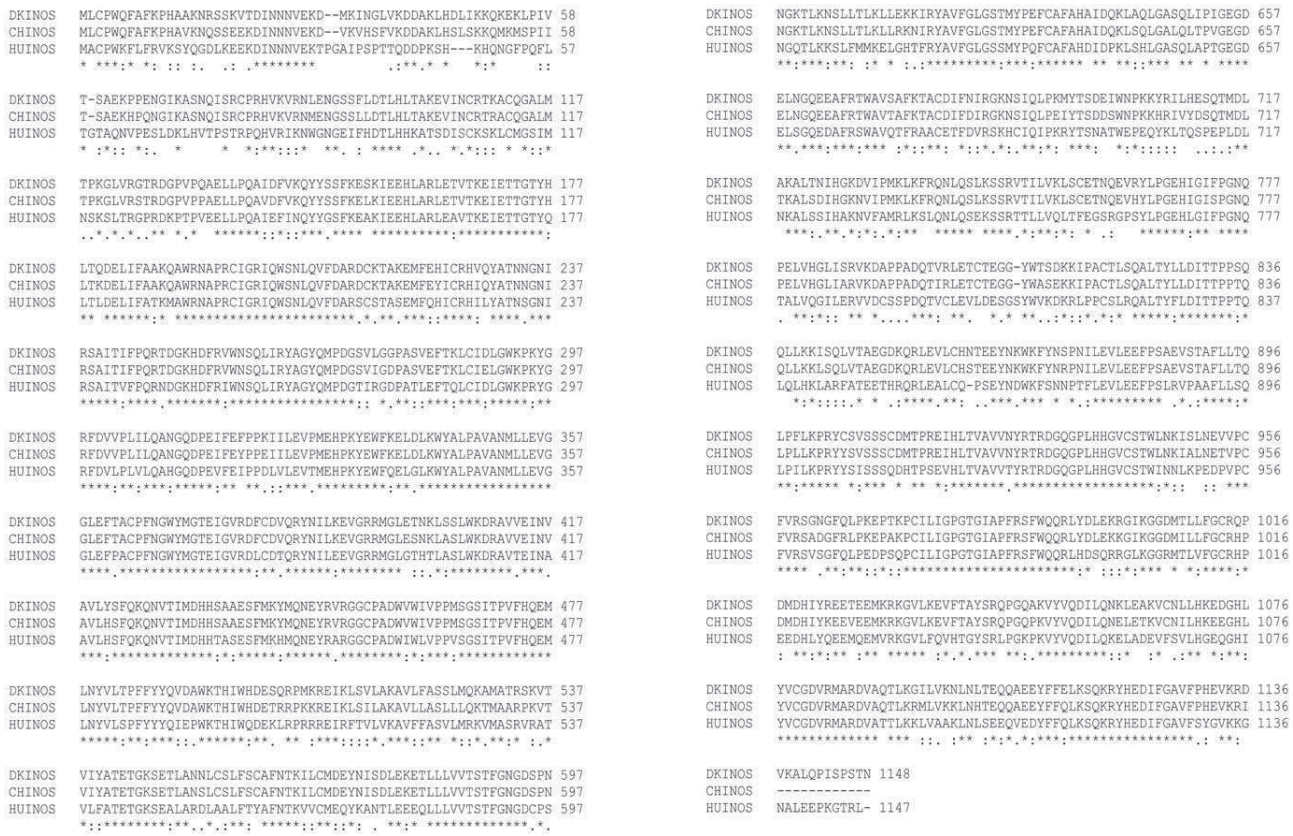

Figure 2. Duck iNOS amino acid sequence alignment. The open reading frame of the duck iNOS (DKINOS) sequence was analyzed through the Clustal W program and the predicted amino acid translation is shown in comparison to that of human (HUINOS) and chicken (CHINOS) iNOS. An asterisk (*) indicates identical amino acid residues while a colon (:) indicates a strongly conserved amino acid substitution and a dot (.) represents a weakly conserved amino acid substitution. Dashed sections (−) represent gaps introduced to optimize the alignment and numbers represent aa number. The nucleotide sequence was subsequently deposited to GeneBank (accession No. FJ966247).
doi:10.1371/journal.pone.0014561.g002

Results

Elevation of NO in the sera of chickens and ducks infected with H5N1 influenza

To establish if NO expression was associated with the immune response following H5N1 influenza virus infection we assayed the concentration of this molecule in the sera of chickens and ducks. Serum was isolated from chickens at the peak of infection, 24 hours post infection (h.p.i,) whilst from ducks serum was isolated after 24 h.p.i, and 72 h.p.i, (with 72 h.p.i) being the relative peak of infection in ducks) and NO levels measured (Fig. 1). H5N1 infected chickens showed a 4-fold increase in NO in sera when compared to uninfected chickens. Ducks initially show no change in NO levels at 24 h.p.i, with H5N1 when compared to uninfected birds. After 72 h.p.i, ducks showed only a 2-fold increase in NO levels compared to uninfected ducks.

Table 2. Similarity of duck iNOS to iNOS in other species.

Organism	GenBank accession No.	No. of aa residues	% Identicle ORF (nucleotides)	% Conserved	Total similarity %
Gallusgallus	NM_204961	1136	91.02	07.21	98.23
Bostaurus	DQ_676956	1156	68.51	22.05	90.57
Mus musculus	NM_010927	1144	65.46	24.73	90.20
Canine	NM_001003186	1154	67.15	22.35	89.51
Rattus novegicus	NM_012611	1147	65.82	23.53	89.36
Homo sapien	NM_000625	1147	65.56	23.71	89.27
Oncorthynchusmykiiss	AJ_300555	1083	59.92	27.88	87.81
Sus suscrofa	NM_001143690	1204	45.34	30.14	75.49

doi:10.1371/journal.pone.0014561.t002

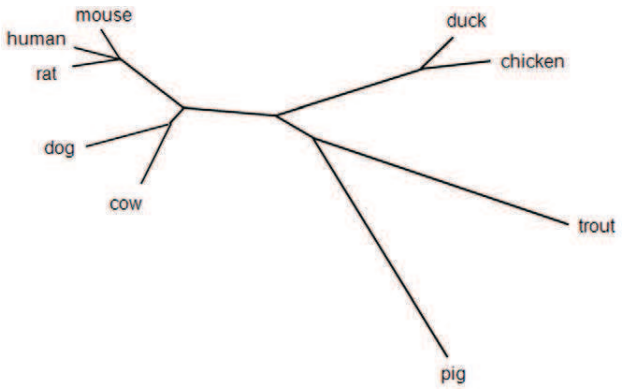

Figure 3. Unrooted phylogenetic tree of the iNOS gene from a range of species. An unrooted tree was constructed based on 1000 bootstrapped amino acid alignments of various iNOS members using the neighbour joining method. GeneBank accession numbers: chicken (*Gallus gallus*) NM_20496, cow (*Bos taurus*) DQ_676956, mouse (*Mus musculus*) NM_010927, dog (*Canine*) NM_001003186, rat (*Rattus novegicus*) NM_012611, human (Homo sapien) NM_000625, trout (*Oncorhynchus mykiss*) AJ_300555, pig (*Sus suscrofa*) NM_001143690.
doi:10.1371/journal.pone.0014561.g003

Molecular cloning and bioinformatic analyses of duck iNOS

As increased levels of NO were observed in the sera of H5N1 infected animals we wanted to further characterise the levels of iNOS, an indicator of NO, in the tissues of infected animals. This is particularly relevant given the different pathogenicity witnessed following H5N1 infection in chickens and ducks. In order to determine the relationship between NO production and iNOS expression, we first had to identify iNOS in the duck. iNOS is produced as part of the oxidative burst in macrophages. Duck splenic leukocytes were therefore stimulated with LPS for 24 hours and PCR amplification with degenerative primers directed at duck iNOS was performed. A partial isoform II NOS (iNOS) cDNA product was obtained which was approximately 500 bp and showed 89% sequence identity with chiNOS cDNA. RLM 3 prime RACE (Invitrogen) was then used to capture the complete iNOS sequence which was deposited in GenBank (accession No. FJ966247). This cloned sequence of 3447 bp encoded a protein of 1148 amino acids with a predicted molecular weight of 130 kDa (Fig. 2). The deduced amino acid sequence of duck iNOS (dkiNOS) was modelled (smart.embl-heidelberg.de) and displayed conserved regions for the binding of iNOS cofactors: heme, calmodulin, FMN, FAD and NADPH. Table 2 shows that dkiNOS has a relatively high amino acid identity to iNOS protein sequences between different species (ClustalW algorithm). The dkiNOS protein was comparable (98%) to the published chiNOS protein [27]. Furthermore, phylogenetic analyses showed dkiNOS clusters near chiNOS, supporting the fact they are similar and may have a conserved function (Fig. 3). With this identification of the duck iNOS gene we then analysed the basal levels of iNOS expression in various tissues and compared these duck levels to the basal iNOS levels observed in chickens. Gene analysis by QRT-PCR showed that iNOS expression (fold increase relative to muscle iNOS levels) in ducks was generally similar to the levels observed in chickens, however, in the lung it appears that the chicken may have higher basal levels of iNOS gene expression (Fig. 4).

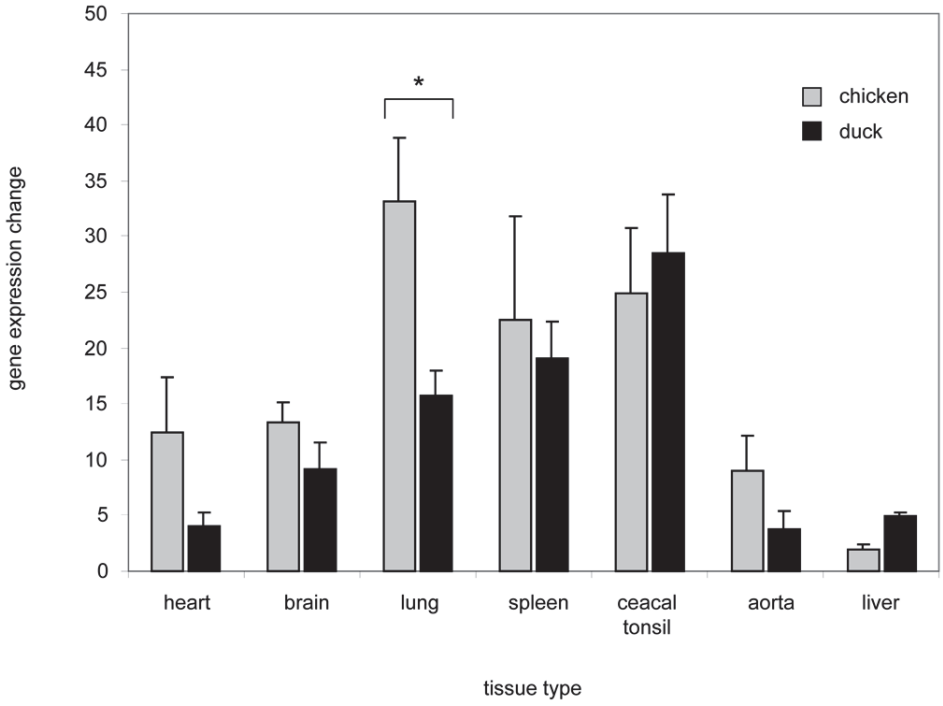

Figure 4. Comparison of basal iNOS levels in chicken and duck tissues measured by QRT-PCR. iNOS mRNA was measured by QRT-PCR in chicken and duck organs. GAPDH was used as a housekeeping gene to standardize results. Expression is shown as the basal fold change increase of iNOS as relative to muscle tissue. Experiments were performed in triplicate with the data representative of 3 independent experiments. An asterisk indicates statistically significant differences of means with $p<0.05$.
doi:10.1371/journal.pone.0014561.g004

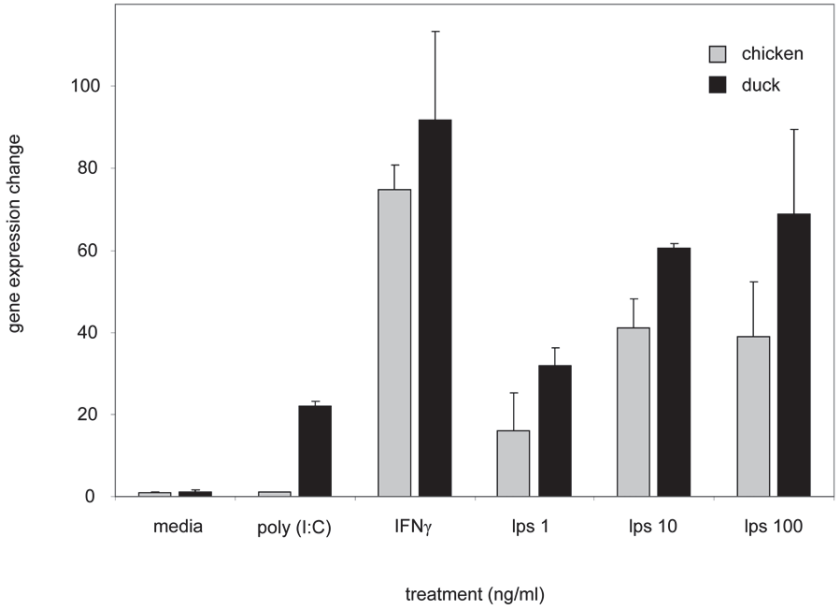

Figure 5. Activated chicken and duck splenocytes measured for iNOS expression levels by QRT-PCR. Chicken and duck splenocytes were cultured for 20 hours with a range of concentrations of LPS (1, 10, and 100 μg/ml) or a single concentration (10 μg/ml) of poly(I:C) or recombinant chicken IFNγ protein. RNA was collected from the cells and quantified iNOS gene levels determined. Obtained iNOS mRNA values were normalized to GAPDH and expression levels are shown as mean fold expression change relative to un-stimulated controls. Experiments were performed in triplicate and data are representative of 3 independent experiments.
doi:10.1371/journal.pone.0014561.g005

iNOS is induced by mitogens in duck and chicken splenic leukocytes

Previous studies have shown that iNOS levels can be elevated following the addition of LPS or IFNγ in cell culture. To assess this newly identified duck iNOS gene, and compare its expression with that of chicken, splenocyte activation cultures were carried out. After 24 h of culture, iNOS levels were elevated in chicken and duck cells stimulated with IFNγ and LPS (increased 80 and 50 fold, respectively). However, the synthetic dsRNA analog poly(I:C) only increased iNOS expression in duck splenic leukocytes compared to chicken (Fig. 5). To test whether increased iNOS expression is associated with increased NO production, splenocytes were cultured with LPS for 48 hours and supernatants were tested using the colorimetric NO assay. At a range of timepoints LPS stimulated NO production in a fashion that correlated with iNOS gene induction (Fig. 6A and B).

Chickens and ducks infected with H5N1 have increased iNOS expression

Since we observed increases in NO in H5N1 infected sera we wanted to assess the levels of iNOS, as a potential indicator of NO, in a range of tissues from infected animals. Therefore, chickens and ducks inoculated with H5N1 influenza (Muscovy duck/Vietnam/453/2006) were sampled and iNOS levels measured at the peak of their relative infection (highest viral titres were at 24 h.p.i, for chickens and at 72 h.p.i, for ducks). The largest change in iNOS expression was observed in the liver of infected chickens (220-fold), with caecal tonsil, spleen and lung tissue also showing increased iNOS mRNA expression (30-fold, 17-fold and 15-fold, respectively). However, the heart and brain showed little significant change in iNOS mRNA expression (Fig. 7). In comparison to chickens, however, infected ducks indicated lower levels of iNOS in all organs except cardiac tissue. Ducks appeared to show an increased iNOS expression in heart tissue (5-fold), with the highest increase in aortic tissue (15-fold). Caecal tonsil tissue also showed some elevation in iNOS expression (5-fold), however, unlike chickens, lung, liver and spleen tissue showed only marginal increases compared to uninfected ducks.

Co-localisation of iNOS and H5N1 influenza virus antigen

As iNOS was upregulated in H5N1 virus infected tissues we assessed the co-localisation of iNOS with regard to virus. Antibody staining for H5N1 antigen was observed in a wide range of chicken organs. In contrast, H5N1 antigen was restricted to fewer sites in infected ducks. In the chicken H5N1 was predominantly found in respiratory and intestinal tissue, spleen, liver as well as heart. However, H5N1 antigen was mainly restricted to the respiratory tract, intestinal tissue and heart of infected ducks (Fig. 8). Antibody staining for iNOS was observed to be present in tissues which were high in H5N1 antigen and was more prevalent in chickens than in ducks (Fig. 9). Uninfected chickens and ducks had little iNOS staining present. During infection with H5N1, chicken lung tissue showed iNOS staining in the respiratory epithelium and the surrounding sub mucosa. In contrast, duck lung tissue showed iNOS staining in the hyaline cartilage, with less staining present in the sub mucosa (Fig. 9A and B). In both chicken and duck caecal tonsil tissue, iNOS was prevalent in the lamina propria and in lymphocyte aggregates (Fig. 9C and D). Nevertheless, tissue from infected chicken liver showed iNOS staining in the lumen of sinusoidal areas, whilst duck liver had little iNOS staining present (Fig. 9E and F). Chicken heart tissue stained positive for iNOS in the myocardium and in endothelial cells surrounding blood vessels. Duck heart tissue had few iNOS positively stained cells present in the myocardium (Fig. 9G and H).

Uninfected tissues stained with either rabbit serum directed against H5N1 influenza virus nucleoprotein or with a rabbit polyclonal antibody directed against iNOS (Abcam ab3523), showed little to no staining in either species. Control antibody staining sections were run following identical protocols but using

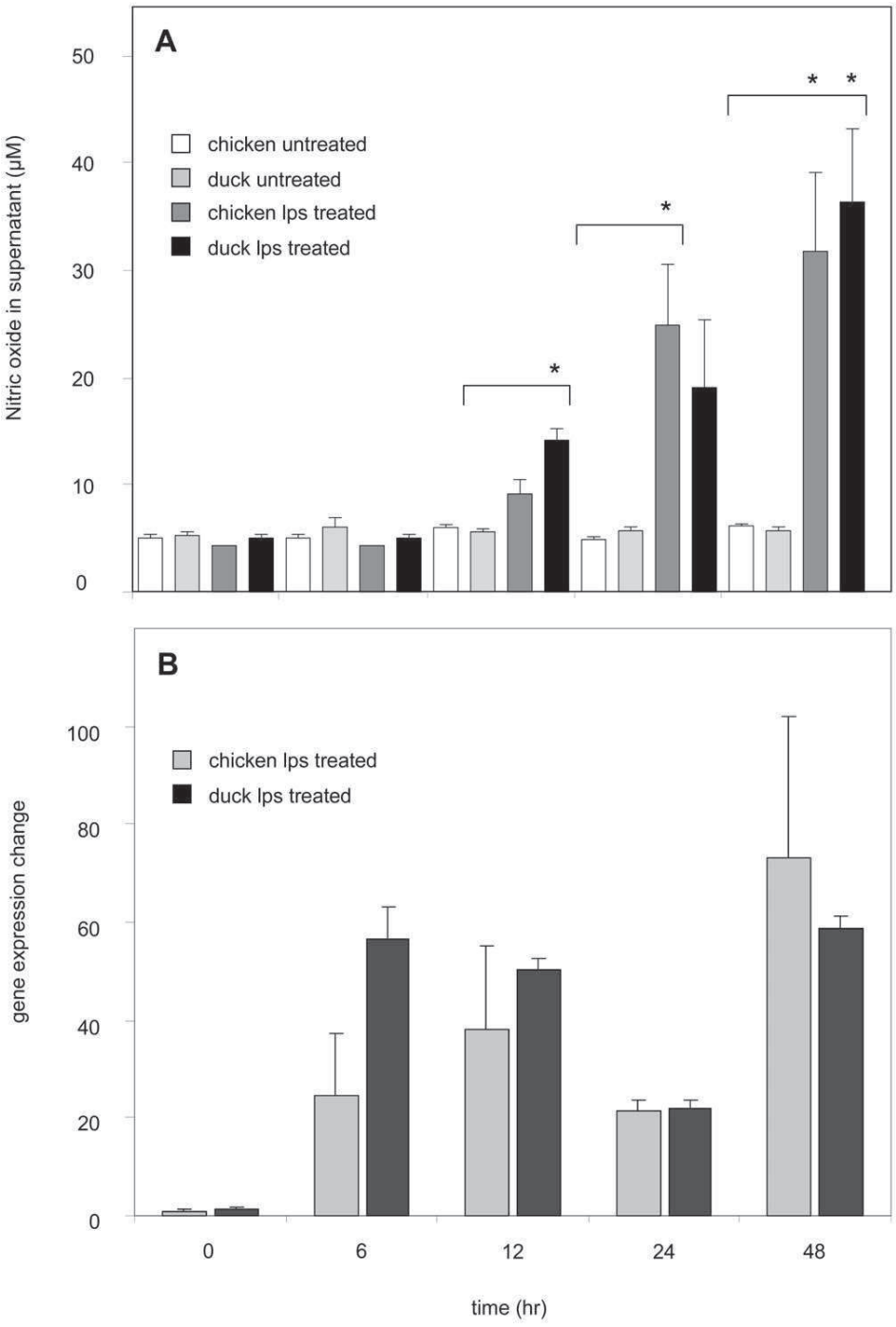

Figure 6. NO and iNOS levels increase in a similar fashion following LPS stimulation of chicken and duck splenocytes. Chicken and duck splenocytes were cultured over a time-course of 6–48 hours with a concentration of LPS (10 μg/mL). (**A**) NO levels, measured by a colorimetric assay, were increased in a similar fashion in chicken and duck cell culture supernatants. (**B**) iNOS gene expression levels, measured by QRT-PCR, were increased in chicken and duck splenocytes. Levels of iNOS gene expression were determined in conjunction with the house keeping gene GAPDH and expression levels are shown as mean fold expression change relative to un-stimulated splenocytes. Experiments were performed in triplicate and data are representative of 3 independent experiments. An asterisk indicates statistically significant differences of means with $p<0.05$.
doi:10.1371/journal.pone.0014561.g006

an irrelevant primary antibody control. To investigate the viral replication of H5N1 virus and confirm H5N1 antigen staining in chicken and duck, tissues were harvested for viral analysis. In chickens H5N1 replicated to high viral titres within 24 h.p.i (between 5–7 $\log_{10}$ $TCID_{50}$) with lung, liver and heart tissue showing the highest viral levels. In contrast, ducks appear to have little or no viral replication 24 h.p.i (data not shown), but rather showed the highest viral replication at 72 h.p.i. Duck heart tissue showed the highest viral replication levels (7 $\log_{10}$ $TCID_{50}$), whilst lung, liver and caecal tonsil had lower viral replication levels.

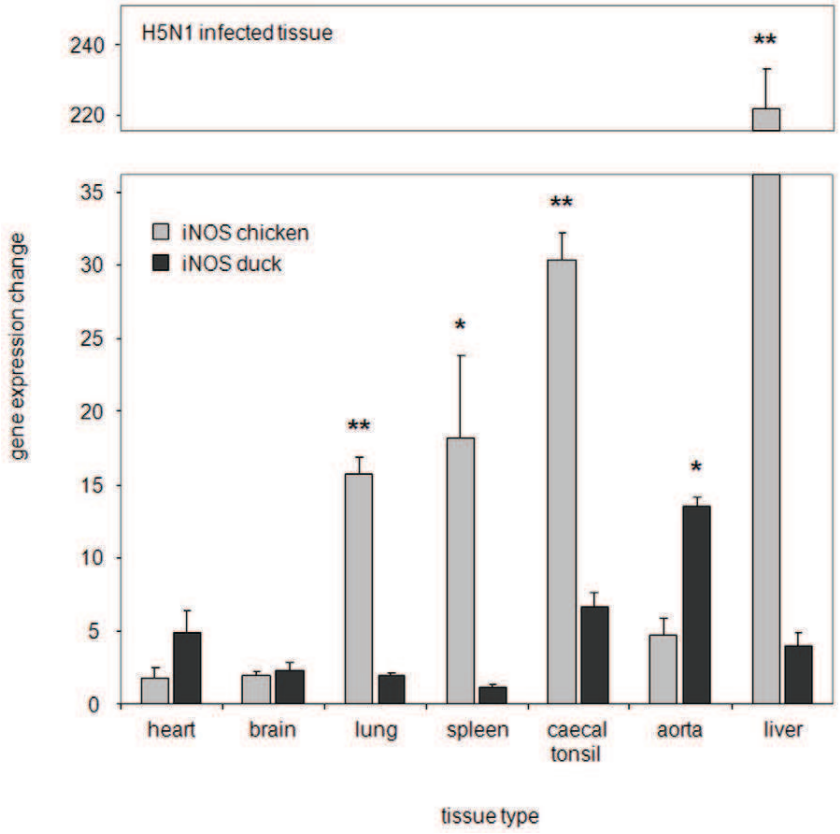

Figure 7. H5N1 influenza infection increases iNOS expression at higher levels in chickens compared to ducks. QRT-PCR was carried out on various tissues from chickens and ducks infected with H5N1 and compared to uninfected controls. Chicken samples were taken at 24 hours post infection, duck samples were taken at 72 hours post infection. Data represents the mean fold expression of either duck or chicken iNOS relative to each uninfected tissue type with GAPDH used as the housekeeping gene. Displayed values are the mean of 2 experiments with 4 birds in each group. Single asterisk indicates statistically significant differences of means with $p<0.05$, double asterisk indicates statistically significant differences of means with $p<0.01$.
doi:10.1371/journal.pone.0014561.g007

Discussion

Continued sporadic outbreaks of H5N1 influenza virus infection highlights the need for alternative strategies to deal with infection. Moreover, without a greater understanding of host-pathogen interactions, the management of H5N1 will continue to be difficult. Nitric oxide is produced primarily as an effector molecule as part of the hosts defence response [33]. However, it has been postulated that the outcomes of this defence response may be either beneficial or detrimental to the host depending on the level of NO produced [12,24]. Therefore, understanding the role that NO and iNOS play during H5N1 infection in chickens and ducks [1,4,34] may provide insights into the underlying mechanisms and differences observed in disease severity. To investigate this we identified the presence of the iNOS gene in ducks and confirmed that dkiNOS acts in a similar fashion to its chicken counterpart [27]. Furthermore, we describe the changes in iNOS levels in H5N1 infected chicken tissues and compared these to iNOS levels in infected duck tissue. Tissue specific increases in iNOS production in the chicken appear to be associated with a higher degree of disease severity in H5N1 infection in chickens when compared to ducks. However, although we have observed this association for the Vt453 H5N1 strain, since there is some degree in pathogenicity between the various isolates of H5N1 virus, there may also be varying degrees of association of iNOS with pathogenicity.

NO is known to be a key molecule in the immune response against viral infection [35,36]. Studies of human and mouse iNOS and the associated NO production, have reported that these molecules are often active in concert with IFNγ, to protect against viral infection [37,38,39], however, their role in avian species is less defined. Moreover, it has been shown that upregulation of IFNγ and the induction of other proinflammatory molecules increase the production of NO [38,40]. The demonstration that dkiNOS expression is increased in a similar fashion to chiNOS, when stimulated by inflammatory mitogens, such as LPS and IFNγ (Fig. 5 and Fig. 6), suggests a comparable role in the innate immune response of avian species. As dkiNOS is structurally similar to other iNOS homologues (Fig. 2) and appears to be associated with NO production, it may be expected that the iNOS response in ducks is similarly conserved. Furthermore, given the growing evidence for the antiviral activity of iNOS in the chicken [41], particularly following influenza infection [34,42], it may be further expected that dkiNOS has the ability to be upregulated during the course of H5N1 influenza virus infection.

It is still unclear what effect iNOS and other proinflammatory molecules have during influenza infection. IFNγ has been shown to upregulate iNOS expression [40] and likewise we observed that the addition of IFNγ to duck cells increased both iNOS mRNA expression and NO production. Correspondingly, H5N1 influenza studies in mammals have shown that IFNγ levels are increased and similarly H5N1 infected chickens have both elevated levels of IFNγ

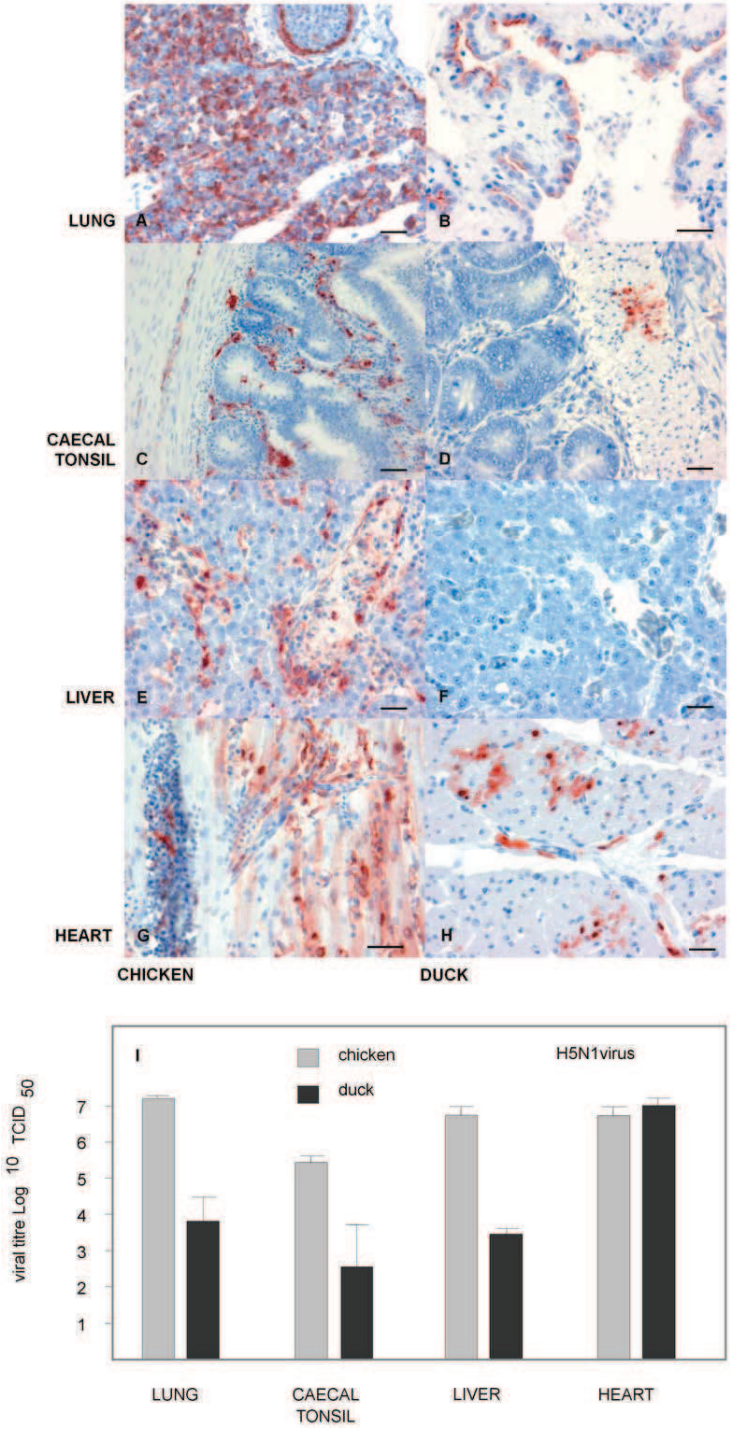

Figure 8. H5N1 infection appears more widespread in chicken tissues in comparison to duck tissues. IHC for H5N1 antigen, left-hand panel shows staining in chicken tissues, right-hand panel shows staining in duck tissues (**A**) Chicken lung 24 hours post infection (h.p.i), with IHC stain showing H5N1 viral antigen as red/brown colour. (**B**) Duck lung 72 h.p.i., H5N1 antigen was less prevalent in duck lung tissue than in chicken. H5N1 antigen was detected in single cells scattered within the lung parenchyma and in the hyaline cartilage. (**C**) Chicken caecal tonsil 24 h.p.i., with H5N1 in caecal lymphoid follicles and submucosa. (**D**) Duck caecal tonsil 72 h.p.i., with similar H5N1 antigen staining. (**E**) Chicken liver tissue 24 h.p.i., with severe H5N1 antigen staining. (**F**) Duck liver 72 h.p.i., showed no signs of viral antigen. (**G**) Chicken heart tissue 24 h.p.i. showed H5N1 staining in the myocardium and typically near blood vessels. (**H**) Duck heart tissue 72 h.p.i., with IHC H5N1 antigen staining the myocardium. All scale bars = 50 μm. (**I**) The graph shows viral replication efficiency between chickens and ducks across a range of organs following H5N1 Vt453 infection. In chickens 24 h.p.i, lung, caecal tonsil, liver and heart tissue showed between 5.5 and 7 $\log_{10}$ $TCID_{50}$. Similarly, ducks had 7 $\log_{10}$ $TCID_{50}$ of virus in heart tissue, but moderately less virus in other tissues, between 2.5 and 4 $\log_{10}$ $TCID_{50}$.
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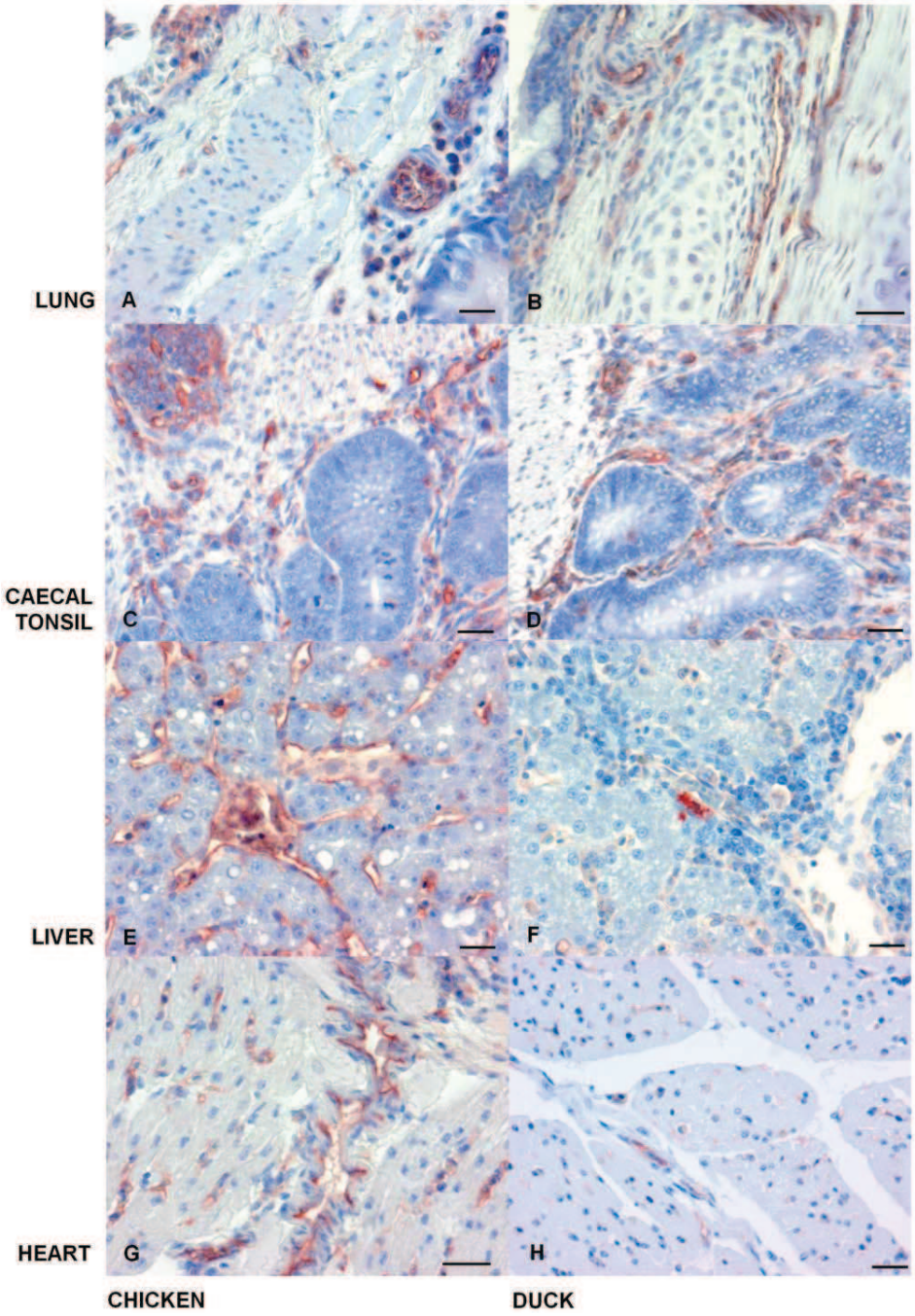

Figure 9. During H5N1 Influenza infection iNOS appears more highly expressed in chicken tissues when compared to duck tissues. IHC for iNOS antigen staining, with left-hand panel showing chicken tissues and right-hand panels showing duck tissues. (**A**) Chicken lung 24 h.p.i., IHC stain (red/brown) for iNOS mainly in the submucosa. (**B**) Duck lung 72 h.p.i., IHC iNOS staining in the submucosa. (**C**) Chicken caecal tonsil 24 h.p.i., with iNOS in lymphoid follicles, the submucosa and lymphoid aggregates. (**D**) Duck caecal tonsil 72 h.p.i., IHC staining of iNOS mainly in the submucosa. (**E**) Chicken liver tissue 24 h.p.i., iNOS present in the sinusoidal endothelium. (**F**) Duck liver 72 h.p.i., little or no iNOS detected. (**G**) Chicken heart 24 h.p.i., iNOS in the myocardium and proximity to blood vessels (presumably endothelial cells). (**H**) Duck heart 72 h.p.i., with low levels of IHC staining for iNOS. All scale bars = 50 μm.
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and other cytokines [11,37,43]. H5N1 viruses are often associated with an increase in inflammatory molecules [44], supporting a possible role for iNOS [34]. With this in mind, we examined NO and iNOS expression during H5N1 infection in chickens and ducks. In H5N1 infected chickens we observed that iNOS expression was increased in the lung, spleen and caecal tonsil by as much as 30-fold. This widespread increase in iNOS may be due to the fact that in chickens H5N1 strains tend to have a broad tissue tropism [8,44,45]. Viral analysis showed high viral titres in chicken lung, caecal tonsil, liver and heart tissue. In chickens, the respiratory

system appears to be the predominant site of infection, however, virus quickly spreads to other organs, such as the heart and liver, with the infection becoming systemic [46]. This may be crucial in the manifestation of excessive levels of NO during infection.

Intriguingly, the levels of iNOS expression were increased over 200-fold in the liver. Basal levels of iNOS gene expression are relatively low in chicken liver tissue compared to chicken lung, spleen and caecal tonsil (Fig. 4) and this may partly account for its high fold increase in this organ. Nevertheless, the high levels of iNOS observed in chicken liver tissue may also be associated with the observed production of acute phase proteins [25]. An acute phase response is involved in many inflammatory infections [13,44,47] whereby proteins, such as serum amyloid A (SAA), have been associated with NO production from macrophages [25]. Furthermore, during studies investigating the acute phase response following H5N1 influenza, we have observed increased levels of SAA in chickens, whilst the levels of SAA in H5N1 infected ducks was comparably low (Burggraaf *et al.*, manuscript in preparation). This higher level of iNOS production in tissues infiltrated with virus raises the question of the role iNOS regulation has in the outcome of viral infections. In chickens, high iNOS production may have an impact on the severity of the pathology witnessed during H5N1 infection.

In contrast to the chicken, H5N1 infection in ducks is characterised by a predilection for viral replication in muscle and lung tissue [29]. We observed the highest viral levels in the heart with less viral replication occurring in the lung, liver and caecal tonsil tissue. We identified that heart and aorta tissue in ducks had the greatest increases in iNOS expression, although IHC for iNOS in duck heart tissue was less prevalent than in infected chickens. Levels of iNOS expression were also increased in the caecal tonsil of ducks and IHC for iNOS expression showed its localisation in the proximity of H5N1 antigen in the lamina propria. This increase in iNOS could be associated with an upregulated inflammatory response during infection. It has been reported that H5N1 infection of duck heart muscle may cause myocarditis and similarly infection of the brain may cause encephalitis [29]. The resultant inflammatory responses in these organs may contribute to some of the observed morbidity in infected ducks. Levels of iNOS may potentially become increased at sites of inflammation, suggesting that in a similar fashion to the chicken, increases in duck iNOS may be associated influenza infection. This may be due to either the action of genes that iNOS stimulates, such as acute phase proteins and cytokines or the actual production of NO and its potential to contribute to apoptosis [24,48]. Nevertheless, NO has the potential to generate free radicals which may damage cells [12] and therefore it is important that NO and iNOS be tightly regulated [49]. Kacergius *et al.*, (2006) indicated that during influenza infection, increases in NO synthesis through iNOS gene induction, contributed to lung damage. For these reasons, iNOS has been implicated in the pathology associated with some viral models [34,35,50]. The question still remains as to the relevance of the association of iNOS and NO in the observed pathology of H5N1 influenza infection. At present there is a clear association, however, the role iNOS plays in viral pathogenicity for each of the species is yet to be determined. Future work will be directed at determining whether the pathological outcomes are a result of iNOS expression and are the cause of disease severity.

Although both H5N1 infected chickens and ducks have increased circulating levels of NO in comparison to uninfected animals, the levels of iNOS gene expression and tissue expression observed by QRT-PCR and IHC are markedly different between the two species. IHC staining for iNOS in chicken H5N1 infected liver tissue showed its presence predominantly in the sinusoidal areas (presumably endothelial cells), whilst in caecal tonsil and lung tissue iNOS was also observed in lymphoid aggregates and in endothelial cells around blood vessels. In comparison to infected chickens, IHC for duck iNOS appears restricted to the caecal toncil, with infrequent staining in upper respiratory and heart tissue. Furthermore, no iNOS staining was found in infected duck liver tissue. This suggests that iNOS may contribute to higher NO production within H5N1 infected chicken organs than in infected duck organs. However, comparably higher NO levels in the chicken may be only a part of the problem for the associated increased severity of H5N1 infection. It is likely other immune genes such, as viral recognition receptors and inflammatory cytokines have a role in the varied pathogenicity following H5N1 influenza infection. For example, Barber *et al.*, (2010), demonstrated that chickens lack the viral sensing receptor RIG-I which when expressed in chicken cells improves the response to H5N1 influenza infection [51]. Furthermore, studies by Okada *et al.*, (2009) suggested that the response to H5N1 influenza in chickens could be improved following reduction of the inflammatory immune cascade [52]. Therefore, it would appear that further research may be required to fully elucidate the mechanisms behind the varied immune response between chickens and ducks.

In conclusion, it may be postulated that since H5N1 viruses are often associated with an increased pathogenicity in chickens, it may be that iNOS expression in chickens becomes dysregulated and this may impact on disease severity through the overproduction of free radicals and the resultant damage to cellular function. With this in mind, further investigation into the inflammatory response during H5N1 viral infection is needed. A better understanding of the inflammatory response following H5N1 influenza infection may help in developing new strategies and approaches for modulating the outcomes of these infections in chickens and ducks.

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Author Contributions

Conceived and designed the experiments: SB JB AGDB. Performed the experiments: SB JB JP. Analyzed the data: SB AGDB. Contributed reagents/materials/analysis tools: SB JB JP AGDB. Wrote the paper: SB WGK JL AGDB.

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Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (*Anas platyrhynchos*), with particular reference to clinical disease, tissue tropism and viral shedding

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Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (*Anas platyrhynchos*), with particular reference to clinical disease, tissue tropism and viral shedding

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Pekin ducks were infected by the mucosal route (oral, nasal, ocular) with one of two strains of Eurasian lineage H5N1 highly pathogenic avian influenza virus: A/Muscovy duck/Vietnam/453/2004 and A/duck/Indramayu/BBVW/109/2006 (from Indonesia). Ducks were killed humanely on days 1, 2, 3, 5 and 7 after challenge, or whenever morbidity was severe enough to justify euthanasia. Morbidity was recorded by observation of clinical signs and cloacal temperatures; the disease was characterized by histopathology; tissue tropism was studied by immunohistochemistry and virus titration on tissue samples; and viral shedding patterns were determined by virus isolation and titration of oral and cloacal swabs. The Vietnamese strain caused severe morbidity with fever and depression; the Indonesian strain caused only transient fever. Both viruses had a predilection for a similar range of tissue types, but the quantity of tissue antigen and tissue virus titres were considerably higher with the Vietnamese strain. The Vietnamese strain caused severe myocarditis and skeletal myositis; both strains caused non-suppurative encephalitis and a range of other inflammatory reactions of varying severity. The principal epithelial tissue infected was that of the air sacs, but antigen was not abundant. Epithelium of the turbinates, trachea and bronchi had only rare infection with virus. Virus was shed from both the oral and cloacal routes; it was first detected 24 h after challenge and persisted until day 5 after challenge. The higher prevalence of virus from swabs from ducks infected with the Vietnamese strain indicates that this strain may be more adapted to ducks than the Indonesia strain.

Introduction

Highly pathogenic avian influenza (HPAI) viruses of the Eurasian lineage H5N1 first appeared in 1996 in mainland China, followed by cases in Hong Kong the following year. After some years of absence, similar viruses reappeared in late 2003 and caused epidemics in south-east Asia, which spread further around Asia, and then to Europe and Africa (Sims *et al*., 2005; Alexander, 2007). H5N1 HPAI viruses have infected a range of bird species, mainly wild waterfowl, domestic ducks and gallinaceous birds (K.S. Li *et al*., 2004; Olsen *et al*., 2006; Webster *et al*., 2007), but have also caused lethal infections of mammals, including humans (Chen *et al*., 2004; Maines *et al*., 2008; World Health Organization, 2008). Viruses of this H5N1 lineage have caused particular concern as, through recombination and mutation, they may contribute to the evolution of the next human influenza pandemic.

H5N1 virus strains are highly pathogenic in gallinaceous birds, causing a severe peracute systemic disease (Perkins & Swayne, 2001; Mase *et al*., 2005b; Zhou *et al*., 2006; Nakamura *et al*., 2008). Infected ducks suffer a more variable disease; with some H5N1 HPAI viruses they may suffer only a mild disease, but continue to shed virus, and it is for this reason that they are thought to be the reservoirs of H5N1 HPAI strains (Hulse-Post *et al*., 2005; Sturm-Ramirez *et al*., 2005; Keawcharoen *et al*., 2008).

The aim of this study was to characterize the pathogenesis of two H5N1 HPAI strains, one from Vietnam (A/Muscovy duck/Vietnam/453/2004) and the second from Indonesia (A/Duck/Indramayu/BBVW/109/2006), in Pekin ducks (*Anas platyrhynchos*). Histopathology, immunohistochemistry and virology on tissues and swabs were conducted to investigate the basis for morbidity and viral shedding.

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Materials and Methods

Animals and husbandry. All procedures conducted on animals in this study were approved by the AAHL Animal Ethics Committee. Five-week-old Pekin ducks (Luv-a-Duck, Victoria, Australia) were identified individually with leg bands and assigned randomly to treatment groups. The ducks were fed with commercial grower chicken pellets, *ad libitum*, and lettuce once a day. Water troughs that were deep enough for the ducks to float and splash in were placed in each room. Each room also had a partially enclosed dry retreat with wood shavings for the ducks to sit on.

The cloacal temperatures of the ducks were measured using digital thermometers (Omron, J.A. Davey Pty Ltd, Victoria, Australia), with a maximum readout of 43.9°C. During infection, the temperature of some ducks exceeded the maximum readout.

Accommodation and biocontainment. The three groups of ducks were housed in separate rooms at microbiological physical containment level 3. The room temperature was held at 22°C and the airflow was set at approximately 15 air changes per hour. At all times after viral challenge, the staff wore disposable overalls, gloves, waterproof boots and breathing air protection. The latter consisted of full-head hoods ventilated with battery-driven filtered air.

Virus strains. Ducks were challenged with either of two strains of H5N1 avian influenza virus: a Vietnamese strain, A/Muscovy duck/Vietnam/453/2004; and an Indonesian strain, A/Duck/Indramayu/BBVW/109/2006 (GenBank accession number EU124084). These viruses belonged to clades 1 and 2.1.3, respectively, as defined by the WHO/OIE/FAO H5N1 Evolution Working Group (Anon., 2008). They were both received directly from the country of origin, the former as a Madin-Darby canine kidney (MDCK) cell culture supernatant and the latter as infective allantoic fluid. Both were passaged twice in embryonating chicken eggs to obtain the working stock virus. The working inoculum consisted of a 1:100 dilution of infective allantoic fluid. A total volume of 0.5 ml was inoculated per duck: each duck dose contained approximately $10^{7.2}$ 50% egg infectious doses (EID_{50}) for the Vietnamese strain and $10^{6.0}$ EID_{50} for the Indonesian strain.

Study design. The ducks were placed into three separate groups. The first group ($n=15$) was challenged with the Vietnamese strain; the second group ($n=15$) was challenged with the Indonesian strain; and the third group ($n=6$) was uninfected controls, used to determine background histopathological lesions and non-specific immunohistochemical staining in the duck tissues. Challenge was done by placing two drops of inoculum into each eye and nostril, and instilling the remainder into the mouth. Oral (deep pharyngeal) and cloacal swabs were taken from each challenged duck before and every day after challenge, placed into 2 ml transport media (phosphate-buffered saline containing penicillin, streptomycin and gentamycin) and stored at −80°C. Cloacal temperatures were taken from each of the challenged ducks, starting before challenge and every day afterwards. Prior to challenge, each duck was bled for serum haemagglutination inhibition antibody titres to confirm lack of prior exposure to antigenically similar viruses. Three ducks from each infected group were killed humanely, according to a pre-designated schedule, on each of 1, 2, 3, 5 and 7 days post infection (d.p.i.) and tissue samples were taken. Ducks displaying moderate to severe disease were killed humanely prior to their designated time points, in accordance with the animal ethics protocol. Uninfected controls were killed humanely coincident with 0 and 7 d.p.i. All euthanasia was carried out by pentobarbital sodium (Lethabarb; Virbac Animal Health, Australia) administration into the wing vein.

Virology. Swabs were thawed from −80°C storage, and 0.1 ml undiluted swab medium was inoculated into the allantoic cavity of three 9-day-old to 12-day-old embryonated fowls' eggs. The embryos were observed for death for 3 days; allantoic fluids from all dead eggs were tested for influenza virus by haemagglutination test using chicken red blood cells (OIE, 2008). The swabs were placed back into −80°C storage for later titration of positive swabs.

Fresh tissues were ground by mortar and pestle and 10% w/v homogenates in phosphate-buffered saline were prepared. These and virus-positive swab media were titrated in Vero cells. Flat-bottomed 96-well micro-titre 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, in 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 carbon dioxide incubator and examined for the presence of cytopathic effect after 5 days. In a previous study (S. Lowther, unpublished data) the sensitivity of Vero cells for influenza H5N1 (A/Vietnam/1203/2004) was examined and results were compared with both MDCK cells and eggs, which are the commonly used substrates for the culture of influenza viruses. Vero cells were found to be slightly less sensitive than eggs, but more sensitive than MDCK cells for this H5N1 strain. The starting dilutions were undiluted for swab media and 1:10 for tissue homogenates. Thus, the lowest possible limit of viral detection, equivalent to a single infected well with optimal cell growth in all wells, was $10^{-0.25}$ and $10^{0.75}$ 50% tissue culture infectious doses per 0.1 ml ($TCID_{50}$/0.1 ml) for swab media and tissue homogenates, respectively. Most tissue samples and cloacal swabs caused non-specific lethal effects to cell cultures at low dilutions; because of this, low titre readings could not be ascertained for many of these samples. In these cases, 1:10 dilutions of the tissue homogenates were inoculated into eggs as described above.

Histopathology and immunohistochemistry. After euthanasia, pieces of tissue from each duck were placed into 4% formaldehyde in neutral-buffered saline. After no more than 3 days of formalin fixation for soft tissues and 21 days for bony tissues, tissues were trimmed and processed into paraffin wax by routine histological methods. Bony tissues were decalcified prior to processing by immersion into a solution of ethylenediamine tetraacetic acid in neutral-buffered formalin for 7 to 14 days. Sections of tissues were cut onto slides and stained using an immunoperoxidase test, as follows. Sections were quenched with 10% hydrogen peroxide for 10 min and digested with 5 to 7 μg/ml proteinase K for 5 min; they were incubated with a rabbit serum directed against a recombinant-expressed purified influenza virus nucleoprotein (produced by the Australian Animal Health Laboratory) for 1 h, followed by horseradish peroxidase-conjugated secondary antibody (DAKO Envision, California, USA) for 45 min. Sections were stained with aminoethylcarbazol substrate chromogen (DAKO Envision) for 5 to 6 min, and counterstained with Mayer's haematoxylin. Duplicate sections were stained with haematoxylin and eosin for visualization of tissue morphology.

Results

Clinical signs. In the present study the clinical signs and viral shedding in challenged ducks were measured, and these parameters were correlated with virus distribution in tissue. Cloacal temperatures were used to assess infection status. Before infection, body temperatures of the ducks averaged 41.6°C (range 40.9 to 42.3°C, $n=23$ ducks) 3 days before challenge and averaged 42.0°C (range 41.4 to 42.6°C, $n=30$ ducks) at the time of challenge. In this study any body temperature of 42.7°C or greater was considered abnormal and taken to indicate pyrexia caused by infection.

With the Vietnamese strain, raised cloacal temperature appeared to be a reliable indicator of infection. At 1 d.p.i. some ducks had temperatures over 43.0°C, indicating that infection caused disease soon after exposure. In most ducks the highest temperature was recorded at 2 d.p.i., but high temperatures persisted until each duck was killed humanely (Table 1). Birds infected with the Vietnamese strain were unusually quiet when handled, but continued to eat, play in water and preen. One duck was found dead 4 d.p.i., after having shown no signs of particular severity during the previous day. Three ducks were killed humanely for welfare reasons 2 to 3 days prior to their designated euthanasia date.

Table 1. *Clinical and pathological findings for the ducks challenged with highly pathogenic avian influenza viruses*

Duck identity	Day of euthanasia	Maximum temperature (day post inoculation)	Temperature at euthanasia (°C)	Summary of clinical signs and gross pathological lesions
A/Muscovy duck/Vietnam/453/2004				
1	1	42.9 (1)	42.9	Normal
2	1	42.6 (1)	42.6	Normal
5	1	42.7 (1)	42.7	Normal
6	2	43.3 (2)	43.3	Normal
9	2	43.7 (2)	43.7	Normal
10	2	43.3 (2)	43.3	Normal
42	3	43.7 (2)	43.0	Depressed
44	3	43.4 (2)	43.0	Depressed
80	3	43.1 (2)	42.7	Depressed, weeping eyes
81	5	>43.9 (2)	42.5	Depressed. Pale spots on pancreas
84	5	43.0 (3)	41.9	Depressed
85	4	43.7 (2)	NA	Found dead 4 days after challenge. Pallor of heart
86	4	43.5 (2)	43.2	Depressed, euthanazed 3 days before designated time
93	5	>43.9 (4, 5)	>43.9	Depressed, euthanazed 2 days before designated time. Pallor of heart
94	5	>43.9 (3)	43.0	Depressed, euthanazed 2 days before designated time
A/Duck/Indramayu/BBVW/109/2006 (Indonesian strain)				
3	1	42.8 (1)	42.8	Normal
4	1	42.1 (1)	42.1	Normal
8	1	42.7 (1)	42.7	Normal
41	2	42.6 (2)	42.6	Normal
45	2	42.9 (1)	42.3	Normal
76	2	42.4 (1)	42.3	Normal
79	3	43.6 (3)	43.6	Normal
83	3	42.4 (1)	42.0	Normal
89	3	42.4 (1, 3)	42.4	Normal
90	5	42.9 (1)	42.4	Normal
91	5	42.4 (5)	42.4	Normal
96	5	43.2 (3)	42.9	Normal
97	7	42.6 (1)	41.4	Normal
98	7	>43.9 (4)	41.4	Normal
99	7	42.2 (3)	41.7	Normal

The ducks were considered pyrexic when the cloacal temperature was above 42.6°C. NA: not applicable.

Cloacal temperature was a less reliable indicator of infection with the Indonesian strain: not all infected ducks, as determined by viral shedding, developed pyrexia—in those that did, it was usually transient. No other abnormal signs were recorded for the ducks infected with this strain. This may be partially due to euthanasia before the expression of clinical signs. In previous trials involving the Indonesian strain, mild neurological signs in low numbers of 5-week-old ducks were recorded (unpublished data).

Viral shedding. Each duck was swabbed every day from the oral and cloacal routes. Virus was detected by inoculation of swab media into eggs (Table 2) and positive swabs were titrated in Vero cells. Viral shedding was detected in all but two (13/15) birds challenged with the Vietnamese strain and in 10 of the 15 ducks challenged with the Indonesian strain. These numbers may have been higher if the birds had lived longer.

In both groups, virus was detected from both the oral and cloacal routes at 1 d.p.i. A high proportion of positive swabs was attained at 2 to 3 d.p.i. for both strains from both routes. Virus in swabs was detected until 5 d.p.i. for both strains. In previous trials, shedding was detected through to day 7 with both strains (unpublished data).

Positive swab fluid was titrated in Vero cells. Virus was isolated in only five oral swabs: these were from both groups on 1, 3 and 5 d.p.i. In these swabs only small quantities of virus was found, between $10^{-0.25}$ to $10^{1.75}$ $TCID_{50}$/0.1 ml. Nearly all cloacal swabs induced non-specific lethal effects on the cells in concentrations around 1:10, and low levels of virus, if present, would have been masked by these effects.

Histopathology and immunohistochemistry. Tissues were taken from each duck; they were examined histologically and were tested for antigen by immunohistochemistry. Six uninfected ducks from the same group were examined for determination of background histopathological lesions and evaluation of non-specific immunohistological staining. No antigen staining was present in any of the six uninfected ducks. Where present, the antigen was quantified on a score of 1 (sparse), 2 (common) or 3 (abundant).

Vietnamese strain (A/Muscovy duck/Vietnam/453/2004). Lesions and antigen were detected in a wide range of cell types from all but one (Duck 2) of the ducks infected

Table 2. *Results of influenza virus detection in oral and cloacal swabs, as determined by egg inoculation and haemagglutination assay*

	Day 1		Day 2		Day 3		Day 5		Day 7	
Duck identity	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
A/Muscovy duck/Vietnam/453/2004										
1	+	−								
2	−	−								
5	−	−								
6	−	−	+	+						
9	−	−	+	−						
10	−	−	+	+						
42	−	−	+	+	+	−				
44	−	−	+	+	+	−				
80	+	−	+	+	+	−				
81	+	−	−	+	+	+	+	+		
84	−	−	+	−	+	−	−	−		
85	−	−	+	+	+	+				
86	+	−	−	+	−	+				
93	−	+	+	+	+	+	+	+		
94	−	−	−	+	+	−	+	−		
A/Duck/Indramayu/BBVW/109/2006 (Indonesian strain)										
3	−	−								
4	+	−								
8	−	−								
41	−	−	−	−						
45	−	−	+	+						
76	−	−	+	−						
79	−	−	−	+	−	+				
83	−	−	−	−	−	−				
89	−	−	+	−	−	−				
90	−	−	−	−	−	−	−	−		
91	−	−	−	−	−	−	+	−		
96	−	+	+	−	+	−	−	−		
97	−	−	+	−	+	−	+	−	−	−
98	+	−	−	+	+	−	−	−	−	−
99	−	−	−	+	+	−	−	+	−	−

+, positive; −, virus not detected.

Table 3. *Influenza virus antigen quantity scores for selected tissue types from infected ducks*

Duck identity	Day of euthanasia	Brain (neurons and neuropil)	Myocardium	Pancreatic acinar cells	Lung	Spleen	Pectoral muscle	Feather follicles	Skull	Bone/cartilage of trachea	Respiratory epithelium[a]
A/Muscovy duck/Vietnam/453/2004											
1	1	−	−	−	++	++	nd	−	−	−	−
2	1	−	−	−	−	−	nd	−	−	−	−
5	1	−	−	−	−	+	nd	−	−	−	−
6	2	+	+	−	+	+	nd	+	++	++	−
9	2	+	+	−	+	+	nd	+	++	++	−
10	2	++	+++	−	++	++	nd	++	++	++	−
42	3	+	++	+	+	−	++	++	++	++	−
44	3	++	+++	+	++	−	+++	++	+	+++	−
80	3	+	+++	++	+	++	++	+	++	+	Air sac, bronchus
81	5	++	+++	+	−	−	++	+	+	+	−
84	5	−	++	+	−	−	−	−	+	−	Air sac
85	4 (d)	++	+++	−	+	−	+++	+++	+++	++	−
86	4	++	+++	+++	++	−	++	+	+	+	Paranasal sinus, airsac, trachea
93	5	++	+++	++	−	−	+	+	+	−	Air sac
94	5	+	++	−	−	−	+	−	+	−	−
A/Duck/Indramayu/BBVW/109/2006											
3	1	−	−	−	−	−	nd	−	−	−	−
4	1	−	−	−	−	−	nd	−	−	−	−
8	1	−	−	−	−	+	nd	−	−	−	−
41	2	−	−	−	−	−	nd	−	−	−	−
45	2	−	−	−	−	−	nd	−	−	−	Air sac
76	2	−	−	−	−	−	nd	−	−	−	Air sac
79	3	+	+	+	−	−	+	−	−	−	Paranasal sinus
83	3	−	−	−	−	−	−	−	−	+	−
89	3	+	+	−	−	−	+	−	+	−	Air sac
90	5	−	−	−	−	−	−	−	−	−	−
91	5	−	+	−	−	−	−	−	+	+	−
96	5	−	−	+	−	−	−	−	+	−	−
97	7	−	−	−	−	−	−	−	−	−	−
98	7	−	−	−	−	−	−	−	−	−	−
99	7	−	−	−	−	−	−	−	−	−	−

−, negative; +, sparse; ++, common; +++, abundant antigen. nd, not done; (d), found dead. [a]Antigen score was+for all cases where antigen was seen in respiratory epithelium.

with the Vietnamese strain (Table 3). The major tissue types affected were heart, skeletal muscle, smooth muscle, brain, pancreas and connective tissue. Virus was detected in early infection (1 d.p.i.) in lymphoid tissues (spleen, thymus and bursa), the liver, the lung, the connective tissue of the trachea and smooth muscle of the proventriculus. By 2 d.p.i., the viral antigen was distributed widely. In most infected tissues, abundant viral antigen persisted until day 5 when the last ducks were killed humanely. In the lung and spleen, viral antigen appeared on 1 d.p.i. but was, in most cases, absent after 4 d.p.i.

The Vietnamese strain used in this study appeared to have a particular predilection for muscle cells of all types. Myocardium was one of the most consistently infected, causing severe, acute, diffuse myocarditis, which was associated with heavy antigen staining (Figure 1a,b). It also had a significant predilection for skeletal muscle including the major muscle masses and muscles in the trachea and oesophagus. Where viral antigen was present in large amounts, as in the pectoral muscles (Figure 1c), it caused a severe, acute, necrotizing myositis (Figure 1d). The strain was also found in single fibres or clusters of smooth muscles of the walls of the gastrointestinal tract and blood vessels (Figure 1e).

The Vietnamese strain caused random foci of antigen staining in neurons and the neuropil (Figure 1f). Often these foci were not associated with lesions. However, when lesions were present they consisted of mild, non-suppurative encephalitis, with mononuclear cell perivascular cuffs, gliotic nodules, oedema of the neuropil and neuronal degeneration.

The bone, cartilage and associated connective tissues contained viral antigen, particularly those around the head and trachea. Antigen was evident in fibrocytes including the perichondrium and periosteum, in chondrocytes of the tracheal rings (Figure 1g) and in internal osteoblasts and the associated marrow of the skull (Figure 1h). Although viral antigen was present in the marrow spaces within the skull, it was not detected in the marrow of the proximal femur. Acute localized necrosis was present in fibrous tissue, and this was associated with heavy antigen staining (Figure 1h). Viral antigen in the tracheal rings was associated with focal chondral necrosis (Figure 1g).

There was a higher prevalence of virus in the oral swabs than in the cloacal swabs. Virus from the oral cavity may have originated from epithelium of either the respiratory tract or the oral cavity. In respiratory tract epithelium, antigen was generally sparse or undetected. When present, it was most prevalent in the epithelium of the air sacs (Figure 2b) and paranasal sinuses. Generally, antigen was not present in the epithelium of the nasal turbinates, trachea, bronchi or parabronchi, nor in the proximal gastrointestinal tract or the surface squamous epithelium of the oral cavity. However, there were exceptions. One small focus of antigen was detected in each of the bronchial epithelium of one bird (Duck 80; Figure 2a) and the tracheal epithelium of another (Duck 86). Occasional small foci of antigen were present in the middle and deep layers of the stratified squamous epithelium of the oral cavity. As these were uncommon and generally did not extend to the superficial layers, they probably would not be significant in contributing to viral shedding.

In no case was antigen abundant in lung tissue. In some cases, antigen was detected in single cells scattered within the lung parenchyma (Figure 2d). No morphological abnormalities were detected that were attributable to the infection. Antigen was not detected in lung samples after 4 d.p.i. and this did not coincide with decline of virus detected in swabs, indicating that the lungs were probably not a major source of shed virus in the later stages of the disease.

Pancreatic acinar cells constituted the main epithelial cell type associated with the intestinal tract that had viral antigen. Antigen was observed in pancreas samples taken from 2 to 5 d.p.i. Lesions consisted of acute focal acinar necrosis, and these lesions were usually associated with sparse amounts of antigen. Renal epithelium and hepatocytes occasionally contained sparse amounts of antigen in small numbers of ducks. No antigen was detected in intestinal epithelium. The origin of the virus in cloacal swabs is unclear.

In addition to the tissues mentioned above, significant quantities of antigen were also detected in the thymus and bursa, in both tissues principally in thymal epithelial cells and connective tissues. Antigen was often detected in the peritoneal membranes, and when present was widely distributed over the serosal surface of the abdominal organs. Antigen was detected in larger blood vessels throughout the organ types and these were mainly in the smooth muscle layers, and occasionally in endothelium. Feather follicle epithelium and the feather pulp frequently contained dense viral antigen, and this was often associated with necrosis of the epithelium and pulp.

Indonesian strain (A/Duck/Indramayu/BBVW/109/2006). Viral antigen in ducks infected with the Indonesian strain showed a generally similar tissue distribution to that seen in ducks infected with the Vietnamese strain (Table 3). However, the quantities of viral antigen were usually considerably lower. In several birds, no viral antigen was detected in any tissues, including all six ducks killed humanely at 1 and 7 d.p.i. Tissue responses associated with viral infection with the Indonesian strain were noticeably less severe than those in ducks infected with the Vietnamese strain.

Viral antigen was detectable in most birds in single cells in myocardium (Figure 3a), skeletal muscle and smooth muscle, in the latter mainly in blood vessel walls (Figure 3b). No cellular reactions were detected in association with heart or skeletal muscle cell antigen staining, although dense viral antigen in arterial walls was associated with mild local cell swelling and degeneration.

In the brain, viral antigen was detected in occasional foci in neurons and in the neuropil (Figure 3c). Occasionally it was detected in ependymal cells, and in these cases the whole of the ventricle lining was infected. Encephalitic lesions, principally mononuclear cell cuffs and gliotic nodules, were mild but prominent from 3 d.p.i. in brains where antigen was detected (Figure 3d).

In most ducks, antigen was also present in connective tissues, including fibrous tissue, chrondocytes and osteoblasts. Small antigen foci in periosteal fibrous tissue were present with focal necrosis and granulocyte infiltration. Sparse antigen was found in lymphoid tissues in several ducks, and this was located in epithelial cells of the thymus and connective tissues of the bursa, in single cells

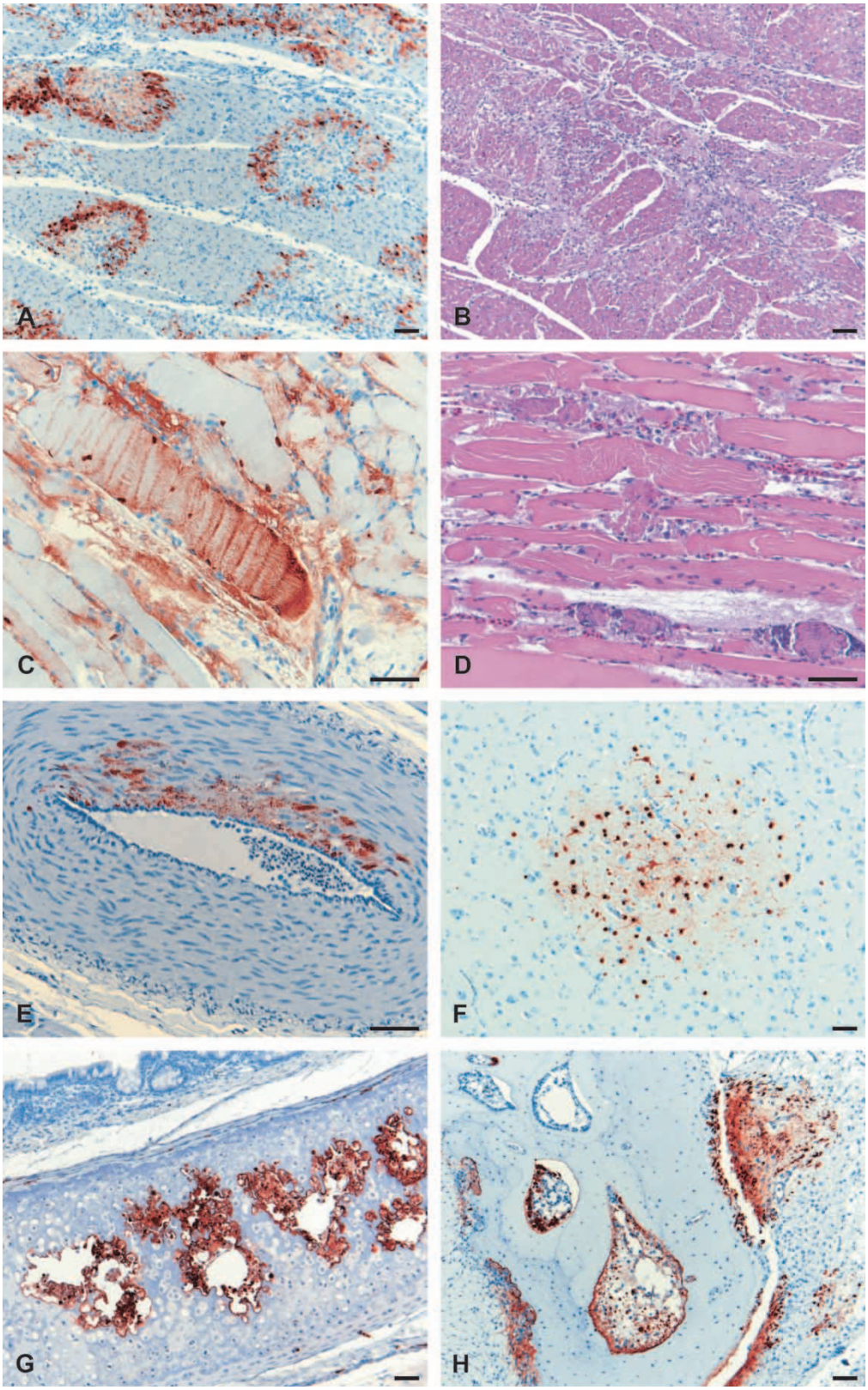

Figure 1. *Infection of A/Muscovy duck/Vietnam/453/2004 in various tissues. 1a: Heart, Duck 81, 5 d.p.i., immunohistochemistry (IHC) stain showing viral antigen as red/brown colour in myocardial fibres. 1b: The same heart as 1a, showing severe, acute myocarditis (haematoxylin and eosin). 1c: Pectoral (skeletal) muscle, Duck 44, 3 d.p.i. showing viral antigen in muscle fibres (IHC). 1d: Pectoral muscle, Duck 85, 4 d.p.i., showing severe acute muscle fibre degeneration (haematoxylin and eosin). 1e: Submucosal artery of oral cavity, Duck 42, 3 d.p.i., showing viral antigen in smooth muscle fibres of artery wall (IHC). 1f: Brain, Duck 44, 3 d.p.i. showing a focus of viral antigen in neurons (IHC). 1g: Tracheal cartilage, Duck 44, 3 d.p.i., showing viral antigen in foci of chondral necrosis (IHC). 1h: Bone and surrounding connective tissues of skull, Duck 44, 3 d.p.i., showing viral antigen in marrow spaces and periosteal fibrous tissue (IHC). All scale bars = 50 μm.*

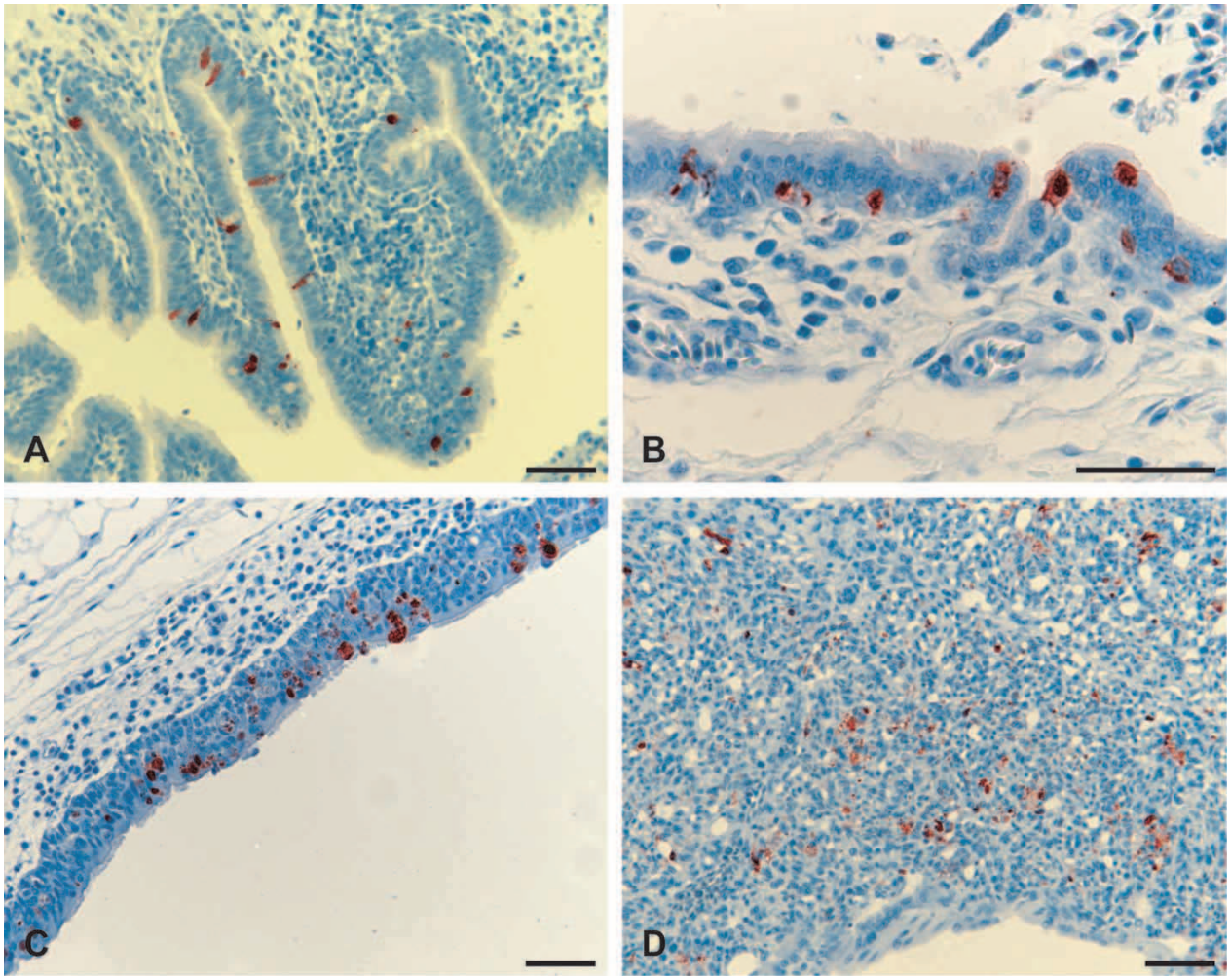

Figure 2. *Viral infection of epithelial tissues. 2a: Bronchial epithelium, Duck 80, 3 d.p.i., Vietnamese strain. 2b: Air sac (pleural surface), Duck 80, 3 d.p.i., Vietnamese strain. 2c: Paranasal sinus, Duck 79, 3 d.p.i., Indonesian strain. 2d: Lung, Duck 10, 2 d.p.i., showing antigen in single cells in lung parenchyma, Vietnamese strain. Immunohistochemistry. All scale bars = 50 μm.*

in one spleen and in one caecal lymphoid follicle. Occasional foci of antigen were present in pancreatic tissue. Focal acinar necrosis occurred in infected pancreatic tissue, often, but not always, associated with antigen. Antigen was also detected in the peritoneal membranes. No antigen was detected in parenchyma of lung, liver and kidney tissues, or in feather follicles.

Epithelial tissues of the respiratory tract that contained viral antigen were the paranasal sinuses (Figure 2c) and the air sacs, which were found in ducks sampled on 2 and 3 d.p.i. One focus of antigen, associated with a submucosal mononuclear cell inflammatory response, was found in turbinate epithelium (Duck 89); this was the only instance, in both groups of ducks, of viral antigen found associated with nasal turbinate epithelium.

Virus quantification in tissues. Tissue virus titration results are presented in Table 4. Virus reached peak levels from 2 to 4 d.p.i., and virus titres were between $10^{2.5}$ and $10^{5.25}$ $TCID_{50}$/0.1 ml for the Vietnamese strain and between $10^{2.5}$ and $10^{4.25}$ $TCID_{50}$/0.1 ml for the Indonesian strain. The lung was the tissue with the most frequent isolation of virus, despite the absence of antigen staining in the corresponding sections. Heart tissue contained the highest titres of virus. No virus was isolated from the pancreas of birds infected with the Vietnamese strain, despite the presence of antigen in sections. In many cases there was no consistent relationship between virus isolation and antigen presence in sections.

Nearly all of the duck tissue samples caused lethal cell degeneration effects at the highest concentration of 1:10. Where virus levels were high and exceeded this dilution, virus levels could be quantified. Many lethal samples were positive when re-inoculated into eggs at 1:10, indicating that virus levels were low but widespread in many tissues.

Discussion

The objectives of the present study were to characterize the disease caused in ducks by two different duck-derived Eurasian lineage H5N1 HPAI virus isolates, and to correlate the disease with virus distribution and shedding patterns. The two H5N1 viruses used in this study caused markedly different morbidity, with the virus of Vietnamese origin causing more severe morbidity compared with the Indonesian isolate. In a previous study, in which infected ducks were observed for 10 days after challenge, the Vietnamese strain caused severe disease, leading to sudden death or necessitating euthanasia, in 80% of ducks (Middleton *et al.*, 2007). The Indonesian strain caused mild disease with recovery and

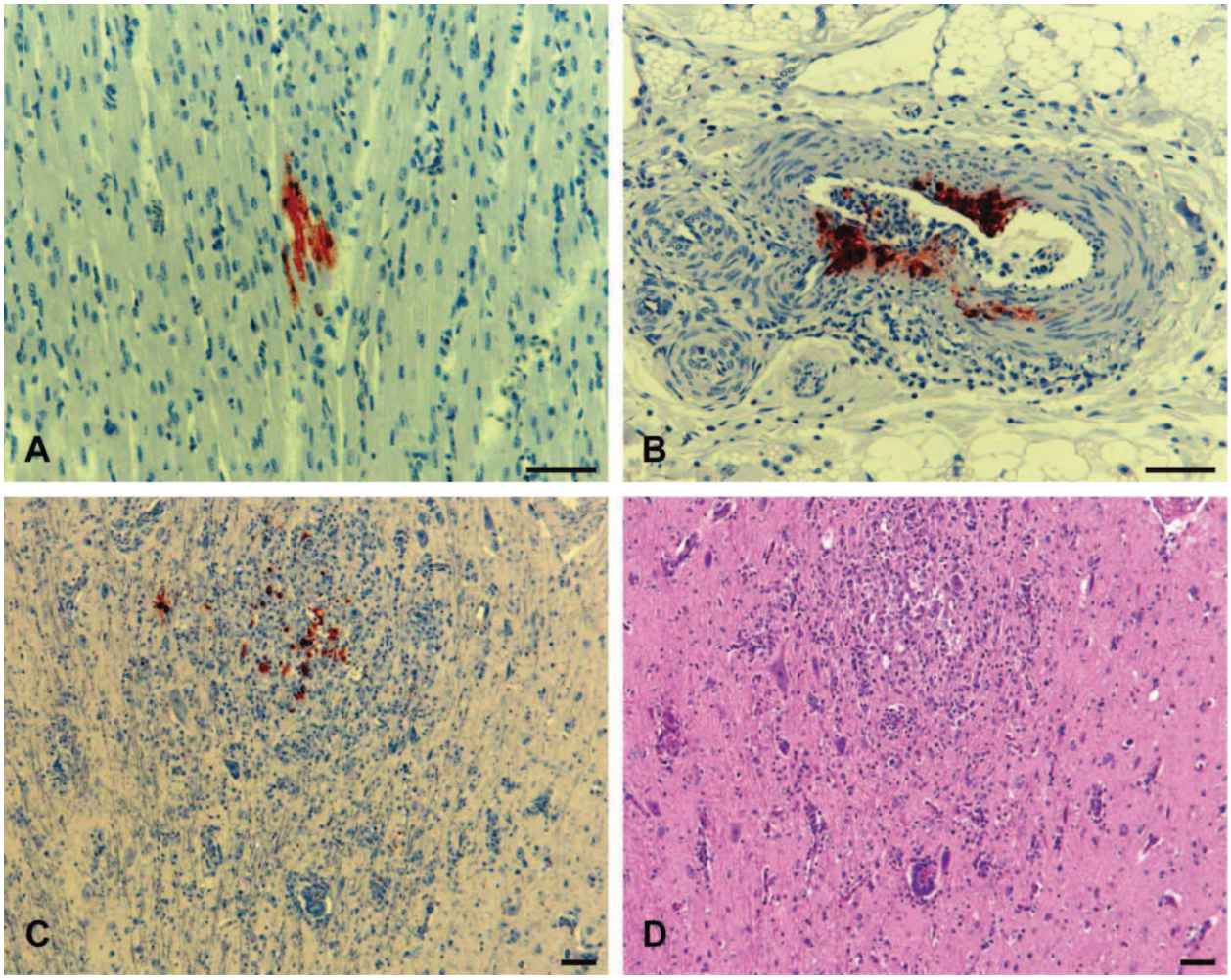

Figure 3. *Duck tissues infected with A/Duck/Indramayu/BBVW/109/2006 (Indonesian strain). 3a: Heart, Duck 79, 3 d.p.i., showing a typical isolated focus of viral antigen in heart muscle (immunohistochemistry [IHC]). 3b: Artery of subcutaneous region of head, Duck 90, 5 d.p.i., showing viral antigen in smooth muscle of vessel wall (IHC). 3c: Brain, Duck 89, 3 d.p.i., showing viral antigen within encephalitic lesion (IHC). 3d: Same brain lesion as in 3a, showing florid encephalitis (haematoxylin and eosin). All scale bars = 50 μm.*

seroconversion; only one bird developed more severe disease, which presented as mild persistent motor neurological disease (unpublished data). In the present study, fever was the only clinical sign observed in many of the ducks; however, as many were killed humanely early in the course of the infection, it is probable that some ducks would have developed more severe disease if they had not been killed. The Vietnamese strain caused prolonged high temperature, whereas the Indonesian strain caused only transient fever.

Other studies have noted the absence of clinical signs in ducks infected with H5N1 HPAI (Songserm *et al.*, 2006). In the present study, ducks showed no or mild signs, apart from pyrexia, in the early stages of the infection: they continued to eat, play in water and interact with eat other. This observation emphasizes the critical role of body temperature as a means of monitoring morbidity in ducks.

The tissue distribution patterns, as determined by immunohistochemistry, were similar to those found in other published studies using other Eurasian lineage H5N1 HPAI isolates in ducks, including various other species of the family Anatidae (Kishida *et al.*, 2005; Kwon *et al.*, 2005; Pantin-Jackwood & Swayne, 2007; Vascellari *et al.*, 2007; Yamamoto *et al.*, 2007; Löndt *et al.*, 2008; Keawcharoen *et al.*, 2008). These studies indicate that Eurasian lineage H5N1 HPAI viruses appear to have a high predilection for a wide range of cell types. There also appears to be reasonable similarity in general tissue tropism amongst various species of domestic and wild ducks, geese and swans, although there does appear to be considerable variability in quantity of antigen staining in different tissues. For example, in infected swans (*Cygnus olor* and *Cygnus cygnus*) virus appeared to have a high tropism for the brain, liver and pancreas and a low tropism for the heart (Teifke *et al.*, 2007; Kalthoff *et al.*, 2008). Similar findings were reported for Canada geese, *Branta canadensis* (Pasick *et al.*, 2007). In black swans (*Cygnus atratus*) virus appeared to have tropism for endothelial cells, leading to peracute death (Brown *et al.*, 2008). In none of these studies was infection of epithelial tissues widely noted. These studies were conducted using a range of virus isolates, so it is possible that the differences observed were due both to virus strain diversity and host species differences. They may also be caused by the range of ages used, as the age of the host may also affect degree of tissue tropism (Pantin-Jackwood *et al.*, 2007).

In the present study, the Vietnamese strain has a particularly high predilection for muscle tissues of all three types: myocardial, skeletal and smooth muscles.

Table 4. *Virus titration results (log_{10} $TCID_{50}$/0.1 ml) from 10% homogenates of duck tissues*

Duck identity	Day of death/euthanasia	Brain	Heart	Pancreas	Lung	Spleen	Feather
A/Muscovy duck/Vietnam/453/2004							
1	1	N	P ($\leq$1.5)	N	P ($\leq$1.5)	N	nd
2	1	N	P ($\leq$1.5)	N	N	N	nd
5	1	N	N	N	P ($\leq$1.5)	N	nd
6	2	$\leq$2.0	2.75	N	3.5	P ($\leq$1.5)	nd
9	2	N	N	N	2.5	2.75	nd
10	2	2.5	5.25	N	4.75	2.5	nd
42	3	3.25	3.75	N	3.5	3.25	>5.5
44	3	nd	5.0	N	3.5	P ($\leq$1.5)	>5.5
80	3	N	4.5	N	4.0	N	>5.5
81	5	–	3.75	2.5	1.25	–	nd
84	5	N	3.25	N	$\leq$1.5	$\leq$1.5	nd
85	4 (d)	3.5	N	N	3.5	N	5.0
86	4	nd	N	N	2.75	N	–
93	5	2.25	3.25	P ($\leq$2.5)	P ($\leq$1.5)	N	nd
94	5	–	1.25	$\leq$1.5	1.75	N	nd
A/Duck/Indramayu/BBVW/109/2006							
3	1	N	N	N	N	N	nd
4	1	N	N	N	P ($\leq$1.5)	N	nd
8	1	N	N	N	P ($\leq$1.5)	N	nd
41	2	P ($\leq$1.5)	P ($\leq$1.5)	N	N	N	nd
45	2	N	N	N	nd	2.25	nd
76	2	N	2.5	4.0	3.75	N	nd
79	3	N	N	N	P ($\leq$1.5)	P ($\leq$1.5)	2.5
83	3	3.25	P ($\leq$1.5)	3.0	3.25	P ($\leq$1.5)	P
89	3	N	$\leq$2.0	2.25	3.75	$\leq$2.0	4.0
90	5	N	N	3.0	P ($\leq$1.5)	N	–
91	5	N	$\leq$1.75	N	4.25	3.25	3.25
96	5	N	$\leq$1.5	2.25	3.0	3.25	5.0
97	7	N	N	N	P ($\leq$1.5)	N	nd
98	7	N	N	N	N	N	nd
99	7	N	N	N	P ($\leq$1.5)	N	nd

Tissue homogenates were titrated on Vero cell cultures, starting at a dilution of 1:10. Where the tissue homogenate was lethal to the cells at this dilution, 1:10 homogenates were inoculated into eggs. 2.25, example of virus titre value (log_{10} $TCID_{50}$/0.1 ml), as determined in cell cultures; $\leq$2.0, virus detected, but exact titre not determinable due to partial lethality in cell cultures; –, no virus detected; N, tissue homogenate at 1:10 was lethal to cell cultures, and negative in eggs; P ($\leq$1.5), tissue homogenate at 1:10 was lethal to cell cultures, but positive in eggs, indicating that virus was less than $10^{1.5}$ $TCID_{50}$/0.1 ml; nd, not done.

The presence of H5N1 virus in skeletal muscle of duck meat has been reported elsewhere (Mase *et al*., 2005a; Pantin-Jackwood & Swayne, 2007; Yamamoto *et al*., 2007). The levels of virus present in some skeletal muscles and the heart—tissues that are used for human consumption—was so high that there is some concern such meat may pose a significant threat of transmission to humans. The risk may be more in the preparation of meat rather than in consumption of cooked meat, as HPAI virus is inactivated during cooking (Thomas & Swayne, 2007). This is particularly relevant as the disease in ducks can be mild and therefore it may be difficult to distinguish infected ducks to exclude them from the human food chain.

The high pathogenicity of the Vietnamese strain is probably due to the viral tropism to the heart and the brain. Infection of these tissues, together with the tissue degeneration and inflammatory responses, would have caused significant morbidity. There was considerably less antigen in the tissues infected with the Indonesian isolate, and this would explain the milder disease seen in ducks infected with this virus. The main clinical observation in ducks infected with this isolate was mild neurological signs in a few individuals (unpublished data). Although presence of virus in brain tissue was low, it did appear to induce mild non-suppurative encephalitis.

Comparison between tissue virus and antigen levels, as determined by virus isolation and immunohistochemistry, respectively, was surprisingly lacking in consistency. Similar inconsistency has been found between immunohistochemistry staining and viral RNA in a study of another Eurasian lineage H5N1 HPAI virus (Löndt *et al.*, 2008). These results may be due to the focal nature of virus distribution within tissues. However, some interesting trends were apparent. For example, no antigen was detected in lung tissue infected with the Indonesian strain, despite significant virus detection. This may be due to the fact that the virus is present in air sac epithelium lining lung tissue, a distinction made on immunohistochemistry but not on virus isolation, and also to the possibility of the presence of virus in blood or tissue fluid, which is removed during histological processing. It may be of value to investigate this further as it may have implications in understanding viral shedding.

One of the principal objectives of the present study was to determine virus shedding patterns in Pekin ducks. Knowledge of shedding patterns is important to the understanding of transmission, and also the capability of the species to act as a maintenance host. Influenza

viruses usually replicate in, and are shed from, the respiratory or gastrointestinal tracts (Webster *et al*., 1978; Alexander, 2001). In the present study it was found that virus was shed from both the oral and cloacal routes, implying viral replication in the epithelia of the gastrointestinal tract, the oral cavity or the respiratory tract. It was somewhat surprising to find that evidence for viral replication was absent or sparse in the epithelial tissues of many of the principal components of the respiratory or gastrointestinal tracts. Viral antigen was most consistently present in air sac and paranasal sinus epithelia, but even in these tissues antigen was not abundant. The possibility exists that even low levels of infection in epithelial tissues are sufficient to induce detectable viral shedding. However, examination of results from other studies indicates that the swab virus levels detected in this study are comparatively low: viral titres of $10^{3.0}$ to $10^{5.0}$ EID_{50}/ml are not unusual for various Anseriform species, particularly from oral swabs (Sturm-Ramirez *et al.*, 2004; Zhou *et al.*, 2006; Brown *et al.*, 2007, 2008; Pantin-Jackwood *et al*., 2007; Kim *et al*., 2008; J. Li *et al*., 2008). It is possible that the low virus levels were due to the two freeze–thaw cycles that they were subjected to during testing. It is also possible that these particular virus strains do not grow well in Vero cells, despite the apparent high sensitivity of these cells for other H5N1 HPAI virus strains. These effects are currently being investigated in more detail.

Since the emergence of Eurasian lineage H5N1 HPAI, it has been hypothesized that ducks are the primary reservoir species of the virus, maintaining the virus over long periods and causing spillover into other avian species (Hulse-Post *et al*., 2005; Sturm-Ramirez *et al*., 2005; Keawcharoen *et al*., 2008). This hypothesis presupposes that the virus would be intimately adapted to ducks, such that infection is effectively established and viral shedding is maximized. The present study showed that ducks infected with the Vietnamese strain were shedding virus more frequently than ducks infected with the Indonesian strain. This indicates that the Vietnamese strain may be better adapted to Pekin ducks than the Indonesian strain. It is even possible that ducks may not be able to effectively maintain the Indonesian strain of avian influenza. This could be investigated further through transmission trials. This does not preclude the possibility that other virus types circulating in Indonesia may be more closely adapted to, and maintained by, ducks.

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Application of chicken microarrays for gene expression analysis in other avian species

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Abstract

Background: With the threat of emerging infectious diseases such as avian influenza, whose natural hosts are thought to be a variety of wild water birds including duck, we are armed with very few genomic resources to investigate large scale immunological gene expression studies in avian species. Multiple options exist for conducting large gene expression studies in chickens and in this study we explore the feasibility of using one of these tools to investigate gene expression in other avian species.

Results: In this study we utilised a whole genome long oligonucleotide chicken microarray to assess the utility of cross species hybridisation (CSH). We successfully hybridised a number of different avian species to this array, obtaining reliable signals. We were able to distinguish ducks that were infected with avian influenza from uninfected ducks using this microarray platform. In addition, we were able to detect known chicken immunological genes in all of the hybridised avian species.

Conclusion: Cross species hybridisation using long oligonucleotide microarrays is a powerful tool to study the immune response in avian species with little available genomic information. The present study validated the use of the whole genome long oligonucleotide chicken microarray to investigate gene expression in a range of avian species.

Background

Gene expression profiling utilising microarrays has become a widely used approach to elucidate biological function in host pathogen interactions. A variety of different platforms and approaches have been developed that have allowed analysis of gene expression in responses to viral infections [1-6] Prior to the sequencing of the chicken genome these microarrays consisted of cDNAs

from many different sources. Sequencing of the chicken genome has increased the range of tools available to investigate gene expression including both short and long oligonucleotide arrays. These gene expression tools have far reaching benefits for the entire poultry industry, including the identification of genes that may play integral roles in determining disease resistance, productivity and quality.

However, identification of such important genes in other avian species is currently inhibited by a paucity of genomic resources. Cross-species gene-expression comparison is a powerful tool that may alleviate the lack of genomic information for a range of different avian species. By utilising existing microarrays it is possible to study gene expression in closely related species; this technique is termed cross species hybridisation (CSH). CSH has been previously used to investigate gene expression across a number of different species including humans and cattle [7], humans and primates [8], sheep and cattle [9] and a number of plant species [10]. CSH highlights the wider unconventional use of microarrays, proving that this technique can be flexible; however one must keep in mind the limits of this application, with regard to species specific genes.

In addition to studying gene expression profiles in closely related species, CSH can be used to explore comparative genomics. Comparative genomics provides an opportunity to ascertain relationships between gene function and location in a range of organisms [11]. Moreover, CSH allows insight into conservation of functional elements and the tracing of evolutionary phylogenies by way of comparing both closely and distantly related species. Comparative genomic studies in birds may help to develop detailed genomic information in a wide range of bird species. The significance of such genomic information is highlighted by the potential it offers in the study of important issues such as the recent outbreaks of avian influenza. Research on such diseases is hindered by the lack of genomic information available for many of the avian species known to be capable of infection by the virus. Alleviating this lack of detailed genomic information will inevitably assist in understanding the differences of immune responses in a range of avian species and allow better treatment and control strategies to be implemented.

We have utilised a whole genome chicken array to determine how useful it may be for the study of gene expression in other bird species. In particular we were interested to test a set of immunological gene probes on our chicken microarrays to see if they provide functionally useful results, thus revealing the utility of the arrays in cross-species studies. Here we test the utility of a whole chicken genome microarray to study immune response in other avian species by investigating spleen tissue expression patterns in a range of bird species and a comparison of spleen tissue from un-infected and H5N1 infected ducks. By understanding the strengths and limitations of cross species microarrays we will be able to elucidate the power of the arrays to address important biological issues in diverse bird species.

Methods

Tissue collection

Spleen samples were collected from a wide range of avian species (Figure 1) (Chicken, *Gallus gallus*; Duck, *Anas platyrhynchos*; Starling, *Sturnus vulgaris*; Magpie goose, *Anseranas semipalmata*; Kookaburra, *Dacelo novaeguineae* and Tawny frogmouth, *Podargus strigoides*) and placed into 10 volumes of RNAlater (Ambion, USA) and stored at -20°C until RNA isolation. All control birds were at adult stage and free from clinical disease. Five-week-old Pekin ducks were challenged with a Vietnamese H5N1 strain (A/Muscovy duck/Vietnam/453/2004); each dose contained approximately $10^{7.2}$ median egg infectious doses (EID_{50}). Spleen samples were collected 2 days post infection. Infected samples were confirmed using viral titres (data not shown).

Total RNA isolation and cDNA preparation

Total RNA for all samples studied was isolated using the Meridian total RNA isolation kit (Cartagen). Five micrograms of total RNA was reverse transcribed into cDNA and indirectly labelled with Cy3 using the ULS cDNA Synthesis and Labelling Kit (Kreatech Technologies). The labelled probes were concentrated using Microcon Ultracel YM-30 Columns (Amicon Bioseparations) and the quality and label incorporation of each sample was verified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

Microarray design and hybridisation

Each Cy3 labelled spleen sample was individually hybridised to the whole genome chicken array (single colour hybridisation). This whole genome chicken microarray was printed with a MicroGrid II spotting robot in house using a set of 20,460 long oligos (65–75 nt) printed in duplicate on each array. The oligo set was designed at the Roslin Institute (Scotland, UK) based upon chicken Ensembl gene transcripts and other genomic information supplied by various research groups around the world http://www.ark-genomics.org/microarrays/bySpecies/chicken/.

Whole genome chicken arrays were pre-hybridised at 42°C for 45 min in pre-hybridisation buffer (25% (v/v) formamide, 5 × SSC, 0.1% (w/v) SDS, 10 mg/ml salmon testes DNA). Following addition of the labelled probe and hybridisation solution (25% (v/v) formamide, 5 × SSC,

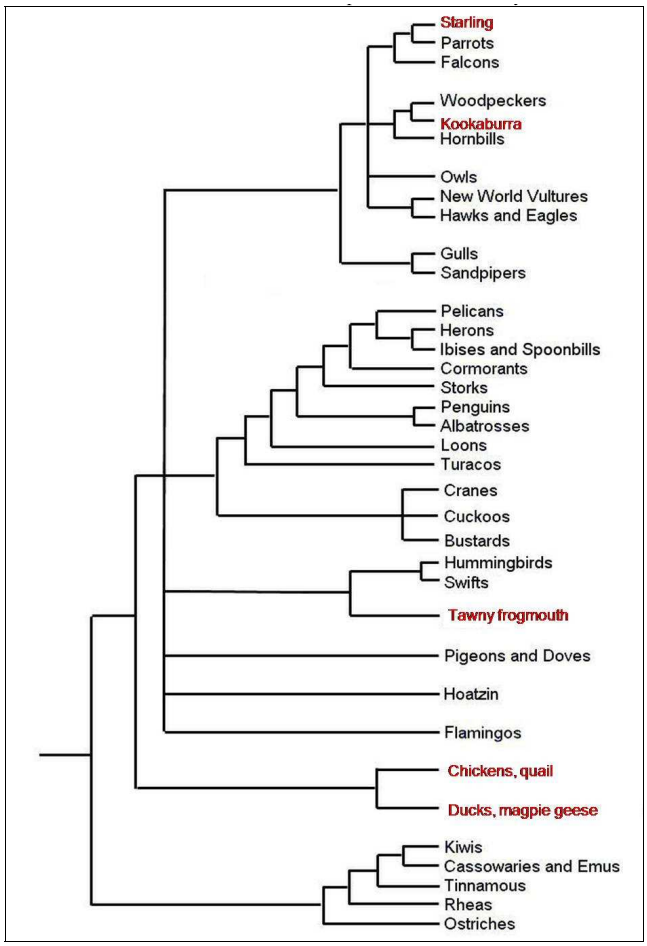

Figure 1
Phylogenetic orders of birds. Phylogenetic tree of all orders of birds displaying the relative evolutionary distance [20]. Red text indicates orders of birds that are represented in this study.

0.1% (w/v) SDS, 25% (v/v) KREAblock), all arrays were incubated for 16 h at 42°C. Post hybridisation, all arrays were washed once in (2 × SSC, 0.1% (w/v) SDS) for 5 min at 42°C, once in (0.1 × SSC, 0.1% SDS) for 10 min at 25°C and three times in 0.1% SSC, each for 1 min at 25°C. For each avian species two independent spleen samples were hybridised to separate arrays to ensure reproducibility, except in the case of the H5N1 duck samples where three independent control spleen duck samples and three independent infected H5N1 spleen duck samples were hybridised.

Microarray analysis

Post hybridisation all arrays were scanned and gene expression signals captured using an ArrayWORXe (Applied Precision) fluorescent scanner. All arrays were background corrected and arrays that had more than 0.0125% saturated spots were discarded. Each array was globally normalised; global normalisation was used as it ensures that the measured intensities are comparable across all slides. This sort of normalisation allows comparison of all arrays without biases when a majority of the spots on the arrays were giving positive hybridisation signals [12]. Subsequent statistical tests were carried out for the duck samples using GeneSpring 7.2 (Silicon Genetics) to determine all genes differentially regulated to a specified cut-off value ($p = 0.05$). A condition tree was constructed to explore the gene expression relationship between all duck samples. Condition trees were performed using standard correlation in GeneSpring 7.2. Spot net intensities were calculated from the median spot and background intensities with all results less than one standard deviation of the respective spot background pixel intensities ignored (set to zero). The net intensities of all arrays were then normalised such that the maximum spot intensity in each array was 10,000.

Results

Cross avian hybridisation of whole chicken genome arrays

A range of avian samples spanning the Neognathae infraclass were used in this experiment (Figure 1). All of these avian samples were successfully hybridised to the whole genome chicken microarray and this is demonstrated in the summary statistics in Table 1. All arrays passed our quality control measures with the magpie geese samples displaying the least amount of spots higher than one standard deviation above background at 67%. As expected chicken spleen samples performed the best with 78% spots passing quality control (it is not expected that hybridising chicken to the whole chicken genome microarray will ever result in 100% spot hybridisation as not all genes in the genome will be expressed in any one sample). Background median intensities were similar for all arrays except for the duck arrays, where the values were higher. The duck spleen samples also displayed a larger net intensity and background standard deviation than all the other microarrays. The distributions of the $\log_{10}$ transformed normalised spot intensities for all control samples are plotted in Figure 2. These plots show similar trends between all samples except the magpie goose and duck arrays. The magpie goose plot shows a shift highlighting the higher number of low intensity spots on these arrays. The duck plot suggests that these samples have a greater number of high intensity spots when compared with chicken. Overall the avian microarrays displayed a significant range of spot intensities across the chicken microarray.

In order to further support the use of the whole chicken genome array for other avian species we specifically investigated the expression levels of a test set of chicken genes with known immunological functions. These results are

Table 1: Summary statistics for avian samples hybridised to the whole genome chicken microarray

Field	Chicken	Duck	Kookaburra	Tawny frogmouth	Magpie goose	Starling
Maximum net intensity	65,245	62,490	64,000	64,259	65,129	64,993
Maximum normalised net intensity	10,000	10,000	10,000	10,000	10,000	10,000
Median net intensity	98	136	90	56	72	55
Median normalised net intensity	15	22	14	9	11	8
Spots above 1 standard deviation* (max 43200)	33,759	32,483	32,659	31,154	28,748	31,390
Percentage of spots above 1 standard deviation*	78	75	76	72	67	73

*One standard deviation above the background intensity.

shown in Table 2. Each of the avian species provided significant net signal intensity for a variety of the genes investigated. A number of the genes were more highly expressed in the other avian species when compared to chicken. The defensin gallinacin 1 gave significant net intensities for all birds studied, with particularly high values for both duck and tawny frogmouth. A number of the genes including IL-8, IFNγ, IFNλ and caspase 3 gave low or no net intensity signals across all samples. In summary, all avian samples tested here provided significant net signal intensities for a number of the chicken genes with known immunological functions.

Differentiating between H5N1 infected and uninfected ducks

A total of 2103 genes were differentially expressed (p = 0.05), 685 genes were up regulated in the H5N1 infected ducks and a further 244 genes were down regulated when compared to the uninfected controls (above 1.8 fold). Details of the specific genes regulated in this experiment can be found in the supplementary data (Additional file 1). Gene expression results are represented as a condition tree (Figure 3) and can be used to clearly differentiate the infected duck samples from the uninfected samples based on their gene expression profiles, which placed each group on separate arms of the tree. This condition tree also highlights the large number of regulated genes in the duck samples, and in particular identified key immune genes IL-1β, GAL4 and MHC class II that are regulated in the H5N1 infected samples.

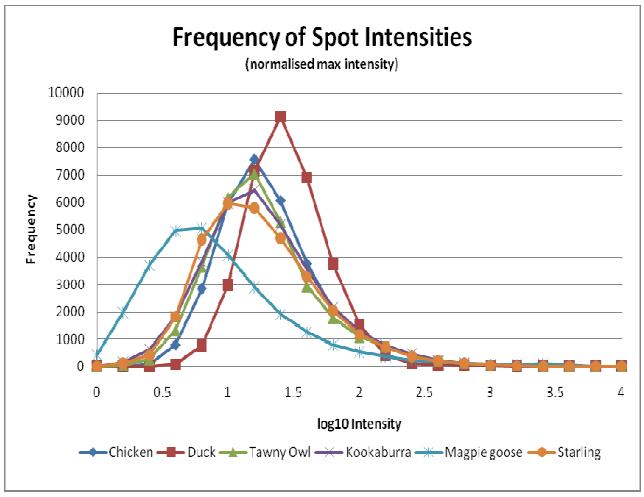

Figure 2
Frequency of spot intensity. Frequency and range of average values of net normalised signals for each avian species tested on the whole genome chicken array are shown. Each plot is the average of at least two independent hybridisations.

Discussion

Microarrays are a powerful tool for studying the gene expression of thousands of genes simultaneously. For species like the chicken where the whole genome has been sequenced it is possible to construct tailored whole genome arrays that allow comprehensive gene expression studies. For other avian species, such as duck, where there is little available genomic information, it is not yet possible to design specific microarrays. In this study, we explored the technical feasibility of utilising cross species hybridisations to identify genes expressed in a broad range of avian species. Our experimental strategy was three-fold. The first goal was to test the hybridisation of a number of phylogenetically diverse avian species on the whole genome chicken microarray. Our second goal was to determine if this microarray platform was able to distinguish between two different biological states in a bird species other than chicken. Thirdly, we were interested in testing the feasibility of the whole genome chicken microarray in avian immunological studies by focusing on the performance of known chicken immune genes.

As documented previously [10], long oligonucleotide microarrays are a reliable tool for CSH as they identify significantly more regulated genes at a lower false discovery rate than microarrays with short oligomers. Long oligonucleotide arrays are also considered to be an improvement over cDNA arrays as they eradicate the problem of clone misidentification and erroneous cross hybridisation [13]. The use of the long oligonucleotide whole genome chicken array as a tool in comparative genomics for other avian species has been validated in this study. We have successfully hybridised a broad range of phylogenetically

Table 2: A selection of chicken immune genes present on the whole genome chicken microarray

		Net Intensity					
Gene Name	**oligo name**	**chicken**	**duck**	**magpie goose**	**starling**	**kookaburra**	**tawny frogmouth**
Interleukins							
IL-1β	RIGG20417	++	+++	++	+	++	+
IL-2	RIGG20032	+	++	+++	+	+	++
IL-6	RIGG20074	++	++	++	++	+	+++
IL-8	RIGG20034	x	++	x	++	x	x
IL-12α	RIGG20057	+++	+	++	+	++	++
IL-13	RIGG20059	+++	++	++	+	+++	++
Interferons							
IFNβ	RIGG20053	+++	+++	+++	+++	+++	+++
IFNγ	RIGG20054	+	++	x	+	+	x
IFNλ	RIGG20055	++	+	+	+	+	+
Chemokines							
K203	RIGG00459	+++	+	+++	+++	+++	+++
CX3C	RIGG00463	++	+++	++	++	+++	++
K60 (CXC chemokine)	RIGG00467	++	+++	+++	++	+	+
CXCL14	RIGG00466	+++	+++	+	++	+++	++
Toll like receptors							
TLR2	RIGG14974	++	++	++	++	+++	++
TLR15	RIGG14349	+++	+++	++	+++	+++	+++
TLR21	RIGG10152	++	+	+++	++	+	++
Defensins							
gallinacin 1	RIGG20043	+++	+++	++	+++	+++	+++
gallinacin 3	RIGG20044	+	+	+++	+	++	++
MHC							
MHC class I	RIGG12432	+++	+++	+++	+++	+++	+++
MHC class II	RIGG16832	++	+	++	+	+	++
Caspases							
caspase 1	RIGG02179	++	+++	+++	++	++	+
caspase 3	RIGG15820	x	++	+	+	x	x

Positive intensity signals are represented by '+' according to the following scale (+ = <50, ++ = 51–100 and +++ = >150. Crosses 'x' represent signals that were deemed unreliable as they did not pass quality control. All results are based on averages of at least two independent hybridisations containing dual representation of each gene.

distinct bird species to this microarray. Each of the avian species had a large number of spots that passed our quality control measures to the extent that there was a low degree of spot drop out (at most 11%) compared to the chicken.

Overall most of the avian samples showed a similar performance to chicken when hybridised to the chicken microarray; however there were some slight shifts in some of the expression profiles. The magpie goose arrays displayed a higher number of spots with lower signals compared with chicken and had the fewest number of genes that passed our quality control measures. This may be explained by a lack of genetic similarity between chicken and magpie goose; however with little sequence information for magpie goose we must be cautious when interpreting our results. These results highlight the potential use of this whole genome chicken long oligonucleotide microarray for gene expression studies in a wide range of avian species.

When performing CSH the degree of sequence complimentarily between probes and targets is variable. Differences in hybridisation levels can be attributed to transcript abundance levels, the presence of sequence mismatches and differences in hybridisation kinetics [13,14]. Due to these limitations, it is not possible to compare gene expression levels for particular genes across species, but comparisons within a species is valid because of the same level of sequence identity and hybridisation kinetics will apply. Thus, we present the experiment utilising the H5N1 infected ducks as a test of the validity of using a chicken microarray to distinguish between two different immune states in ducks. This serves as a tool to carry out more in depth analysis of H5N1 infection in ducks. By contrasting such results with findings in the chicken it may be possible to determine the basis for the very different outcomes of viral infection in each species [15].

The whole genome chicken microarrays were successful in differentiating the infected ducks and control ducks from

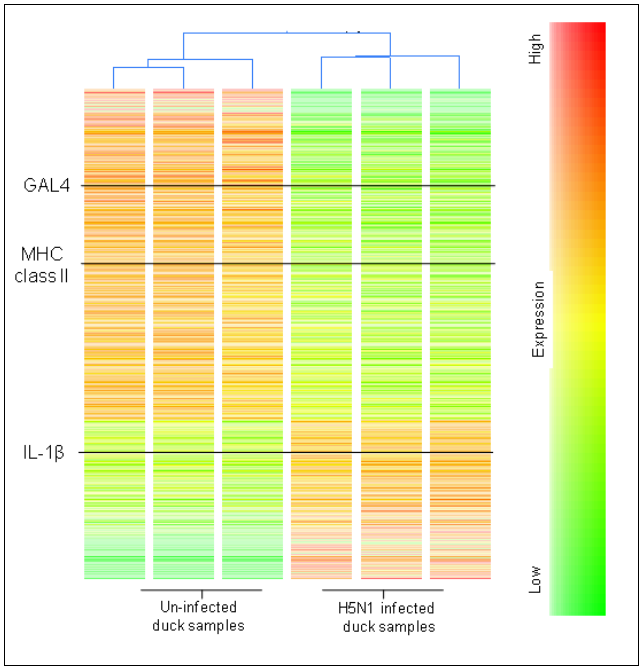

Figure 3
Condition tree of H5N1 infected and uninfected ducks. Average signal log intensities are presented, highlighting the strong relationship between the H5N1 infected samples compared to the uninfected duck controls. Immune genes of particular interest are shown in black.

one another based on their overall gene expression profiles. In addition, the chicken microarrays were able to identify a number of key immune genes that were regulated in the H5N1 infected ducks. These genes include IL-1β, MHC class II alpha chain and the defensin gene GAL4. These genes have been previously shown to be involved in a chicken's response to infection [16] and thus it is plausible that these genes play a similar role in ducks. Interestingly the sequence similarity of IL-1β in ducks and chicken is 99% and it has recently been shown to be structurally and functionally homologous [17-19]. Moreover, the use of chicken microarrays to investigate gene expression in other birds may provide a new way of identifying conserved genes with known immunological functions in other avian species. In particular, CSH may be used to investigate H5N1 infection in a range of other bird species including swans, crows, turkeys, quail and geese. Cross species hybridisation is a feasible approach because of the relatively close evolutionary distance between avian species. While CSH has great potential for use in a wide range of avian species, it must also be noted that this method of studying gene expression will neglect to capture species specific genes that are involved in the host response.

Conclusion

This is the first study to show the benefits of CSH for avian species using a whole genome chicken long oligonucleotide microarray. We have successfully demonstrated hybridisation of a number of phylogenetically diverse bird species to this chicken array. In so doing we have been able to identify a range of immune genes that can be detected in the avian species studied. We have also demonstrated the use of this chicken array as a tool to investigate gene expression in closely related immune genes. This study provides avian immunologists with a new tool to investigate gene expression in avian species with little or no genome information.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TMC performed the RNA extractions, microarray hybridizations and analyses and drafted the manuscript. VRH was instrumental in the cross species microarray analysis. SB helped with the total RNA isolation and collected the H5N1 infected duck samples. RJM is the principal investigator of the laboratory and was involved in the conceptualisation of the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

***All genes regulated in the duck H5N1 experiment**. all genes that were regulated greater than 1.8 fold (p = 0.05 and p = 0.01) between uninfected control duck samples and duck samples infected with H5N1.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-S2-S3-S1.pdf]

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