Enhancing Host Immunity to Avian Influenza Virus using Toll-like Receptor Agonists in Chickens

By

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ABSTRACT

ENHANCING HOST IMMUNITY TO AVIAN INFLUENZA VIRUS USING TOLL-LIKE RECEPTOR AGONISTS IN CHICKENS

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University of Guelph, 2012

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Toll-like receptors (TLRs) are evolutionarily conserved pattern recognition receptors that mediate host-responses to pathogens. In mammals, TLR ligands promote cellular activation and the production of cytokines. Several TLR ligands have been employed prophylactically for the control of bacterial or viral diseases in the mouse model. However, the TLR-mediated responses in chickens have not been well described. Importantly, the utility of TLR agonists for the control of viral pathogens, such as avian influenza virus (AIV), has not been fully explored in chickens. To this end, the studies described in this thesis characterized the kinetics of in vivo responses in chickens to the TLR4 ligand lipopolysaccharide (LPS) and the TLR21 ligand CpG ODN. It was demonstrated that both of these ligands induced the up-regulation of several immune system genes in the spleen, including those associated with pro-inflammatory and antiviral responses, as well antigen presentation. By harnessing the immunostimulatory properties of TLR ligands, it was also demonstrated that the prophylactic administration of either poly I:C (a TLR3 ligand), LPS or CpG ODN may confer immunity to a low pathogenic avian influenza virus, as determined by a reduction in both oropharyngeal and cloacal virus shedding in infected birds. Furthermore, transcriptional analysis of genes in the spleen and lungs identified interleukin (IL)-8, interferon (IFN)-α and IFN-γ as
correlates of immunity. In conclusion, TLR ligands may modulate several aspects of the chicken immune system to induce an anti-viral state, thereby conferring immunity to AIV.
ACKNOWLEDGEMENTS

First, I would like to thank my advisor Dr. Shayan Sharif. Your guidance, mentoring and support have been invaluable in providing me with a foundation for a successful career in immunology. I also greatly appreciate the fact that you were always very supportive of my many side projects, even though a few of them were definitely out in left field!

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I would also like to thank my friends and colleagues in the Pathobiology department and elsewhere. I would also like to thank Claudia for her endless encouragement, patience and support. No matter what happened with my research, you were always able to show me the bright side of things. I am truly grateful and appreciative of all the help and support that you have provided me.

Lastly, I would like to thank my family. You have always been there to support me during my studies and I could not have achieved this without you.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AIV</td>
<td>Avian influenza virus</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>d.p.i.</td>
<td>Days post-infection</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HPAI</td>
<td>Highly pathogenic avian influenza virus</td>
</tr>
<tr>
<td>HPI</td>
<td>Hours post-injection</td>
</tr>
<tr>
<td>IBV</td>
<td>Infectious bronchitis virus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LPAI</td>
<td>Low pathogenic avian influenza virus</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M1</td>
<td>Matrix protein 1</td>
</tr>
<tr>
<td>M2</td>
<td>Matrix protein 2</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-activated protein</td>
</tr>
<tr>
<td>MD-2</td>
<td>Myeloid differentiation protein-2</td>
</tr>
<tr>
<td>Mda5</td>
<td>Melanoma differentiation-associated gene 5</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin Darby Canine Kidney Cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid Differentiation Factor 88</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NDV</td>
<td>Newcastle disease virus</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NS1</td>
<td>Non-structural protein 1</td>
</tr>
<tr>
<td>NS2</td>
<td>Non-structural protein 2</td>
</tr>
<tr>
<td>OAS</td>
<td>Oligoadenylate synthetase</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>PA</td>
<td>Polymerase acidic protein</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PB1</td>
<td>Polymerase basic protein 1</td>
</tr>
<tr>
<td>PB1-F2</td>
<td>Polymerase basic protein 1-F2</td>
</tr>
<tr>
<td>PB2</td>
<td>Polymerase basic protein 2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Post-infection</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>Polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse-transcription PCR</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid inducible gene I</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% Tissue culture infectious dose</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>T-helper</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-IL 1 Receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon- β</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Declaration of work performed

The studies described in this thesis are original and the experiments were performed by Michael St. Paul. Technical assistance was provided by in alphabetical order: Kamran Haq, Amirul I. Mallick, Shahriar Orouji, Payvand Parvizi, Leah R. Read and Alexander Ian Villanueva, with the intellectual assistance of Dr. Mohamed Faizal Abdul-Careem and Dr. Éva Nagy. All work was carried out under the supervision of Dr. Shayan Sharif at the Department of Pathobiology, University of Guelph.
INTRODUCTION

Harnessing the power of the innate immune system holds promise in protecting chickens against viral pathogens. Traditionally, the innate immune system is defined as having several classic hallmarks, namely the potential to respond to a broad range of pathogens immediately, as well as lacking specificity and immunological memory. As such, the innate immune system serves as the first line of defence against pathogens and microbes. This is largely achieved through a multilayered approach, consisting of anatomical, physiological and cellular barriers (Kindt et al., 2006). For example, anatomical barriers include the skin, and mucous membranes, while physiological barriers include stomach acid, fever, complement, lysozyme and anti-microbial peptides. However, if these two barriers are overcome, the innate immune system relies on the cellular barrier, which is constituted by a variety of cell subsets including macrophages, natural killer (NK) cells, heterophils/neutrophils, mast cells, basophils and eosinophils. Together, these cell subsets protect the host through a variety of mechanisms, such as by phagocytosis of foreign particles and microbes as well as through the production of cytokines that may induce a pro-inflammatory state. Importantly, certain cells of the innate immune system, such as macrophages, play a critical role in initiating and modulating the adaptive immune response through antigen presentation. Adaptive immune responses are mediated both by antibodies and cell-mediated mechanisms, relying on B cells and T cells, respectively. The adaptive immune system is thought to have specificity, diversity, self/non-self recognition and memory. However, recent discoveries have clearly shown that members of the innate immune system may also share at least some of the above features with the adaptive immune system.
In mammals and chickens, there is a high degree of integration and cross-talk between the innate and adaptive arms of the immune system. For example, cytokines produced by dendritic cells, such as interleukin (IL)-12, and by mast cells or other cells, such as IL-4, influence the polarization of T-helper (T_H) cells into either a T_H1 or T_H2 phenotype, which are important for mediating responses against intracellular or extracellular pathogens, respectively. Conversely, cytokines, such as interferon (IFN)-γ, as well as signaling molecules, such as CD40 ligand, produced by T cells activate innate immune system cells and enhance their effector functions (Buhtoiarov et al., 2005). Together, this integration and crosstalk confers both flexibility and robustness to the immune response, thereby conferring protection against a variety of pathogens. Therefore, following the administration of compounds that activate the innate immune system, the resulting cellular activation and cytokine production may be exploited to protect chickens against viral pathogens, including avian influenza virus.
CHAPTER 1:

Literature Review

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Modified versions of the different sections of the Literature Review will be submitted for publication:

St. Paul, M., Brisbin, J. and Sharif, S. Immunomodulatory properties of Toll-like receptor ligands in chickens. To be submitted to *Veterinary Immunology and Immunopathology*
Toll-Like Receptors

To identify potential invaders and discriminate self from non-self, cells of the innate immune system rely on pattern recognition receptors (PRRs) to recognize and bind conserved motifs found in bacteria and viruses, known as pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997). Although there are several different types of PRRs, the one type that has been studied the most is the Toll-like receptor (TLR). In 1996, it was found that the Toll receptor was required to protect *Drosophila* from a fungal pathogen (Lemaitre et al., 1996). It was soon after this discovery that Medzhitov and colleagues showed that this receptor is evolutionarily conserved, as numerous TLRs were subsequently detected in humans, mice and chickens (Medzhitov et al., 1997; Roach et al., 2005). To date, at least 12 TLRs have been identified in mice and 10 in chickens (Temperley et al., 2008). Together, these molecules mediate responses to pathogens by binding different types of PAMPs. In humans and mice for example, TLRs 3 and 9 recognize double-stranded RNA and CpG DNA motifs, respectively, which are a characteristic of certain viral and bacterial nucleic acids, while TLR4 recognizes lipopolysaccharides (LPS) found in the cell wall of Gram-negative bacteria (Chow et al., 1999; Akira and Takeda, 2004). Although mice and chickens share many TLR orthologs including TLRs 3 and 4, chickens possess unique TLRs as well, namely TLRs 15 and 21. While recognizing the same CpG DNA ligand as mammalian TLR9, TLR21 is evolutionarily distinct from TLR9 and is thus classified as a separate TLR (Keestra et al., 2010). Although most of the chicken TLRs are expressed on the cell surface, TLRs 3, 7, and 21 are typically located intracellularly, and are recruited to the endosomes following the endocytosis/phagocytosis of a pathogen (Keestra et al., 2010). This phenomenon may allow for more frequent interactions with their ligands, which are all some type of nucleic
acid, and as such are not exposed to TLRs until the pathogen has been digested in the endosome. A summary of chicken TLRs and their ligands may be found in Table 1.

**Table 1.** Chicken Toll-like receptors and their ligands

<table>
<thead>
<tr>
<th>TLR</th>
<th>Structure recognized</th>
<th>Agonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TLR1b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TLR2a</td>
<td>Bacterial cell wall components</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>TLR2b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TLR3</td>
<td>Double stranded RNA</td>
<td>Poly I:C</td>
</tr>
<tr>
<td>TLR4</td>
<td>Bacterial cell wall components</td>
<td>LPS</td>
</tr>
<tr>
<td>TLR5</td>
<td>Bacterial Flagellin</td>
<td>Flagellin</td>
</tr>
<tr>
<td>TLR7</td>
<td>Single-stranded RNA</td>
<td>Imiquimod, Resiquimod</td>
</tr>
<tr>
<td>TLR15</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>TLR21</td>
<td>CpG DNA</td>
<td>CpG ODN</td>
</tr>
</tbody>
</table>

TLRs are expressed by a wide variety of cells including macrophages, dendritic cells, neutrophils, NK cells and mast cells (Hopkins and Sriskandan, 2005). While largely considered to be an innate receptor, recent evidence has also detected TLRs in lymphoid cells, such as B cells, T cells and invariant natural killer T cells (Hanten et al., 2008). Stimulation of TLRs on these cell subsets results in cellular activation, maintenance of memory cells and exertion of effector functions (Kulkarni et al., 2010). In chickens, TLRs have been detected in several cell subsets including in heterophils (Kogut et al., 2005b), monocytes and macrophages (He et al., 2006), as well as in B cells and T cells (Iqbal et al., 2005). Furthermore, chicken TLR transcripts have been detected in several
tissues including in the spleen, the bursa of Fabricius, the lungs, and several regions of the gastrointestinal tract such as the duodenum and jejunum (Iqbal et al., 2005).

**TLR Structure and Function**

TLRs are transmembrane proteins consisting of three domains: the extracellular domain containing leucine rich repeats, the transmembrane domain, and the intracellular Toll/IL-1 receptor (TIR) domain (Akira et al., 2001). Following binding of a ligand to the extracellular domain, the TIR domain recruits adaptor molecules, two key ones being Myeloid Differentiation Factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) (Moynagh, 2005). In mammals, it has been shown that all TLRs, except TLRs 3 and 4, utilize MyD88 dependent pathways exclusively, recruiting solely MyD88 and not TRIF. Moreover, TLR3 utilizes the MyD88-independent pathway, recruiting TRIF and not MyD88, while TLR4 utilizes both the MyD88-dependent and independent pathways. Nonetheless both pathways are similar in that they lead to the downstream activation of mitogen-activated protein (MAP) kinases and nuclear factor (NF)-κB (Kawai and Akira, 2010). NF-κB is a transcription factor, which when activated, translocates into the nucleus promoting transcription of pro-inflammatory genes, including IL-1β, IL-6 and tumor necrosis factor (TNF)-α, in addition to the up-regulation of co-stimulatory molecules and the maturation of antigen presentation cells (Akira and Hoshino, 2003).

In chickens, the MyD88 and TRIF genes have both been identified and cloned by our group (Wheaton et al., 2007). Similar to mammals, it appears that many of the chicken TLRs signal through the MyD88-dependent pathway (Keestra and van Putten,
Although it has not yet been demonstrated whether chicken TLR3 signals through the TRIF pathway, there is some debate as to whether TLR4 signals through both pathways. A study by Keestra and colleagues (2008) suggested that MyD88-independent signaling does not occur with chicken TLR4, as demonstrated by the lack of type I interferon up-regulation in response to LPS. In contrast, it has recently been proposed that TLR4 may in fact signal through the TRIF pathway, as chicken macrophages produce type I IFNs in response to LPS (Lian et al., 2012). Taken together, early evidence suggests that both mammalian and chicken TLRs utilize many of the same signaling pathways, however more studies are needed to determine the extent of the utilization of the TRIF pathway in chickens.

As all of the studies that are described in the present thesis involved the use of ligands for TLRs 3, 4 and 21, the immunomodulatory properties of only these TLRs in chickens will be discussed in detail.

**Chicken TLR3**

Transcripts for chicken TLR3 have been detected in several tissues, such as the spleen and lungs, as well as in several cell subsets including heterophils, B cells and T cells (Iqbal et al., 2005). As mentioned earlier, the PAMP associated with TLR3 is double-stranded RNA, and the most well studied TLR3 ligand used in mammals is the synthetic double-stranded RNA, polyinodinic:polycytidylic acid (Poly I:C). Indeed, it appears that poly I:C is also stimulatory for chicken TLR3 as well (Schwarz et al., 2007), and its immunomodulatory effects have been documented in previous studies. In heterophils for example, treatment with poly I:C significantly increased degranulation,
while down-regulating the production of the pro-inflammatory cytokines IL-1β, IL-6 and IL-8 (Kogut et al., 2005b). Similarly, chicken macrophages and fibroblasts treated with poly I:C up-regulated TLR3 transcripts as well as transcripts for IFN-α (Karpala et al., 2008). The study by Karpala and colleagues (2008) also found that using RNA interference targeting TLR3 in chicken fibroblasts reduced the amount of IFN-α produced in response to poly I:C. As such, it is evident that poly I:C is recognized by chicken TLR3 and mediates cellular activation and the production of cytokines. It should be noted that poly I:C is not the only immunostimulatory synthetic double-stranded RNA in chickens. Studies in our lab have compared the efficacy of the dsRNA ligands β-Galactosidase 728, poly U:G, SARS coronavirus-targeting dsRNA molecule, poly I:C and the dsRNA molecule targeting enhanced green fluorescent protein, in up-regulating TLR3 transcripts as well as certain cytokines, such as the type I IFNs, in chicken splenocytes (Villanueva et al., 2011). All of these molecules were indeed determined to be immunostimulatory, with poly I:C inducing the most robust and rapid cytokine response. In contrast, the responses to poly U:G were lower compared to responses to poly I:C, however the duration of this response was more prolonged when compared to that of poly I:C-induced responses.

In addition to in vitro studies, our group has also demonstrated that poly I:C is immunostimulatory for chickens in vivo (Parvizi et al., 2012). In a recent study, poly I:C was administered through three different routes: aerosol, intra-airsac and intramuscular (i.m.), and subsequently gene expression in both the spleen and lung was examined. It was found that poly I:C administered through the i.m. route induced the greatest up-regulation of IFN-α and IFN-β in both the spleen and lungs (Parvizi et al. 2012). Taken
together, it is clear that poly I:C is indeed immunostimulatory for chickens, and responses to poly I:C are mediated in part by chicken TLR3.

**Chicken TLR4**

The chicken TLR4 gene was sequenced in 2003 by Leveque and colleagues, where they also noted that allelic variation in TLR4 was linked to susceptibility to *Salmonella enterica* serovar Typhimurium. It was later confirmed that similar to mammals, LPS is the ligand for chicken TLR4 and binding of LPS in chickens is mediated by the formation of the TLR4/myeloid differentiation protein-2 (MD-2) complex, as well as by the accessory proteins lipopolysaccharide binding protein and CD14 (Kogut et al., 2005a; Keestra and van Putten, 2008). Transcripts for TLR4 have been detected in numerous cell subsets, such as in macrophages, heterophils, thrombocytes and B cells, as well as in tissues including the spleen and bursa of Fabricius (Iqbal et al., 2005), where they help to mediate host-responses to several pathogens including species of *Salmonella* (Leveque et al., 2003; Abasht et al., 2008; Chaussé et al., 2011) and *Clostridium perfringens* (Lu et al., 2009).

The *in vitro* responses to LPS have been characterized in several immune system cell subsets in chickens. For example, our group has shown that chicken B cells respond to LPS by up-regulating several immune system genes including TLR4 transcripts and CD164 (Sarson et al., 2007). Furthermore, thrombocytes up-regulate several transcripts associated with pro-inflammatory responses including the cytokines IL-1β and IL-6, as well as increasing the production of cyclooxygenase-2 and prostaglandin E2 in response to LPS (Ferdous et al., 2008; Scott and Owens, 2008). In addition, LPS treatment has
been shown to induce the production of nitrite in avian macrophages (He, Genovese, et al., 2011) and promote the respiratory burst and degranulation of heterophils (Kogut et al., 2005).

Although well studied in vitro, only a few studies have characterized the immunomodulatory effects of LPS in vivo. Previously, it has been shown that LPS administered intravenously increases the concentration of pro-inflammatory cytokines in the serum at 1 hour post-treatment, in addition to increasing the numbers of circulating leukocytes, such as heterophils (Boever et al., 2009). In addition, intravenous administration of LPS has been shown to induce the up-regulation of some immune system genes in the spleen, namely IFN-γ, IL-6, IL-8 and IL-15, at 2 hours post-treatment (Sijben et al., 2003).

**Chicken TLR21**

Chicken TLR21 is a unique TLR that functions similar to mammalian TLR9, as they both mediate responses to CpG oligodeoxynucleotides (ODNs) (Keestra et al., 2010). Similar to mammals, it has been shown in chickens that certain sequences of the ODNs are more immunostimulatory than others. Depending on the sequence of the ODN, it may be grouped into one of 3 classes, A, B or C (Vollmer et al., 2004). Class A ODNs are highly immunostimulatory to plasmacytoid dendritic cells (pDC) and thereby induce the production of IFN-α (Krug et al., 2001). In contrast, class B ODNs weakly induce IFN-α production, but are highly immunostimulatory for B cells, thereby resulting in enhanced IFN-γ production (Krug et al., 2001). Lastly, class C ODNs are a mixture of Class A and B motifs, thereby moderately stimulating both pDCs and B cells. In
chickens, the vast majority of previous studies have used the class B CpG ODN 2007, as it has been shown to be one of the most immunostimulatory ODN sequences for chicken TLR21 (He et al., 2003; Brownlie et al., 2009).

Transcripts for TLR21 have been detected in several immune system cell subsets and tissues including B cells, macrophages and heterophils, as well as in the spleen and bursa of Fabricius (Brownlie et al., 2009; Wattrang, 2009; Keestra et al., 2010). Responses mediated by TLR21 include cellular activation and proliferation of chicken B cells (Wattrang, 2009) and the production of IL-6 in splenocytes (Jenkins et al., 2009). Furthermore, CpG ODNs up-regulate pro-inflammatory cytokines in chicken macrophages and enhance their nitric oxide production (He et al., 2003; Brownlie et al., 2009). In an in vivo study by Patel and colleagues (2008), it was revealed that after treatment with CpG, some immune system genes, including IFN-γ and IL-10, were up-regulated in both the spleen and bursa of Fabricius.

Responses to TLR21 ligands may be synergistically enhanced when administered with ligands for TLR3. This has been demonstrated in chicken monocytes that were stimulated with CpG ODN and poly I:C, as they demonstrated a significant increase in effector functions as well as a 100 to 1000-fold greater up-regulation of IFN-γ transcripts when compared to treatment with either ligand alone (He et al., 2007a, 2012).
Exploiting TLR ligands to enhance host-resistances to pathogens

Owing in part to their immunostimulatory properties, TLR ligands have been employed prophylactically as stand-alone agents to protect against several pathogens in mammals. In humans for example, ligands for TLRs 3, 7 and 9 are the subject of numerous clinical trials aimed at protecting against viral infections such as treating hepatitis C virus, genital herpes and human papilloma viruses (Kanzler et al., 2007). In addition to humans, a similar phenomenon has also been observed in mice. It was found that the prophylactic administration of TLR ligands may also confer immunity to several types of pathogens, such as influenza virus, as several studies have shown that poly I:C, LPS and CpG ODN are all effective in protecting mice against lethal influenza virus challenges (Wong et al., 1995, 2009; Shinya et al., 2012). From this, it is clear that the prophylactic administration of TLR ligands has the potential to protect mammals against a variety of pathogens,

Based on their success in mammals, several studies over the past decade have been aimed at investigating whether this phenomenon may be extended to chickens. Indeed, it appears that this is the case, as it has been shown that TLR ligands can protect chickens from bacterial, viral or parasitic organisms as outlined in Table 2. For the most part, the majority of TLR ligands used in this context are those which bind to TLRs 3, 7 or 21. As all three of these TLRs recognize viral PAMPs, these TLRs modulate the immune system towards an anti-viral state mediated in part through the production of a robust type I or type II interferon response.

Type I interferons play an important role in mediating protection against many viral pathogens, such as avian influenza virus (AIV). In addition to up-regulating the
interferon inducible genes (ex. 2’-5’ oligoadenylate synthetase), these cytokines inhibit viral transcription and translation, promote the apoptosis of infected cells (Samuel, 2001). In this regard, ligands that induce a robust IFN-α and IFN-β response make them an attractive strategy for enhancing immunity against AIV. Although signaling through either the MyD88 or TRIF pathways culminate in type I IFN production, a greater magnitude of type I IFN responses are typically associated with the TRIF pathway. To this end, TLR3 ligands have been used in vitro and in vivo to protect chickens and chicken cell populations against both low and high pathogenic AIV. For example, it was demonstrated by Stewart et al. (2011) that incorporating a 5’-UGUGU-3’ motif into small interfering RNAs (siRNA) inhibited the replication of highly pathogenic AIV H5N1 in chicken macrophages. In this scenario, it is likely that the protection conferred by TLR3 ligands was mediated through two mechanisms: 1) the direct activation of macrophages, thereby enhancing their effector functions such as phagocytic capabilities and the production of nitric oxide, and 2) the production of type I IFN. In fact, type I interferons, especially IFN-α, are particularly efficacious in enhancing host immunity to AIV, as IFN-α acts to contain the spread of the virus through pre-activating uninfected cells, thereby reducing their susceptibility to infection (Jiang et al., 2011; Meng et al., 2011).

As mentioned previously, the induction of a robust type I IFN response is not limited to only those ligands that signal through the TRIF pathway. In particular, TLR7 ligands have been shown to induce the up-regulation of these cytokines in chicken splenocytes (Stewart et al., 2011a). To this end, it was demonstrated that stimulating chicken macrophages in vitro with the TLR7 ligand loxoribine significantly inhibited PR8 influenza virus replication and reduced virus titre in the supernatant (Stewart et al.,
Moreover, it was found that injecting loxoribine into embyonated chicken eggs 24 hours prior to infection with PR8 significantly reduced the amount of virus found in the allantoic fluid. As such, it is clear that IFN-α, and the TLR ligands that induce its up-regulation, present themselves as an attractive prophylactic strategy against viral infections. However, as the studies done so far in chickens involving AIV were conducted in vitro, it remains to be determined whether similar protective effects occur in vivo.

It is not only type I interferons that demonstrate anti-viral properties, cytokines associated with Th1 responses, such as IFN-γ, may also induce an anti-viral state. This is mediated in part through the activation of NK cells and CD8+ T cells. Together, these cell subsets exert their anti-viral effects partly through the targeted release of cytotoxic granules towards infected cells, thereby promoting their apoptosis. Moreover, IFN-γ may also up-regulate the interferon inducible genes, thereby protecting uninfected cells. In this regard, CpG ODN, which induces a Th1-like response in chickens (Patel et al., 2008), has been shown to be efficacious in protecting against several viral pathogens, such as infectious bronchitis virus (IBV) (Dar et al., 2009b). Indeed, as shown by Dar and colleagues (2009), a reduction in IBV load was correlated with an up-regulation of IFN-γ and the interferon inducible gene 2’-5’ oligoadenylate synthetase (OAS), which initiates the RNase L pathway and promotes the cleavage of viral RNA.

It is also important to note that the efficacy of CpG ODN prophylaxis is not just limited to viruses, as it may also confer immunity against bacterial and parasitic pathogens. A study by Dalloul et al. (2004) demonstrated that CpG ODN administered intravenously decreases the amount of oocyst shedding in chickens infected with the
parasite *Eimeria*, while at the same time improving weight gain in chickens. Moreover, several studies have focused on the protective effects of CpG ODN against bacterial pathogens such as *Escherichia coli* in chickens (Gomis et al., 2003, 2004). This is despite the fact that *E. coli* is not an intracellular pathogen and therefore the activation of CD8+ T cells and the induction of many of the interferon inducible genes are not as relevant in this context. This suggests that other TLR21 mediated mechanisms may be involved, such as the direct activation of monocytes and heterophils, thereby increasing their phagocytic uptake of bacteria. Another possibility is through the direct activation of B cells, as CpG ODN is highly immunostimulatory for chicken B cells and enhances their effector functions including cytokine production and the up-regulation of both major histocompatibility complex (MHC) class II and the relevant co-stimulatory molecules (Wattring, 2009; St. Paul et al., unpublished results). As such, it is clear that CpG ODN has the potential to confer immunity against a broad spectrum of pathogens.
<table>
<thead>
<tr>
<th>TLR</th>
<th>Agonist</th>
<th>Pathogen</th>
<th>Outcome</th>
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<tr>
<td></td>
<td>5'-UGUGU-3'</td>
<td>AIV (H5N1)</td>
<td>Reduced virus replication in vitro</td>
<td>(Stewart et al., 2011b)</td>
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<td>Poly I:C</td>
<td>Avian reovirus</td>
<td>Reduced plaque formation in infected CEFs</td>
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<td>Poly I:C</td>
<td>Newcastle disease virus</td>
<td>Reduced plaque formation in infected CEFs</td>
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<td>Flagellin</td>
<td><em>Salmonella enteritidis</em></td>
<td>Reduced mortality</td>
<td>(Genovese et al., 2007)</td>
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<td>TLR7</td>
<td>Loxoribine</td>
<td>PR8 influenza virus (H1N1)</td>
<td>Reduced virus replication in vitro and in ovo</td>
<td>(Stewart et al., 2011a)</td>
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<td></td>
<td>CpG ODN</td>
<td><em>Eimeria acervulina</em></td>
<td>Decreased oocyst shedding</td>
<td>(Dalloul et al., 2004)</td>
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<tr>
<td>TLR21</td>
<td>CpG ODN</td>
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<td>Reduced virus replication in chick embryos</td>
<td>(Dar et al., 2009b)</td>
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<td></td>
<td>CpG ODN</td>
<td><em>Escherichia coli</em></td>
<td>Reduced mortality</td>
<td>(Gomis et al., 2004; Taghavi et al., 2009)</td>
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<td></td>
<td>CpG ODN</td>
<td><em>Salmonella</em> spp.</td>
<td>Reduced mortality or bacterial load</td>
<td>(He et al., 2005; Taghavi et al., 2008; Mackinnon et al., 2009)</td>
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**TLR Ligands demonstrate adjuvant potential**

The protective benefits of TLR ligands are not limited to prophylactic administration prior to a microbial challenge, as many ligands have shown promise as vaccine adjuvants to enhance antibody production and T cell responses. In this regard, CpG ODN has been the most widely used TLR ligand in chickens to enhance the immunogenicity of vaccines against several pathogens including AIV. Our group has shown that CpG ODN demonstrates superior adjuvant potential compared to recombinant
IFN-γ or diluent, in eliciting antibody-mediated and cell-mediated responses to avian influenza virosomes (Mallick et al., 2011). A similar finding has been extended towards vaccines against the highly pathogenic H5N1, as Wang and colleagues (2009) found that adding CpG ODN to the oil and water emulsion significantly enhanced antibody responses and hemagglutination inhibition (HI) antibody titres, while increasing the amount of serum IFN-γ. Although the exact immunological basis behind CpG ODN’s superior adjuvant potential has not been well described in chickens, it is hypothesized that this may be a result of 1) a robust IFN-γ response, which acts to mature antigen presenting cells and enhance their antigen presenting capabilities (Patel et al., 2008) and 2) the direct activation of B cells, thereby promoting the up-regulating of co-stimulatory molecules, MHC II and cytokine transcripts (St. Paul et al., submitted).

In addition to CpG ODN, a few of the other TLR ligands have been shown to possess adjuvant potential in chickens, such as poly I:C, Pam3CSK4, LPS and flagellin (Erhard et al., 2000, Chaung et al., 2011, Parvizi et al., submitted). In mice, it has been well established that the TLR5 ligand flagellin enhances mucosal immune responses and this property has been exploited to enhance the immunogenicity of vaccines against pathogens which enter through mucosal surfaces, such as influenza virus (Wang et al., 2010). A similar phenomenon has recently been demonstrated in chickens, as Chaung et al. (2011) discovered that an adjuvant consisting of flagellin mixed with aluminum gel significantly enhanced secretory IgA antibodies following intranasal vaccination with an inactivated H5N2 AIV. The increase in secretory IgA may be attributed in part to the T_{H}2-like response observed in chicken splenocytes following treatment with flagellin (St. Paul et al., submitted).
Not all TLR ligands can serve as an adjuvant to enhance antibody responses, as it has been shown that LPS may in fact decrease antibody responses. In a study by Parmentier et al. (2004) administration of lipoteichoic acids (LTA) 24 hours prior to immunization with keyhole limpet hemocyanin (KLH) significantly augmented anti-KLH antibodies. In contrast, pre-treatment with LPS significantly down-regulated the antibody responses to KLH when compared to PBS-treated control birds. A similar phenomenon was observed following an intratracheal administration of Newcastle disease virus (NDV) co-encapsulated in a liposome with LPS, as liposomes without LPS induced significantly higher antibody titres and increased survival rates after a single immunization (Tseng et al., 2009). In contrast, Parmentier et al. (2008) found that antibody responses were significantly increased following an intratracheal immunization of chickens with HSA along with LPS as an adjuvant. The mechanisms behind this discrepancy is not known at this time, and it may be related to the source of LPS, the dose and the route of administration, as all three of these factors may in fact influence the expression of immune system genes in chicken lymphoid tissues (Pulendran et al., 2001; Parvizi et al., 2012). Taken together, these studies demonstrate the potential for LPS to both augment or decrease antibody responses, and future studies may be aimed at optimizing LPS formulations as adjuvant.

Having a proven track record of enhancing immune response to avian influenza vaccines, as well as enhancing immunity to avian influenza virus \textit{in vitro}, administration of TLR ligands prophylactically may serve as a novel approach to protect chickens against AIV \textit{in vivo}. 
Influenza Virus

Influenza virus is an enveloped, negative-sense single-stranded RNA virus belonging to the family Orthomyxoviridae. There are three types of influenza viruses, each classified to a corresponding genus of the same name: Type A, Type B and Type C (Suarez and Schultz-Cherry, 2000). Unlike types B and C, which are confined mostly to humans, influenza A viruses have the largest host range, infecting a variety of animal species including swine, humans, poultry and waterfowl (Suarez and Schultz-Cherry, 2000). Influenza A viruses are further classified into subtypes according to the presence of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA and 9 NA subtypes have been identified (Rappuoli and Del Giudice, 2008).

The influenza genome is composed of 8 segments that encode 11 viral genes (Samji, 2009). The glycoproteins HA and NA, are found on the surface of the virion, while the matrix protein 2 (M2) is a proton channel integrated into the viral envelope, which is composed of the host’s plasma membrane. Approximately 80% of the surface proteins are HA, with 17% being NA and the remainder being M2 (Samji, 2009). The internal proteins of the virus consist of the polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase basic protein 1-F2 (PB1-F2), the nucleoprotein (NP), the matrix protein 1 (M1) and the non-structural protein 2 (NS2). The viral genome also encodes the non-structural protein 1 (NS1), which is produced in large quantities but not packaged into the virion.

Viral Replication Cycle
The replication cycle of influenza virus begins with the HA of the virion binding to sialic acid expressed on the surface of host cells. In humans, cells in the respiratory tract contain α(2,6) linkages between the sialic acid and the carbohydrate galactose, while in avian species, cells contain α(2,3) linkages (Rappuoli and Del Giudice, 2008). The determining factor for host and cell tropism of an influenza virus strain depends on the sialic acid specificity of the HA expressed. For example, avian influenza viruses have a preference for α(2,3) linkages, while human viruses have an affinity for α(2,6) linkages. Interestingly, porcine cells express both α(2,3) and α(2,6) linkages, making them ideal 'mixing pots' for virus recombination (Rappuoli and Del Giudice, 2008). Nevertheless, following the binding of HA to sialic acid, the virus is endocytosed via receptor-mediated endocytosis by the host cell, enclosing the virus in an endosome. The low pH of the endosome (pH 5-6) promotes a conformational change in the HA exposing HA1 and HA2, the latter of which mediates fusion between the host membrane and viral envelope. In addition, the low pH also allows for passage of ions into the virion via M2, thereby acidifying the viral core and promoting the release of the viral ribonucleoproteins (vRNPs) into the cytoplasm of the host cell (Samji, 2009).

The vRNPs is composed of the viral RNA in conjunction with NP and the polymerase proteins PB1, PB2 and PA (Samji, 2009). Together, these proteins provide the vRNP with nuclear localization signals and facilitate its entry into the nucleus. In the nucleus, the viral RNA is used as a template to generate positive sense RNA, which is mediated by the viral polymerases. Some positive sense viral RNA strands are polyadenylated and serve as a template for the translation of viral proteins, while other full-length positive sense viral RNA strands are not polyadenylated and act as a template.
for the replication of new viral RNA strands to be packaged into newly forming virions. The viral protein M1 then mediates virion assembly, and the progeny virions exit the cell by budding from the host cell plasma membrane (Samji, 2009). To prevent re-agglutination of the progeny virus to the host cell, the NA on the progeny virus subsequently cleaves off sialic acid from the host cell.

**Viral Pathogenesis**

Based on their pathogenesis, avian influenza viruses can be classified into one of two types, highly pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI). While the majority of subtypes are classified as LPAI, a few strains belonging to either the H5 or H7 subtypes are considered to be highly pathogenic, such as H5N1. The major difference between LPAI and HPAI is the ability of the virus to replicate locally or systemically, respectively (Lee and Suarez, 2005). This is achieved largely by mutations in HPAI viruses allowing the HA to be cleaved by a wider range of proteases found throughout the host tissues. Furthermore, these proteases may be expressed in the cell cytoplasm therefore cleaving HA of virus progeny before they are released from the cell, resulting in an increase in their cell tropism (Suarez, 2010). As such, following a HPAI challenge, infectious virus can be detected in a variety of tissues including in the brain, heart and blood vessels (Brown et al., 1992), while LPAI tend to remain confined to the mucosal surfaces including the lungs and the gastrointestinal tract (Slemons and Swayne, 2010). The clinical signs associated with LPAI infection in chickens are mild and often remain undetected, and include ruffled feathers as well as a drop in egg production (Reynolds, 2006). However, infection with HPAI strains results in a rapid spread within
and between flocks, leading to severe respiratory and neurological signs, multi-organ failure and 90-100% mortality (Chmielewski and Swayne, 2011). Therefore, in order for an avian influenza virus subtype to be officially classified as HPAI according to the United States Department of Agriculture, the virus must be lethal to 75% of the infected birds upon intravenous administration of the virus.

**Host Responses to Influenza Virus in Mammals**

TLRs and other PRRs play an important role in mediating host-responses to influenza virus. Following infection of murine lung epithelial cells, influenza virus is recognized by the cytosolic PRR retinoic acid inducible gene (RIG)-1, which binds RNA containing 5’ triphosphate ends (Le Goffic et al., 2007). Furthermore, it has been hypothesized that epithelial TLR3 recognizes the dsRNA intermediary occurring during viral replication, however the extent of contributions made through TLR3 stimulation to host-responses against influenza virus is unclear and is subject to debate (Guillot et al., 2005; Le Goffic et al., 2007). Nevertheless, TLR7 is thought to be the main TLR responsible for initiating host-responses to influenza virus through binding its ssRNA (Ehrhardt et al., 2010). As TLR7 is located intracellularly in macrophages and dendritic cells, this TLR encounters its ligand upon endocytosis of whole influenza virions or of virally infected dead epithelial cells. Here, TLR7 stimulation facilitates the maturation of dendritic cells, which subsequently migrate to the draining lymph node to present virus antigens to T cells (Dzupalic et al., 2010). In addition, macrophages, along with infected epithelial cells, produce certain cytokines and chemokines, including the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, as well as the anti-viral type-1 IFNs (IFN-
α/β), which serve to recruit and activate other leukocytes in addition to controlling the early spread of the virus through activating epithelial cells as well as promoting the apoptosis of infected epithelial cells (Peiris et al., 2010).

Within 48 h following infection, natural killer cells and neutrophils are recruited into the lungs to control early viral replication (Rappuoli and Del Giudice, 2008). Here, NK cells play an important role in controlling the virus during the early stages of infection through the production of IFN-γ and the lysis of virally infected cells. As such, it is not surprising that mice depleted of NK cells have an increased virus load following an influenza challenge, when compared to mice that have normal NK cell numbers and functions (Nogusa et al., 2008). In addition to NK cells, neutrophils also contribute to the containment of the virus during the early stages of infection. This is achieved in part through the phagocytosis of virions as well as producing anti-microbial peptides that contribute to the early host defenses against influenza virus (Gaudreault and Gosselin, 2008). Neutrophils also produce cytokines in response to influenza virus (Wang et al., 2008) and help sustain subsequent CD8+ T cell responses (Tate et al., 2011). Furthermore, mice depleted of neutrophils have a higher titre of influenza virus in the lung and demonstrate signs of an increased severity of disease (Tate et al., 2008).

T cells activated in the draining lymph node by dendritic cells play an important role in viral clearance. Activated CD4+ T cells are able to provide the necessary help for antigen-specific B cells through TCR stimulation and the production of cytokines and CD40L, providing the signals required to differentiate B cells into plasma cells (Thomas et al., 2006). Antibodies secreted from these plasma cells are generally targeted toward the HA and NA surface glycoproteins and are important for immunity to infection, as
well as neutralizing extracellular virions to help contain the spread (Rappuoli and Del Giudice, 2008). In addition to antibody-mediated responses, CD8+ cytotoxic T lymphocytes also contribute to containment and clearance of the virus, where they arrive in the lungs on days 5-7 post-infection and kill virus-infected cells. To this end, it has been shown that adoptive transfer of CD8+ T cells from mice vaccinated with influenza virus into naive mice may confer immunity to these naïve mice when challenged with the same strain of virus (Ulmer et al., 1998).

During the course of the immune response a wide array of cytokines are produced, with one group of essential cytokines being the type I interferons. Therefore, to antagonize the host IFN response, influenza viruses produce NS1 protein to prevent its viral RNA from binding to intracellular sensors, among other functions (Kochs et al., 2007). Moreover, NS1 aids in the pathogenesis of influenza viruses through the inhibition of the interferon inducible genes, which hinder viral replication and promote the early containment of the virus (Min and Krug, 2006). The importance of NS1 in the pathogenesis of highly pathogenic AIV is evident through its identification as a key virulence factor of H5N1 HPAI, thereby further supporting the significance of type I IFNs in host immunity to influenza viruses (Li et al., 2006). However, even though type I IFNs and other cytokines in the milieu work to protect the host, deleterious responses can occur following infection with highly pathogenic strains of influenza, in what is described as a ‘cytokine storm’. The cytokine storm leads to prolonged and extensive inflammation and recruitment of immune cells, in addition to edema, leading to respiratory distress and potential death of the host (Rappuoli and Del Giudice, 2008).
Immune responses to AIV in chickens

There is a wealth of knowledge about mechanisms of immunity against influenza viruses in mammals. However, little is known about these mechanisms in chickens. Moreover, the importance of the contributions made by certain chicken cell subsets, PRRs and cytokines has not been thoroughly investigated, partly due to the lack of reagents. However, some aspects of the host-response towards AIV in chickens have been elucidated, and for the most part are similar to what occurs in mammals.

The initial site of virus replication in chickens for both LPAI and HPAI viruses is in the nasal cavity and lungs. Here, both types of viruses promote necrosis of the parabronchial epithelium, edema and fibrin exudation in airways and air capillaries, with the pathological lesions induced by HPAI infections being more severe (Degen et al., 2006; Pantin-Jackwood and Swayne, 2009). During this time, both types of AIV are detected by host pattern recognition receptors, namely TLRs and melanoma differentiation-associated gene 5 (Mda5), but not RIG-I, as this PRR has not been identified in chickens (Karpala et al., 2011b). Nevertheless, stimulation of PRRs initiates the host immune response, mediated in part through the production of chemokines and cytokines (Karpala et al., 2011b; Liniger et al., 2012). These cytokines and chemokines serve to recruit and promote the influx of the early innate responders into the lungs, such as macrophages and monocytes (Rebel et al., 2011). It is thought that these cell subsets phagocytose virus-infected cells and process viral antigens for presentation to T cells, as well as produce cytokines to contain the spread of the virus. These cytokines include type I interferons, which act to up-regulate the interferon inducible genes, among other functions. The importance of these interferon inducible genes was observed in chickens
that had a variant of the myxomavirus-resistance (Mx) gene. The protein encoded by the Mx gene in these chickens did not demonstrate anti-viral activities, and as a result, these chickens had a higher virus load and mortality upon AIV infection, when compared to those with the functional protein (Ewald et al., 2011). In addition to Mx, it was determined through microarray analysis that another major interferon inducible gene, 2’-5’ OAS, correlates with immunity to AIV in the lungs (Uchida et al., 2012). As such, it is not surprising that IFN-α pre-treatment may protect chickens from AIV, again mediated in part through the up-regulation of both Mx and 2’-5’ OAS (Jiang et al., 2011; Meng et al., 2011).

In addition to type I IFNs, other cytokines and immune system genes are up-regulated in the lungs of AIV-infected chickens. These include the pro-inflammatory cytokines such as IL-6 as well as inducible nitric oxide synthase, which may both be attributed to the influx of activated macrophages (Degen et al., 2006; Burggraaf et al., 2011). Importantly, cytokines are produced in the lung that may bias the immune response towards a Th1-phenotype, as demonstrated by the up-regulation of both IFN-γ and IL-12, and not IL-4 (Karpala et al., 2011a). As a result, this leads to a robust CD8+ T cell response and an influx of CD8α+ into the lungs of infected chickens (Rebel et al., 2011). In conjunction with CTLs, anti-influenza antibody responses are detected at 1 week post-challenge, with antibody-secreting cells found in the lungs, spleen and bone marrow (De Geus et al., 2011). Importantly, it appears that the CTL response is specific against highly conserved epitopes spread across several influenza strains, raising the possibility that the CTLs may be cross-protective (Seo and Webster, 2001; Haghighi et al., 2009). Indeed, this is the case, as it was found that the adoptive transfer of H9N2
specific CTLs into naïve chickens protected them from a lethal challenge with H5N1 (Seo and Webster, 2001). Similarly, it was found that chickens depleted of H9N2 specific CD8+ T cells, but not CD4+ T cells, died as a result of lethal challenge with HPAI H5N1 (Seo et al., 2002). As such, it is clear that both antibody-mediated and cell-mediated responses promote immunity against the virus.

Following the initial replication in the respiratory tract, both LPAI and HPAI viruses spread to other organs. In the case of LPAI, this spread is limited to the gastrointestinal tract. Here the host responds through up-regulating transcripts for TLRs and cytokines such as IFN-β and IFN-γ, but not IFN-α (Nang et al., 2011). In contrast, HPAI disseminate from the respiratory tract to the intestines as well as to several other organs including the brain, kidneys, pancreas and the heart. As a result, the host-responses to HPAIV occur in these tissues as well. For example, in the brain, a significant up-regulation of heat shock proteins and chemokines such as IL-8, were observed, likely serving to induce a pro-inflammatory response and promote the recruitment of leukocytes (Balasubramaniam et al., 2012). However, the pro-inflammatory response occurring in the brain and other organs results in immune-mediated pathological reactions, thereby exacerbating the signs of disease. As a result, necrosis and apoptosis of cells in the brain, as well as in the other organs lead to multi-organ failure, haemorrhage, edema and thrombosis and finally death (Pantin-Jackwood and Swayne, 2009).

**Vaccination**

Poultry in regions where H5N1 is endemic are being vaccinated as a part of the strategy to contain and limit the spread of the virus. The desired role of an ideal vaccine
is to prevent or reduce infection and disease, to reduce the amount of virus being shed, to allow easy differentiation between infected and vaccinated chickens, and to raise the minimum threshold of exposure required for infection (Lee and Suarez, 2005). Currently, two types of vaccines are being used commercially: inactivated whole virion oil-in-water emulsion vaccines, and a fowlpox recombinant vaccine (Capua and Marangon, 2006). Conventional inactivated vaccines typically induce a predominant antibody-mediated response with antibodies specific towards surface HA and NA, while recombinant fowlpox vaccine induces both antibody-mediated and cell-mediated immune response (Lee and Suarez, 2005; Haghihghi et al., 2010). However, despite eliciting antibodies and T cells specific against HA and NA, current vaccines do not prevent infection and thus do not completely eliminate shedding of the virus into the environment (Swayne, 2009).

To address the limited efficacy in reducing virus shedding, new types of vaccine platforms are currently being developed, with one approach being influenza virosomes. Influenza virosomes are reconstituted virus particles that are devoid of nucleic acids and some of the viral protein, although they contain surface HA and NA. As a result, these virosomes may bind to, and fuse with, the host cell membrane. Indeed, vaccination with virosomes derived from the LPAI H4N6 has been shown to induce robust antibody-mediated and cell-mediated immune responses against H4N6 virus, thereby reducing the viral load in experimentally infected chickens (Mallick et al., 2011, 2012). However, it remains to be elucidated if the virosome vaccine platform may protect chickens against HPAIV.

In addition to virosomes, another promising AI vaccine platform is DNA vaccines. Previously, it has been shown that DNA vaccines may induce neutralizing
antibodies against highly pathogenic H5N1 and LPAI H5N2 in chickens, and subsequently protect them from mortality and signs of disease (Kodihalli et al., 1997; Jiang et al., 2007b; Rao et al., 2008). In addition, it was found that the antibody response elicited by DNA vaccines may be broadly neutralizing against heterologous subtypes as determined by a virus neutralization assay (Rao et al., 2008). However, one potential drawback to DNA vaccines is the requirement of a gene gun in order to induce a more robust immune response when compared to i.m. injection as determined by Rao and colleagues (2008), and thus may serve as potential barrier for their widespread adoption.

In summary, it is clear that AIV vaccines may limit the spread of the virus and protect chickens from mortality. However, existing commercial vaccines do not eliminate virus shedding into the environment from infected birds. Although potentially promising new vaccine platforms are under development, it remains to be determined how efficacious they are outside of an experimental setting. As such, it is clear that novel approaches should be identified to reduce the virus load and shedding in chickens infected with AIV, with one potential approach being the prophylactic use of TLR agonists.
STATEMENT OF RATIONALE

It is evident that Toll-like receptor ligands modulate several aspects of the chicken immune system by enhancing cell effector functions and inducing the production of cytokines. These cytokines include the pro-inflammatory IL-1β and IL-8, and more importantly, those associated with antiviral responses, namely type I IFNs. However, although the TLR-mediated responses have been well studied in vitro, few studies have examined how TLR ligands may modulate the chicken immune system in vivo.

Secondly, the prophylactic administration of TLR ligands in chickens has been shown to enhance immunity to several types of pathogens, including E. coli. Moreover, TLR ligands have been shown to inhibit avian influenza virus replication and reduce virus load in vitro and in ovo. However, it remains to be elucidated as to whether a similar phenomenon occurs in vivo,
STATEMENT OF HYPOTHESIS

In the studies described in the present thesis, it was hypothesized that *in vivo* administration of TLR ligands influences the expression of immune system genes in the spleen. These genes likely include those associated with pro-inflammatory responses, antigen presentation as well as anti-viral activity. Secondly, it was hypothesized that when administered prophylactically, prior to challenge with a low pathogenicity avian influenza virus, TLR ligands confer immunity to the host and significantly reduce virus shedding. It was also hypothesized that TLR ligands elicit immunity against AIV that is mediated by cytokines associated with anti-viral and T\textsubscript{H}1 responses, such as the type I IFNs and IFN-\textgamma.
EXPERIMENTAL APPROACH

Objective 1:
To elucidate the mechanisms through which TLR4 and TLR21 stimulation modulate the chicken immune system in vivo.

Key steps involved

- Administered both a low and high dose of the TLR4 ligand LPS and the TLR21 ligand CpG ODN intramuscularly. Control groups received non-CpG ODN or PBS.
- Spleen tissue was collected at 2, 6, 12 and 24 hours post-treatment from 6 chickens in each group for RNA extraction and cDNA synthesis.
- The relative expression of several immune system genes was quantified using real-time PCR

Objective 2:
To identify a TLR agonist that enhances host-immunity to avian influenza virus when administered prophylactically.

Key steps involved

- Administered both a low and high dose of the TLR3 ligand poly I:C, the TLR4 ligand LPS, and the TLR21 ligand CpG ODN intramuscularly. One control group received non-CpG ODN while the other two received PBS.
- Twenty-four hours post-injection, chickens were infected with the low-pathogenic AIV H4N6. One of the PBS-treated control groups remained un-infected.
- Oropharyngeal and cloacal swabs were collected on days 4 and 7 post-infection.
• Spleen and lung tissue were collected on days 2, 4 and 7 post-infection for RNA extraction and cDNA synthesis.

• Virus shedding was assessed by titrating swab fluid in MDCK cells.

• Potential correlates of immunity in the spleen and lung of TLR-ligand treated birds were identified and quantified using real-time PCR.
CHAPTER 2:

*In vivo* administration of ligands for chicken Toll-like receptors 4 and 21 induces the expression of immune system genes in the spleen

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Abstract

Toll-like receptors (TLRs) are a group of conserved proteins that play an important role in pathogen recognition in addition to the initiation and regulation of innate and adaptive immune responses. To date, several TLRs have been identified in chickens, each recognizing different ligands. TLR stimulation in chickens has been shown to play a role in host-responses to pathogens. However, the mechanisms through which TLRs modulate the chicken immune system have not been well examined. The present study was conducted to characterize the kinetics of responses to TLR4 and TLR21 stimulation in chickens following intramuscular injections of their corresponding ligands, lipopolysaccharide (LPS) and CpG oligodeoxynucleotides (ODNs), respectively. To this end, relative expression of cytokine genes in the spleen was determined at 2, 6, 12 and 24 hours after injection of TLR ligands. The results indicated that LPS strongly induced the up-regulation of some immune system genes early on in the response to treatment, including interferon (IFN)-γ, interleukin (IL)-10, and IL-1β. Furthermore, treatment with CpG ODN promoted the up-regulation of major histocompatibility complex (MHC)-II, IFN-γ and IL-10. The response to CpG ODN appeared to be somewhat delayed compared to the response to LPS. Moreover, we found a significant increase in IFN-α gene expression in response to LPS but not CpG ODNs. Future studies may be aimed to further characterize the molecular mechanisms of TLR activation in chickens or to exploit TLR agonists as vaccine adjuvants.
Introduction

Toll-like receptors (TLR) are highly conserved ligand binding proteins present in numerous species including humans, mice and chickens (Roach et al., 2005). Being a pattern recognition receptor (PRR), TLRs are responsible for the recognition of conserved motifs found on bacteria, fungi, parasites and viruses known as pathogen associated molecular patterns (PAMPS) (Medzhitov, 2001). In mammals, TLR4 recognizes lipopolysaccharide (LPS) found in the outer membrane of Gram-negative bacteria (Chow et al., 1999), whereas TLR9 recognizes CpG DNA motifs found in certain bacterial and viral nucleic acids (Akira and Takeda, 2004). Several TLRs have been identified in chickens to date, with many of them being homologous to mammalian TLRs (Fukui et al., 2001; Leveque et al., 2003; Iqbal et al., 2005; Temperley et al., 2008; Keestra et al., 2010).

Host responses elicited by interactions between TLRs and their ligands are achieved through the differential recruitment of adaptor molecules by the intracellular domain of TLRs, an example of which is myeloid differentiation factor 88 (MyD88). MyD88 recruits additional adaptor molecules, which subsequently lead to the downstream activation of mitogen-activated protein kinases and transcription factor nuclear factor (NF)-kappaB (Wesche et al., 1997). NF-kappaB induces the transcription of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α, and promotes the transcription of co-stimulatory molecules and the maturation of antigen presentation cells (APCs) (Akira and Hoshino, 2003). The MyD88-dependent pathway is used by all of the mammalian TLRs except for TLR3, which signals through the MyD88-independent pathway instead.
The chicken TLR4 is an orthologue of mammalian TLR4, thereby binding LPS following the formation of the TLR4/myeloid differentiation protein-2 (MD-2) complex (Leveque et al., 2003; Keestra and Van Putten, 2008). Previously, the expression of chicken TLR4 has been detected in many cell subsets, such as macrophages and B cells, as well as in tissues including the spleen and bursa of Fabricius (Iqbal et al., 2005). LPS treatment has been shown to induce the production of nitrite in avian macrophages (He et al., 2011a) and promote the respiratory burst and degranulation of heterophils (Kogut et al., 2005b). Moreover, LPS has previously been shown to induce the up-regulation of cytokines, such as interferon (IFN)-γ in chicken B cells (Sarson et al., 2007) and pro-inflammatory cytokines in the spleen (Sijben et al., 2003) and serum (Boever et al., 2009). Furthermore, the possible role of TLR4 in eliciting host immune responses to pathogens such as species of *Salmonella* (Leveque et al., 2003; Abasht et al., 2008; Chaussé et al., 2011) and *Clostridium perfringens* (Lu et al., 2009) has been reported.

In mammals, responses to CpG ODN are largely mediated through TLR9. However unlike TLR4, no orthologue of TLR9 has been identified in chickens to date. Despite that, early research by Vleugels and colleagues (2002) established that CpG ODN are immunostimulatory for chickens, while Gomis and colleagues (2003) identified their immunoprotective nature. Further studies identified TLR21 as the TLR that interacts with CpG ODN (Brownlie et al., 2009; Keestra et al., 2010). It has been demonstrated that TLR21 may share a similar ligand specificity to that of TLR9, as the responses to a given ODN may be influenced by the sequences flanking the CpG motif (He et al., 2003; Ciraci and Lamont, 2011). In this regard, the class B CpG ODN, which preferentially activates mammalian B cells in an IFN-α independent manner, has been shown to
promote the proliferation of chicken B cells (Wattrang, 2009), and up-regulation of cytokines such as IL-10 in the bursa of Fabricius (Patel et al., 2008) and IL-6 in splenocytes (Jenkins et al., 2009). Furthermore, prophylactic treatment of chickens with class B CpG ODN has been shown to enhance host immunity to numerous pathogens such as *Escherichia coli* (Gomis et al., 2003; Gomis et al., 2004) and avian influenza virus (St. Paul et al., in preparation), in addition to enhancing the immunogenicity of avian-vaccines when administered as an adjuvant (Ameiss et al., 2006; Mallick et al., 2011).

Although it is thought that stimulation with TLR4 and 21 agonists will promote a pro-inflammatory response and up-regulation of IFNs as previously observed *in vitro*, few studies have examined the kinetics of host responses in chickens to TLR stimulation *in vivo* (Sijben et al., 2003; Patel et al., 2008). Therefore, the present study was an attempt to understand and elucidate the mechanisms through which TLR4 and TLR21 stimulation modulates the chicken immune system. This was done by transcriptional analysis of several immune system genes that represent various aspects of host responses to TLR ligands. The results indicated that when TLRs 4 and 21 were stimulated, they induced a dose-dependent up-regulation of IFN-γ, IL-8, inducible nitric oxide synthase (iNOS), IL-10, MyD88, IL-1β and MHC-II, and in the case of TLR21, a down-regulation of IL-13.
Materials and Methods

Experimental chickens and housing

One-day-old broiler chickens \((n=144)\) were procured from Maple Leaf Hatchery (Hamburg, Ontario, Canada), and housed in the isolation facility of the Ontario Veterinary College, University of Guelph (Guelph, ON). This research was approved by the University of Guelph Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care.

TLR ligands

Lipopolysaccharide (LPS) from \(Escherichia coli\) 0111:B4 was purchased from Sigma-Aldrich-Canada (Oakville, ON), while synthetic class B CpG ODN 2007 \([5’-TCGTCGTTGTCGTTTTGTCGTT-3’]\) and non-CpG ODN \([5’-TGCTGCTTGTGCTTTTGTGCTT-3’]\) (Brownlie et al., 2009) were purchased from Eurofins MWG Operon (Ebersberg, GER). All of the ligands used were re-suspended in sterile phosphate buffered saline (PBS, pH 7.4).

Experimental design

Fourteen-day-old chickens were randomly divided into 6 groups of 24 birds each. Chickens were immunized intramuscularly (i.m) in the pectoral muscle with either a high or low dose of LPS or CpG ODNs, while two control groups received either non-CpG ODNs or PBS. The “LPS low” and “LPS high” groups received 100 μg or 500 μg of LPS, respectively, while the groups “CpG low” and “CpG high” received a 10 μg or 50 μg of CpG ODN 2007, respectively. The two control groups received 50 μg of non-CpG ODNs.
or PBS. Doses were selected based on previous experiments in chickens involving these TLR agonists (Gomis et al., 2004; Parvizi et al., manuscript in preparation). Sampling was done at 2, 6, 12 and 24 hours post injection, and at each time point, 6 chickens from each group were euthanized and spleen tissue was stored in RNAlater (Qiagen, Valencia, CA) for RNA extraction. The spleen is a secondary lymphoid organ and was selected as the sampling tissue as previous studies have demonstrated responses in the spleen following i.m. injection of TLR agonists (Patel et al., 2008).

RNA extraction and cDNA synthesis

Total RNA was extracted from 50 mg of spleen tissue using TRIzol® (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Following extraction, 10 μg of RNA was treated with DNA Free® (Ambion, Austin, TX) DNAse, and 1 μg of purified RNA was reverse transcribed to cDNA using Superscript® II First Strand Synthesis kit (Invitrogen, Carlsbad, CA) and oligo-dT primers, according to the manufacturers recommended protocol. The resulting cDNA was subsequently diluted 1:10 in DEPC treated water.

Real-time PCR

Quantitative real-time PCR using SYBR Green was performed on diluted cDNA using the LightCycler® 480 II (Roche Diagnostics GmbH, Mannheim, GER) as previously described (Villanueva et al., 2011). Briefly, each reaction involved a pre-incubation at 95°C for 10 min, followed by 45 cycles of 95 °C for 10 min, 55 °C -64 °C (T_A as per primer) for 5 s, and elongation at 72 °C for 10 s. Subsequent melt curve analysis was performed by heating to 95°C for 10 s, cooling to 65°C for 1 minute, and heating to
97°C. Primers were synthesized by Sigma-Aldrich-Canada (Oakville, ON), and their specific sequences, references and accession numbers are outlined in Table 3.

Data Analysis

Relative expression levels of all genes were calculated relative to the housekeeping gene β-actin based on the formula developed by Pfaffl (Pfaffl, 2001). Gene expression was displayed as fold changes relative to the PBS-treated control group. Fold changes, standard error and statistical significance were calculated using the software REST 2009 (Qiagen, Valencia, CA) (Pfaffl et al., 2002). The software REST 2009 employs random relocation and bootstrapping techniques to test for statistical significance between treatment groups and the PBS-treated control group. Results were considered statistically significant if p ≤ 0.05 (*) and p ≤ 0.01 (**).

Table 3. Primer sequences and accession numbers used for quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence</th>
<th>Accession Number</th>
<th>Reference</th>
</tr>
</thead>
</table>
| MHC II | F: 5'-CCACGGACGTGATGCAAGA-3'  
R: 5'-ACCAGCAGGAACAGAAGA-3' | 113206149 | (Thanthrige-Don et al., 2010) |
| IFN-α | F: 5'-ACACGGACGTGATGCAAGA-3'  
R: 5'-ACCAGCAGGAACAGAAGA-3' | AB021154 | (Villanueva et al., 2011) |
| IFN-γ | F: 5'-ACACGGACGTGATGCAAGA-3'  
R: 5'-ACCAGCAGGAACAGAAGA-3' | X99774 | (Brisbin et al., 2010) |
| IL-1β | F: 5'-ACACGGACGTGATGCAAGA-3'  
R: 5'-ACCAGCAGGAACAGAAGA-3' | Y15006 | (Abdul-Careem et al., 2009) |
| IL-8 | F: 5'-ACACGGACGTGATGCAAGA-3'  
R: 5'-ACCAGCAGGAACAGAAGA-3' | AJ009800 | (Abdul-Careem et al., 2009) |
| IL-10 | F: 5'-ACACGGACGTGATGCAAGA-3'  
R: 5'-ACCAGCAGGAACAGAAGA-3' | AJ621614 | (Haq et al., 2010) |
| IL-13 | F: 5'-ACACGGACGTGATGCAAGA-3'  
R: 5'-ACCAGCAGGAACAGAAGA-3' | AJ621250 | (Abdul-Careem et al., 2008b) |
| iNOS | F: 5'-ACACGGACGTGATGCAAGA-3'  
R: 5'-ACCAGCAGGAACAGAAGA-3' | NM 204961 | (Abdul-Careem et al., 2007) |
| MyD88 | F: 5'-ACACGGACGTGATGCAAGA-3'  
R: 5'-ACCAGCAGGAACAGAAGA-3' | NM 001030962 | (Villanueva et al., 2011) |
| β-Actin | F: 5'-ACACGGACGTGATGCAAGA-3'  
R: 5'-ACCAGCAGGAACAGAAGA-3' | X00182 | (Haq et al., 2010) |
Results

Expression of cytokine transcripts in the spleen following LPS treatment

The changes in gene expression levels among the chickens treated with different doses of LPS are expressed as fold changes relative to the PBS treated control group (Figure 1).

To shed some light on the early type I interferon response, we quantified transcripts of IFN-α. IFN-α transcripts increased by 2.06 fold in chickens receiving the low dose (100 μg) LPS treatment, whereas these transcripts rose by 4.04 fold (p ≤ 0.05) at 2 hours post injection (h.p.i) in those receiving the high dose (500 μg) treatment (Figure 1A). By 6 h.p.i., transcript levels in both treatment groups were not statistically different from the control group.

To assess cytokines associated with the modulation and regulation of the immune response, we examined IFN-γ, IL-13 and IL-10, which are associated with T-helper (T_H)1, T_H2 and anti-inflammatory responses, respectively. Treatment with high dose or low dose of LPS resulted in a 236.65 (p ≤ 0.01) and 188.55 (p ≤ 0.01) fold increase of IFN-γ transcripts, respectively, in the spleen at 2 h.p.i. (Figure 1B). From 6 h.p.i., the transcript levels of IFN-γ for both the high and low dose groups decreased, with the high dose having a 1.64 fold increase (p ≤ 0.05) at this time point. There was no statistically significant difference in either group when compared to the control group at 12 and 24 h.p.i. Transcript levels of IL-13 were increased by 2.67 fold (p ≤ 0.05) in only the high dose treatment group at 2 h.p.i. (Figure 1C). However, at 6 h.p.i, both the high and low dose LPS treated groups had a 2.21 fold (p ≤ 0.01) and 2.30 fold (p ≤ 0.05) decrease in
IL-13 transcripts, which remained reduced at both 12 and 24 h.p.i.. IL-10 transcripts were significantly up-regulated at 2 h.p.i., with a 61.95 (p ≤ 0.01) and 32.58 (p ≤ 0.05) fold increase, for the high and low dose treated groups, respectively (Figure 1D). By 6 h.p.i., transcript levels remained elevated with a 7.31 fold (p ≤ 0.01) increase for the high LPS dose treatment, and a 3.12 fold (p ≤ 0.01) increase for the low LPS dose treatment. For the low dose group, the transcript levels were not statistically different from those of the control group at 12 h.p.i., in contrast to the high dose treated group whose IL-10 transcripts remained higher compared to the control group (1.91 fold, p ≤ 0.01). However, by 24 h.p.i., transcript levels in the high dose treated group were subsequently down-regulated by 2.42 fold (p ≤ 0.01).

To gain some insight into the pro-inflammatory response, we quantified transcripts of two pro-inflammatory cytokines, IL-1β and IL-8. IL-1β transcripts were significantly increased by 74.68 (p ≤ 0.01) and 54.72 (p ≤ 0.01) fold at 2 h.p.i. following high and low dose LPS treatments, respectively (Figure 1E). Levels of IL-1β transcripts declined by 6 h.p.i to 3.35 fold (p ≤ 0.01) for the high dose LPS treatment group, and 3.21 fold (p ≤ 0.01) for the low dose LPS treatment group. Gene expression remained significantly higher in the high dose group at 24 h.p.i., 2.15 fold higher when compared to controls (p ≤ 0.05). IL-8 transcripts were increased by 25.84 (p ≤ 0.01) and 21.92 (p ≤ 0.01) fold, at 2 h.p.i. for both low and high dose LPS treatments, respectively (Figure 1F). At 24 h.p.i., IL-8 transcripts present in the spleen of the low dose treated chickens were not statistically different from those in the control group, though the high dose treated group still had a 2.87 (p ≤ 0.01) fold increase in IL-8 transcripts.
Lastly, we examined the transcriptional responses of relevant proteins and transcription factors which are suggestive of TLR stimulation and APC activation. MyD88 transcripts were significantly up-regulated by 4.30 fold (p ≤ 0.05) at 2 h.p.i. following the high dose LPS treatment, while the low dose LPS treated chickens showed a 3.75 fold increase (p ≤ 0.05) (Figure 1G). At 6 h.p.i., transcript levels of MyD88 in both treatments groups were not statistically different from the PBS treated control group. Furthermore, iNOS transcripts were significantly up-regulated at 2 h.p.i. by 18.01 (p ≤ 0.01) and 19.76 (p ≤ 0.01) fold following high and low dose LPS treatment, respectively (Figure 1H). Furthermore, at 6 h.p.i., the low dose LPS treated group was not statistically different from the PBS control group, whereas the high dose treated group remained up-regulated with a 1.92 fold increase (p ≤ 0.05). However by 24 h.p.i., iNOS transcript levels in both the high and low dose treated groups were significantly down regulated by 4.07(p ≤ 0.05) and 3.45 (p ≤ 0.01) fold, respectively. Additionally, MHC-II transcripts were increased by 1.80 fold (p ≤ 0.01) at 2 h.p.i. for the high dose treated group (Figure 1I). By 6 h.p.i., expression levels in both groups were not statistically different from the control group. However, at 24 h.p.i transcripts in the high dose treated group were reduced by 3.12 fold (p ≤ 0.01).

Expression of cytokine transcripts in the spleen following CpG and non-CpG ODN treatment

The changes in gene expression levels among chickens treated with low dose (10 μg) or high dose (50 μg) of CpG ODNs, in addition to non-CpG ODNs (50 μg) are expressed as fold changes relative to the PBS treated control group (Figure 2). For all
genes examined, there were generally no statistically significant differences between the non-CpG treated group and the PBS treated control group.

With respect to the type I interferon, IFN-α, we observed no significant differences of IFN-α transcript levels among any of the treatment groups at any time point (Figure 2A).

In contrast to IFN-α, we observed significant differences in transcripts of the cytokines associated with the modulation and regulation of the immune response, namely IFN-γ, IL-13 and IL-10. IFN-γ transcripts were up-regulated by 1.91 fold (p ≤ 0.01) for the low dose treated group (Figure 2B). At 6 h.p.i., transcript levels in all groups were not statistically different from the PBS control group. Moreover, IL-13 transcripts were down-regulated at 2 h.p.i. for the high dose treated group by 1.93 fold (p ≤ 0.05) (Figure 2C). While transcript levels for the high dose treated group were not statistically different from the PBS treated control group at 6 h.p.i., the low dose treated group had a 4.39 fold (p ≤ 0.01) decrease, and remained decreased at 12 h.p.i. Nonetheless, at 24 h.p.i., both the high and low dose CpG treated groups showed significant down-regulation in IL-13 transcripts by 1.96 (p ≤ 0.05) and 1.57 (p ≤ 0.05) fold, respectively. Furthermore, IL-10 transcripts were up-regulated at 2 h.p.i. by 2.37 fold (p ≤ 0.05) for the high dose treated group (Figure 2D). However, at 6 h.p.i., transcript levels were not statistically different from the control group, while transcript levels in the low dose CpG treated group decreased by 2.6 fold (p ≤ 0.01). At 12 h.p.i., transcript levels in all treatment groups were not statistically different from the PBS control group, but at 24 h.p.i., IL-10 transcripts were up-regulated for the high dose treated group by 1.61 fold (p ≤ 0.05).
Similar to LPS treatment, we observed an up-regulation in both of the studied pro-inflammatory cytokines, IL-1β and IL-8. Interleukin-1β transcripts were significantly up-regulated at 2 h.p.i., with a 1.82 fold (p ≤ 0.05) and 2.18 fold (p ≤ 0.05) increase for low and high dose CpG treated groups, respectively (Figure 2E). However, at 6 h.p.i., transcript levels for the low dose treated group were not statistically different compared to PBS treated controls, while the high dose treated group still had a 2.01 (p ≤ 0.01) fold increase in transcripts. However, at 12 h.p.i., IL-1β transcript levels in all groups were not statistically different from the PBS treated control group. With respect to IL-8, transcripts were not up-regulated for any of the treatment groups until 12 h.p.i., where there was a 2.23 fold (p ≤ 0.05) increase for the low dose treated group (Figure 2F). This cytokine in the high dose treated group was increased by 1.99 fold, but this was not statistically significant. There was no statistically significant up-regulation of gene expression following the 12 h.p.i. time point.

Lastly, we examined the transcriptional responses of relevant proteins and transcription factors which are suggestive of TLR stimulation and APC activation. MyD88 transcripts were significantly up-regulated by 2.0 fold (p ≤ 0.05) at 2 h.p.i. for the high dose CpG treated group (Figure 2G). However at 6 h.p.i., transcript levels among all treatment groups were not statistically different from the PBS treated control group. Furthermore, at 6 h.p.i. iNOS transcripts were down-regulated by 1.8 fold (p ≤ 0.01) and by 3.05 fold (p ≤ 0.01) for the low dose treated group and the non-CpG treated group, respectively (Figure 2H). However, at 12 h.p.i., iNOS transcripts were increased by 1.66 (p ≤ 0.05) fold for the low dose treated group. At 24 h.p.i., transcript levels were decreased by 3.05 (p ≤ 0.01) and 2.5 (p ≤ 0.01) fold for low and high dose treated groups,
respectively. Finally, MHC-II transcripts were not statistically different from the PBS control group at all time points, except at 6 h.p.i., where the high dose treated group had a 16.94 fold ($p \leq 0.01$) increase (Figure 2I).

A summary of all transcriptional responses of the examined genes in response to LPS and CpG ODN treatments is presented in Table 4.
**Table 4.** Summary of transcriptional responses in the spleen following LPS and CpG ODN treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hours</th>
<th>IFN-α</th>
<th>IFN-γ</th>
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A "+++"] indicates a significant up-regulation of at least 20 fold, "++" indicates a significant up-regulation of at least 5-fold, while a “+” indicates a significant up-regulation when compared to the PBS treated control group. Furthermore, a “-” indicates a significant down-regulation and a “0” indicates no significant change observed when compared to the PBS treated control group.
Discussion

Recognition of PAMPS by TLRs triggers innate host responses, promoting secretion of cytokines, which leads to elicitation of innate and adaptive immune responses (Medzhitov, 2001). In chickens, TLR agonists have been successfully used as vaccine adjuvants (Linghua et al., 2007; Wang et al., 2009; Mallick et al., 2011) and as prophylactic agents to prevent infection or ameliorate disease (Gomis et al., 2004; Dar et al., 2009b). However, little is known about the dynamics and repertoire of chicken responses to TLR agonists. Therefore, the present study was aimed to determine the effects of stimulation with TLR4 and TLR21 ligands in chickens, by assessing transcriptional regulation of a few relevant immune system genes in vivo.

Based on our findings, it appears that LPS elicits an overall greater response in comparison to CpG ODNs. This could be a result of the shorter in vivo half-life of nucleic acids as they are more prone to extracellular degradation. Moreover, similar to mammalian TLR9, TLR21 may be expressed primarily in intracellular vesicles. Therefore, it is possible that only a small fraction of the administered CpG ODN was endocytosed and reached TLR21, therefore reducing the frequency of CpG-TLR21 interactions. To increase the intracellular availability of CpG ODNs, future studies should consider incorporating CpG ODN into a protective vehicle, such as liposomes, to prevent extracellular degradation and promote efficient delivery of CpG ODNs into the endosome. Furthermore, our results indicate a temporal difference in peak responses to CpG ODN and LPS. Following treatment with LPS, responses typically peaked by the earliest time point, 2 hours post-treatment, while responses to CpG ODN peaked at varying time points, both early and late. These findings agree with previously published
time-series reports comparing the responses to CpG ODN and LPS in mammals (Hartmann and Krieg, 1999). The increased response time to CpG ODN treatments may again be related to the time required for uptake and interiorization of CpG ODNs by host cells.

In mammals, TLRs are expressed by a variety of cell types including those of the innate and adaptive immune system such as macrophages, dendritic cells (DCs), natural killer (NK) cells, mast cells, T cells and B cells. Furthermore the response of these cells to TLR stimulation has been well characterized. For example, treatment with TLR agonists induces the proliferation of murine splenocytes and human peripheral blood mononuclear cells, in addition to the up-regulation of cytokines, including type I IFNs, pro-inflammatory cytokines as well as cytokines associated with T_h1, T_h2 and T regulatory cells (Weeratna et al., 2005; Ghosh et al., 2007). Macrophages and DCs express a wide array of TLRs in mammals and, therefore, play an important role in the overall host response to TLR agonists. Indeed, it has been shown that TLR-activated macrophages and DCs up-regulate expression of MyD88, co-stimulatory molecules and MHC-II, in addition to up-regulating pro-inflammatory cytokines such as IL-1β and IL-8 (Ucla et al., 1990). These activated macrophages also increase their expression of iNOS, leading to production of nitric oxide (Mosser, 2003). These findings may also be extended to chickens, as previous studies have demonstrated production of nitric oxide and pro-inflammatory cytokines by chicken heterophils and monocytes following stimulation with TLR ligands (Kogut et al., 2005; He, MacKinnon, et al., 2011).

IFN-α and IFN-β belong to a class of antiviral cytokines that promote an antiviral state through the production of interferon-inducible genes (Samuel, 2001). Typically,
IFN-α is up-regulated during the early phases of a viral infection by virus-infected cells and by TLR stimulated plasmacytoid dendritic cells (Doly et al., 1998). In addition to their direct anti-viral effects, type I interferons promote the differentiation of naïve CD4+ T cells into Th1 cells (Brinkmann et al., 1993), enhancing their effector functions against viruses. In the case of LPS, there was an early up-regulation of IFN-α, raising the possibility that LPS may directly and indirectly activate anti-viral responses through IFN-γ and IFN-α.

Regulation of an immune response is achieved through a variety of molecular and cellular mechanisms, among which regulatory cytokines such as TGF-β and IL-10 are of note. IL-10 has anti-inflammatory properties which down-regulate activated dendritic cells and macrophages, in addition to suppressing both Th1 and Th2 responses (Saraiva and O’Garra, 2010). The results of the present study suggest that LPS up-regulates IL-10 immediately following treatment. Importantly, LPS stimulation induced a significant increase (50-100 fold increase depending on LPS dose) in IL-10 transcripts. It is speculated that this immediate up-regulation of IL-10 may serve as a protective mechanism to regulate the activation of responding cells. It is not clear which cells were the predominant source of IL-10, however, it is plausible that macrophages, DCs, T cells and B cells, were the main producers of this cytokine, as they have high expression of TLR4 and can respond to TLR agonists by producing IL-10 (Barr et al., 2007; Conroy et al., 2008).

Although LPS is common to all Gram negative bacteria, kinetics of host response to LPS is partly dependent on the bacterial species the LPS is derived from. As demonstrated by Keestra and colleagues (2008), chicken TLR4 responds more robustly to
certain LPS including those derived from *E. coli* and *Salmonella spp* as determined by a NF-kappaB reporter assay. Furthermore in mammals, treatment with LPS derived from *Salmonella spp* and *Escherichia coli* promotes a T_H1 biased response characterized by the up-regulation of IL-12 and IFN-γ, while treatment with LPS derived from *Porphyromonas gingivalis* does not up-regulate either of these two cytokines, but promotes the production of IL-5, which is suggestive of a T_H2-like response (Pulendran et al., 2001). Although responses to multiple sources of LPS were not characterized in the present study, our results indicate that LPS derived from *E. coli* up-regulates both IFN-γ and IL-13 in the spleen, raising the possibility of a more balanced T_H1/ T_H2-like phenotype, although further studies are needed to confirm this. Furthermore, IL-13 may synergize with IL-10 to promote the resolution of the immune response, as previous studies have demonstrated the anti-inflammatory properties of IL-13 (Marie et al., 1996; Watson et al., 1999).

A similar ligand specific response applies to TLR21 as well, as the sequences flanking the CpG motif differentially modulate the kinetics of the host response. There are three major classes of CpG ODN that are grouped according to their flanking sequence motifs, classes A, B and C (Vollmer et al., 2004). The class A CpG ODNs are potent activators of mammalian TLR9 expressed by plasmacytoid dendritic cells (pDC), thereby promoting the production of IFN-α (Krug et al., 2001). This is in contrast to class B CpG ODN which weakly induces IFN-α. However, this ODN is strongly immunostimulatory for B cells, thereby enhancing proliferation and antigen presentation capabilities, in addition to the promoting the production of cytokines such as IFN-γ (Krug et al., 2001). Class C CpG ODN contains a mixture of Class A and Class B flanking
sequences, thereby moderately activating both pDCs and B cells. Although the responses to different classes of CpG ODN in chickens remain to be elucidated, it has been shown that chicken B cells proliferate in response to Class B and Class C CpG ODNs (Wattrang, 2009), which may support the notion of a flanking-sequence specific response demonstrated by chicken TLR21. In the present study, we administered the class B CpG ODN 2007 and subsequently observed no up-regulation of IFN-α in the spleen, which is similar to IFN-α-independent responses that occur in mammals in response to class B CpG ODN (Liu et al., 2010). Furthermore, we observed a significant up-regulation of IFN-γ and MHC II in the spleen, which may be attributed potentially to a robust TLR-mediated B cell response (Jiang et al., 2007; Booth et al., 2010). The biological significance of IFN-γ has been well established in mammals, such as promoting the activation of cell subsets with anti-viral capacity including cytotoxic T lymphocytes, macrophages and natural killer cells (Samuel, 2001). Additionally, IFN-γ possesses anti-viral properties as well, inducing virus-infected cells to produce and activate anti-viral proteins such as RNase L, which inhibit viral replication and promote apoptosis of the infected cell (Samuel, 2001). Furthermore, IFN-γ is indicative of a T_{H1}-biased response, and therefore our data raise the possibility that treatment with Class B CpG ODN biases the immune response towards a T_{H1} phenotype in chickens, as characterized by the up-regulation of IFN-γ and down-regulation of IL-13, although further studies are needed to confirm this.

Lastly, we observed an up-regulation of IL-10 in response to CpG ODN treatment, likely functioning to promote resolution of the immune responses and regulate the activation of responding cells. These findings support previous in vitro studies, which
suggested that dendritic cells and B cells produce IL-10 in response to CpG ODN (Lenert et al., 2005; Flores et al., 2007).

In conclusion, the present study highlights some of the underlying mechanisms of induction of host responses by TLR agonists in chickens. Future studies may employ these findings to select TLR ligands for enhancing immune responses in chickens, for example for enhancing immunity conferred by vaccines.
Figure 1. Gene expression in the spleens of chickens at 2, 6, 12 and 24 hours post injection (h.p.i.) with 100 μg or 500 μg of LPS. Gene expression was assessed using quantitative RT-PCR, and was calculated relative to the house keeping gene β-actin. Graphed values are expressed as fold change relative to the PBS treated control group ± standard error. Results were considered statistically significant from the PBS treated control group if p ≤ 0.05 (*) and p ≤ 0.01 (**).
Figure 2. Gene expression in the spleens of chickens at 2, 6, 12 and 24 hours post-injection (h.p.i.) with 10 μg or 50 μg of CpG ODNs, or 50 μg of non CpG ODNs. Gene expression was assessed using quantitative RT-PCR, and was calculated relative to the house keeping gene β-actin. Graphed values are expressed as fold change relative to the PBS treated control group ± standard error. Results were considered statistically significant from the PBS treated control group if p ≤ 0.05 (*) and p ≤ 0.01 (**).
CHAPTER 3

Prophylactic treatment with Toll-like receptor ligands enhances host immunity to avian influenza virus in chickens


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Abstract

Avian influenza viruses (AIV) pose a threat towards the health of both poultry and humans. To interrupt the transmission of the virus, novel prophylactic strategies must be considered which may reduce the shedding of AIV. One potential is the prophylactic use of Toll-like receptor (TLR) ligands. Many cells of the immune system express TLRs, and cellular responses to TLR stimulation include activation and the production of cytokines. TLR ligands have been employed as prophylactic treatments to enhance host resistance to pathogens both in mammals and chickens. Therefore, the present study was conducted to determine whether TLR ligands may be used prophylactically in chickens to enhance host immunity to AIV. Chickens received intramuscular injections of either low or high doses of the TLR ligands poly I:C, lipopolysaccharide (LPS) and CpG ODN. Twenty-four hours post-treatment, chickens were infected with the low pathogenic avian influenza virus H4N6, and both oropharyngeal and cloacal virus shedding were assessed on days 4 and 7 post-infection. To identify potential correlates of immunity, spleen and lungs were collected on days 2, 4 and 7 post-infection for RNA extraction. The results suggested that all of the TLR ligand treatments induced a significant reduction in virus shedding, with the TLR3 ligand poly I:C conferring the greatest AIV immunity compared to control birds, followed by CpG ODN and LPS. Furthermore, transcriptional analysis of gene expression in the spleen and lungs suggest IFN-α and IL-8 as correlates of immunity conferred by poly I:C, and IFN-γ for CpG ODN and LPS. In conclusion, TLR ligands, have the ability to enhance host immunity against AIV, and future studies should consider exploring the combinatory effects of poly I:C and CpG ODN prophylaxis in conjunction with AIV vaccination.
1. Introduction

Avian influenza viruses (AIV) are enveloped, single stranded RNA viruses belonging to the family *Orthomyxoviridae*. AIV are typically classified according to their pathogenicity; whether they are a low pathogenic avian influenza (LPAI) virus or a highly pathogenic avian influenza virus (HPAI) (Suarez and Schultz-Cherry, 2000). Although HPAI viruses, such as H5N1, are of great concern to the poultry industry and public health, outbreaks of LPAI viruses have also become problematic. For example, the recent spread of H7N3 among poultry in England led to the culling of 35,000 chickens (Nguyen-Van-Tam et al., 2006), while an outbreak of H5N2 in Japan affected 5.78 million chickens spread across 41 farms (Okamatsu et al., 2007). Importantly, some LPAI strains such as H9N2, have the ability to transmit to other species including to humans and pigs (Lin et al., 2000). To interrupt the inter- and intra-species transmission of AIV from infected poultry, several prophylactic strategies may be considered, including vaccination, administration of antiviral compounds such as interferons, as well as the use of Toll-like receptor ligands.

Toll-like receptors (TLRs) are an evolutionarily conserved group of pattern recognition receptors that play an important role in the initiation and modulation of host responses to pathogens. Ten TLRs have been identified in chickens, each recognizing conserved structural motifs of pathogens termed pathogen-associated molecular patterns (PAMPs) (Temperley et al., 2008). For example, TLR3 recognizes double stranded RNA, a product of some viruses during their replication cycle, as well as synthetic molecules such as polyinosinic:polycytidylic acid (poly I:C), while TLR4 recognizes lipopolysaccharide (LPS) found in the outer membrane of Gram-negative bacteria, and
TLR21, the functional homologue of mammalian TLR9, recognizes CpG DNA motifs found in certain bacterial and viral nucleic acids (Medzhitov, 2001; Brownlie et al., 2009). TLRs are expressed by a variety of immune system cell subsets where they mediate cellular activation and the production of cytokines (Hopkins and Sriskandan, 2005; Akira et al., 2006).

TLR ligands have been employed prophylactically in laboratory animal models to enhance host resistance to pathogens including hepatitis C virus, *Burkholderia mallei* and influenza virus (Waag et al., 2006; Kanzler et al., 2007; Wong et al., 2009). Moreover, TLR ligand prophylaxis has been extended to chickens, enhancing host immunity to pathogens including *Salmonella enterica* serovar Enteritidis (Genovese et al., 2007; He et al., 2007b), *Escherichia coli* (Gomis et al., 2004; Taghavi et al., 2009), Marek’s disease virus (P. Parvizi, unpublished data) and infectious bronchitis virus (Dar et al., 2009b). Therefore, we hypothesized that TLR ligands may enhance immunity to AIV as well, thereby reducing viral load and shedding from chickens that are experimentally infected with AIV. The results of our study suggest that the ligands for TLR3, 4 and 21 can reduce virus load and shedding in infected birds, with the TLR3 ligand poly I:C conferring the greatest host immunity towards AIV.
2. Materials and Methods

2.1 Experimental chickens and housing

Specific pathogen-free (SPF) eggs \( (n=108) \) were procured from the Animal Disease Research Institute, Canadian Food Inspection Agency (Ottawa, ON), hatched at the Arkell Poultry Research Center, University of Guelph (Guelph, ON) and housed in the isolation facility of the Ontario Veterinary College, University of Guelph. This research was approved by the University of Guelph Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care.

2.2 Avian influenza virus

Chickens were infected with the LPAI A/Duck/Czech/56 (H4N6) which was propagated in 11-day-old embryonated chicken eggs as described in (Mallick et al., 2011).

2.3 TLR ligands

Poly I:C and LPS from \( E. \ coli \) 0111:B4 were purchased from Sigma-Aldrich, Canada (Oakville, ON), while synthetic class B CpG ODN 2007 [5’-TCGTCGTTGTCGTTTTGTCGTT-3’] and non-CpG ODN [5’-TGCTGCTTGTGCTTTTGTGCTT-3’] were purchased from Eurofins MWG Operon (Ebersberg, GER). All of the ligands used were re-suspended in sterile phosphate buffered saline (PBS, pH 7.4).
2.4 Experimental design

Fourteen-day-old chickens were randomly divided into 9 groups of 12 birds each. Chickens were injected intramuscularly (i.m.) in the pectoral muscle with either a low (80 μg) or high (400 μg) dose of poly I:C, a low (100 μg) or high (500 μg) dose of LPS or a low (10 μg) or high (50 μg) dose of CpG ODN, while control groups received either non-CpG ODN (50 μg) or PBS (St Paul et al., 2011; Parvizi et al., 2012). Twenty-four hours post-treatment (Renis, 1970; Ashkar et al., 2003; Dar et al., 2009b), in all of the groups except for one of the two PBS treated control groups, chickens were infected with 1x10^6 50% tissue culture infectious dose (TCID\textsubscript{50}) of H4N6 virus through the oculo-nasal route. To assess virus shedding on days 4 and 7 post-infection (p.i.), oropharyngeal and cloacal swabs were collected from 4 to 6 chickens in each group, depending on the number of chickens remaining. These time points represent the peak in oropharyngeal and cloacal shedding, respectively (Le Gall-Reculé et al., 2007; Spackman et al., 2010). To identify correlates of immunity, 4 chickens from each group were euthanized on days 2, 4 and 7 p.i., and spleen and lung tissue were stored in RNA later (Qiagen, Valencia, CA). Of the two doses of TLR ligands administered to the chickens, only mRNA expression in the spleen and lung tissue of the most efficacious dose of each TLR agonist (poly I:C high dose, LPS low dose and CpG ODN high dose) was quantified.

2.5 Virus titration

Oropharyngeal and cloacal swabs were collected as described (WHO, 2002). A virus titration assay using Madin-Darby canine kidney (MDCK) cells (University of Guelph
Animal Health Laboratory) was performed to assess viral shedding from both oropharyngeal and cloacal swabs as described previously (Szretter et al., 2006). Viral titres were expressed as log_{10} TCID_{50}/mL of swab sample.

2.6 RNA extraction, cDNA synthesis and real-time PCR

Total RNA extraction and cDNA synthesis was performed as previously described (St Paul et al., 2011). Real-time PCR using SYBR Green was performed on diluted cDNA using the LightCycler® 480 II (Roche Diagnostics GmbH, Mannheim, GER) as previously described (Villanueva et al., 2011). Primers were synthesized by Sigma-Aldrich, Canada (Oakville, ON), and their specific sequences have been previously published elsewhere (Abdul-Careem et al., 2006, 2008a, 2009; Brisbin et al., 2008; Villanueva et al., 2011).

2.7 Data analysis

The log_{10} TCID_{50}/mL of virus present in the oropharyngeal and cloacal swab fluid of each chicken was calculated based on the formula by Reed-Muench (Szretter et al., 2006). To examine if each TLR ligand, both high and low doses, differed from the PBS control, separate ANOVA for each TLR ligand were computed. Any statistically significant main effects were examined with post-hoc Tukey’s test. With respect to quantitative real-time PCR, relative expression of all target genes was calculated relative to the housekeeping gene β-actin using the LightCycler® 480 Software (Roche Diagnostics GmbH,
Mannheim, GER), based on the formula developed by Pfaffl (Pfaffl, 2001). Statistical
difference was calculated using an un-paired student’s t-test. All results were considered
statistically significant from the infected, PBS-treated control group if $p \leq 0.05$ (*) and $p
\leq 0.01$ (**), considered statistically significant from the uninfected PBS control group if
$p \leq 0.05$ (#), and lastly in the case of CpG ODN, considered statistically significant from
the non-CpG ODN group if $p \leq 0.05$ (~).
3. Results

3.1 Treatment with TLR ligands reduces oropharyngeal and cloacal virus shedding.

On days 4 and 7 p.i., oropharyngeal and cloacal virus shedding was assessed by virus titration in MDCK cells (Figure 3). Treatment with the high dose of poly I:C resulted in the lowest amount of virus being shed orally on day 4 p.i. (Figure 3A), with a \( \log_{10} \text{TCID}_{50}/\text{mL} \) of 1.84 (\( p \leq 0.01 \)), followed by, from lowest to highest, treatment with the low dose of poly I:C (2.92 \( \log_{10} \text{TCID}_{50}/\text{mL} \), \( p \leq 0.01 \)), the high dose of CpG ODN (3.17 \( \log_{10} \text{TCID}_{50}/\text{mL} \), \( p \leq 0.01 \)), the low dose of LPS (4.59 \( \log_{10} \text{TCID}_{50}/\text{mL} \), \( p \leq 0.01 \)), and lastly, treatment with the low dose of CpG ODN (5.50 \( \log_{10} \text{TCID}_{50}/\text{mL} \), \( p \leq 0.01 \)).

Of the two control groups which were infected, the non-CpG ODN treated group had a viral titre of 4.59 \( \log_{10} \text{TCID}_{50}/\text{mL} \) (\( p \leq 0.01 \)), while the PBS treated group had a viral titre of 6.92 \( \log_{10} \text{TCID}_{50}/\text{mL} \). Although a similar pattern was observed for cloacal shedding on day 4 p.i. (Figure 3B), virus shedding in none of the treatment groups resulted in a statistically significant difference from the PBS treated control group.

There was no detectable oropharyngeal shedding on day 7 p.i. in any of the treatment and control groups (data not shown). However, shedding was detected in the cloaca on day 7 p.i., and treatment with the high dose of CpG ODN resulted in the lowest amount of virus being shed (Figure 3C), with a titre of 1.75 \( \log_{10} \text{TCID}_{50}/\text{mL} \) (\( p \leq 0.01 \)), followed by, from lowest to highest, treatment with the high dose of poly I:C (2.17 \( \log_{10} \text{TCID}_{50}/\text{mL} \), \( p \leq 0.01 \)), the low dose of poly I:C (2.75 \( \log_{10} \text{TCID}_{50}/\text{mL} \), \( p \leq 0.01 \)), the low dose of LPS (3.09 \( \log_{10} \text{TCID}_{50}/\text{mL} \), \( p \leq 0.05 \)) and lastly, treatment with the low dose of CpG ODN (3.25 \( \log_{10} \text{TCID}_{50}/\text{mL} \), \( p \leq 0.01 \)). Of the two control groups that were
infected, the non-CpG ODN treated group had a viral titre of 3.25 log_{10} TCID_{50}/mL (p \leq 0.01), while the PBS treated group had a viral titre of 5.92 log_{10} TCID_{50}/mL.

3.2 Correlates of immunity in the spleen and lungs of chickens treated with TLR ligands.

To identify immunological correlates of protection, gene expression in the spleen and lungs was examined on days 2, 4 and 7 p.i. using real-time PCR (Figures 4-7). Statistical significance described in the results is in reference to the infected, PBS treated control group unless otherwise indicated.

Interferon (IFN)-α transcripts were down-regulated in the spleen on day 7 p.i. in the CpG ODN treated group (p \leq 0.01) (Figure 4A), while IFN-β transcripts were up-regulated in the LPS treated group on day 4 p.i. (p \leq 0.01) (Figure 4B). With respect to IFN-γ, transcripts were down-regulated in the spleens of the poly I:C, LPS, CpG and non-CpG ODN treated groups on day 7 p.i. (p \leq 0.01) (Figure 4C). Transcripts of the interferon inducible gene 2’-5’-oligoadenylate synthetase (OAS) were up-regulated (p \leq 0.05) in the CpG ODN treated group in the spleen on day 4 p.i., (Figure 4D).

In the spleen on day 2 p.i., transcript levels of IL-1β were down-regulated in the CpG ODN treated group (p \leq 0.05) (Figure 5A), As for IL-8, there were no statistically significant differences in transcript levels for any of the treatment groups in the spleen (Figure 5B). Transcript levels of IL-18 were significantly down-regulated in the spleen on day 7 p.i. in the poly I:C treated group (p \leq 0.05), and in the LPS, CpG ODN and non-CpG ODN treated groups (p \leq 0.01) (Figure 5C).
In the lungs, IFN-α transcripts were up-regulated in the poly I:C treated group on day 4 p.i. (p≤ 0.05) and day 7 p.i. (p≤ 0.05), and up-regulated in the LPS treated group on day 7 p.i. as well (p≤ 0.01) (Figure 6A). Similarly, IFN-β transcripts were up-regulated in the lungs on day 2 p.i. in the LPS, poly I:C, CpG ODN and non-CpG ODN treated groups (p≤ 0.05) (Figure 6B). However, on day 4 p.i., transcripts were down-regulated in the poly I:C and non-CpG ODN treated groups (p≤ 0.05). In the case of IFN-γ, transcripts were up-regulated in the lungs on day 2 and 4 p.i. for the CpG ODN treated group (p≤ 0.01), while being up-regulated only on day 4 p.i. for the LPS and non-CpG ODN treated groups (p≤ 0.05) (Figure 6C). On day 7 p.i., IFN-γ transcripts in the poly I:C treated group were up-regulated (p≤ 0.05). Transcripts for OAS were up-regulated in the lungs on day 4 p.i., in the CpG ODN treated group (p≤ 0.05) (Figure 6D).

In the lung, transcripts for IL-1β were up-regulated in the CpG ODN treated group on day 7 p.i. (p≤ 0.05) (Figure 7A), while IL-8 transcripts were up-regulated on day 2 p.i. (p≤ 0.01) in the poly I:C treated group (Figure 7B). Lastly, IL-18 transcripts were up-regulated in the lungs on day 2 p.i. in the poly I:C treated group (p≤ 0.05), while being down-regulated in the LPS, CpG ODN and non-CpG ODN treated groups on day 7 p.i. (p≤ 0.05) (Figure 7C). A summary of virus shedding and mRNA expression is found in Table 5.
Table 5. Summary of virus shedding and transcript levels in the lungs on days 4 and 7 p.i.

<table>
<thead>
<tr>
<th>TLR ligand</th>
<th>Day P.i.</th>
<th>Reduction in Oropharyngeal Shedding</th>
<th>Reduction in Cloacal Shedding</th>
<th>IFN-α</th>
<th>IFN-β</th>
<th>IFN-γ</th>
<th>OAS</th>
<th>IL-1β</th>
<th>IL-8</th>
<th>IL-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly I:C (high dose)</td>
<td>4</td>
<td>5.08</td>
<td>0.96</td>
<td>+</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>ND</td>
<td>3.75</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LPS (low dose)</td>
<td>4</td>
<td>2.33</td>
<td>0.33</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>ND</td>
<td>2.83</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>CpG ODN (high dose)</td>
<td>4</td>
<td>3.75</td>
<td>0.71</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>ND</td>
<td>4.17</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Non-CpG ODN</td>
<td>4</td>
<td>2.33</td>
<td>0.12</td>
<td>0</td>
<td>-</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>ND</td>
<td>2.67</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

The amount reduction in virus shedding was determined by subtracting the mean log_{10} TCID_{50}/mL for each treatment group from that of the infected, PBS treated control group. A “ND” indicates no shedding was detected. A “+” and “++” indicate a significant up-regulation of p ≤ 0.05 and p ≤ 0.01, respectively, when compared to the infected, PBS treated control group. A “0” indicates no significant up-regulation and a “-” indicates a significant down-regulation compared to the infected, PBS treated control group.
4. Discussion

The immunomodulatory properties of TLR ligands have been exploited to enhance host immunity to several pathogens in chickens. In the present study, we report that TLR ligands may also confer protection against an LPAI virus.

When used prophylactically, our findings indicate that all three of the TLR ligand treatments reduced AIV shedding, with the TLR3 ligand poly I:C being the most efficacious followed by CpG ODN and LPS. The anti-viral role of poly I:C has been well established in mammals, especially in the context of enhancing influenza virus immunity (Ichinohe et al., 2005; Wong et al., 2009). This anti-influenza property of poly I:C may be attributed to the unique signaling pathway utilized by TLR3, which signals solely through the TIR domain-containing adaptor inducing IFN-β (TRIF) pathway and thereby promotes the production of the type I interferons IFN-α and IFN-β (Matsumoto and Seya, 2008). Consistent with these findings, we observed an up-regulation of type I IFNs in the lungs of the poly I:C treated chickens, possibly mediated in part by TLR3 and other pattern recognition receptors that also bind dsRNA, such as Mda5 (Karpala et al., 2011b). Type I interferons demonstrate many anti-viral functions, including inhibiting viral transcription and translation, promoting the apoptosis of infected cells and activating antigen presenting cells (Samuel, 2001). Furthermore, IFN-α has been shown to protect ferrets (Kugel et al., 2009) and chickens (Meng et al., 2011) against influenza viruses, and may also contribute to viral clearance during the natural course of infection, as our data indicate an up-regulation of IFN-α in the spleens of the challenged control group. In addition to IFN-α, our results suggest that poly I:C significantly up-regulated IL-8 in the lungs, raising the possibility that heterophils were recruited to the lung, due to the fact
that IL-8 is a chemoattractant for heterophils (Kogut, 2002). Although the importance of heterophils in AIV immunity is not clear, in mammals neutrophils promote the clearance of influenza virus in part through the production of cytokines and anti-microbial peptides (Gaudreault and Gosselin, 2008; Wang et al., 2008). Taken together, our results suggest that IFN-α and IL-8 both serve as correlates of AIV immunity for the poly I:C treatment.

In chickens, CpG ODN has previously been shown to induce a T_{H}1 biased immune response characterized by the up-regulation of IFN-γ and down-regulation of IL-13 (Patel et al., 2008; St Paul et al., 2011). Although not as efficacious as poly I:C, our results showed that treatment with both low and high doses of CpG ODN significantly reduced AIV shedding. Moreover, our data suggest that immunity conferred by CpG ODN correlates with IFN-γ, which promotes an anti-viral state mediated in part by CD8+ T cells and natural killer (NK) cells. Furthermore, IFN-γ induced the up-regulation of the interferon inducible gene 2’-5’ OAS thereby suggesting the initiation of the RNase L pathway, which acts to cleave viral RNA and promote the apoptosis of infected cells (Liang et al., 2006). Interestingly, our results suggest that treatment with non-CpG ODN enhanced immunity to AIV as well, although it was not as efficacious as an equivalent dose of CpG ODN. Similar immunostimulatory effects of non-CpG ODN have been observed both in mammals as well as in chicken B cells (Senn et al., 2005; Wattrang, 2009). Although our findings raise the possibility that the immunity conferred by non-CpG ODN was mediated in part through the production of IFN-γ, further studies are needed to elucidate the mechanisms in which non-CpG ODN modulate the chicken immune system.
In contrast to the other examined TLR ligands, LPS is strictly a bacterial PAMP. Chickens treated with low dose LPS (but not high dose LPS) displayed reduced virus shedding. Although the basis behind this observation is unknown, a similar phenomenon has been observed in mice. Mice challenged with influenza virus PR8 strain and treated with high doses of LPS (i.e. 20 μg/g) demonstrated an increased virus load which was attributed to an influenza virus associated encephalopathy (Tanaka et al., 2010). This is in contrast to mice treated with lower doses of LPS (i.e. 1.25 μg/g), as they are protected against challenges with PR8 and the highly pathogenic H5N1 (Shinya et al., 2011). As such, future studies may consider employing a lower range of LPS doses, or perhaps even substituting LPS with another TLR4 ligand, such as a synthetic lipid A or FimH, which have previously been shown to protect mice against influenza virus infection (Cluff et al., 2005; Abdul-Careem et al., 2011). Our results suggest that type I interferons and IFN-γ serve as potential correlates of immunity conferred by LPS, which is also supported by our previous study which indicated an up-regulation of these cytokines in response to LPS treatment (St Paul et al., 2011). In mice, it has previously been shown that immunity to H5N1 conferred by LPS is dependent on signaling both through the myeloid differentiation factor 88 (MyD88) independent pathway, as well as the presence of the accessory molecules cluster of differentiation (CD)14 and myeloid differentiation protein-2 (MD-2) (Shinya et al., 2012). In chickens, it has been suggested that responses mediated by TLR4 is dependent in part on signaling through the MyD88 independent pathway (Lian et al., 2012) as well relying on the presence of both CD14 and MD-2 (Kogut et al., 2005a; Keestra and van Putten, 2008). As such, we speculate that immunity
to AIV conferred by LPS in chickens may be mediated by the same pathways and accessory molecules identified in mice.

Through transcriptional analysis of relevant immune system genes, we discovered that several of the genes were up-regulated in the lungs while being down-regulated in the spleen. This finding might suggest that a greater proportion of the immune response to AIV occurs in the lungs rather than in the spleen, which is in agreement with previous studies (Degen et al., 2006; De Geus et al., 2011). Having said that, it is possible a contributing factor towards the efficacy of an intramuscularly injected TLR ligand is its ability to up-regulate immune system genes in the lungs. Previously, our group has demonstrated that an i.m. injection of poly I:C in chickens induces a significant up-regulation of many immune system genes in the lungs (Parvizi et al., 2012) however it remains to be elucidated whether i.m. injections of LPS and CpG ODN up-regulate genes in the lungs as well, and if so, to what extent when compared to that of poly I:C.

Although TLR ligand prophylaxis may be efficacious in an experimental setting, there are some limitations that need to be addressed prior to their widespread adoption. For example, it has previously been shown in chickens that the protective benefits conferred by the ligands is highest when the challenge occurs within 3 days of treatment, and decreases if this time period is extended to 6 days and is absent at 9 days and onwards (Gomis et al., 2003). Therefore, we propose that future research should be aimed at investigating ways to increase the duration of efficacy of TLR ligands while at the same time facilitating their administration. One potential strategy may be to protect the TLR ligands from degradation by encapsulating them in protective vehicles, such as nanoparticles, which can then be aerosolized (Evans et al., 2011).
In conclusion, TLR ligands are able to reduce the viral load following infection with an LPAI virus, with the TLR3 ligand poly I:C being the most efficacious in terms of reducing the quantity of virus shed. Furthermore, transcriptional analysis of genes in the spleen and lungs identified IL-8, IFN-α, IFN-β and IFN-γ as correlates of immunity. Future studies should explore the prophylactic use of TLR agonists in conjunction with vaccination against AIV, as this may potentially lead to a greater reduction in virus shedding conferred by either treatment alone.
Figure 3. Titre of AIV present in oropharyngeal and cloacal swabs on days 4 and 7 post-infection. Twenty-four hours prior to infection, chickens received intramuscular injections of poly I:C (80 μg or 400 μg), LPS (100 μg or 500 μg), CpG ODN (10 μg or 50 μg), non-CpG ODN (50 μg), or PBS. Viral titre was assessed by virus titration in MDCK cells. Graphed values represent the mean log_{10}TCID_{50}/mL of swab fluid in each treatment group, with error bars representing standard error. Statistical significance between treatment groups compared to the infected, PBS treated control group was calculated using an ANOVA followed with a post-hoc Tukey’s test, and results were considered statistically significant if p ≤ 0.05 (*) and p ≤ 0.01 (**) and in the case of CpG ODN, statistically significant from the non-CpG ODN group if p ≤ 0.05 (~).
Figure 4. Relative gene expression of interferons and the interferon inducible gene 2’-5’-oligoadenylate synthetase (OAS) in the spleen on days 2, 4 and 7 post-infection as determined by quantitative RT-PCR. Twenty-four hours prior to infection, chickens received intramuscular injections of poly I:C (400 μg), LPS (100 μg), CpG ODN (50 μg), non-CpG ODN (50 μg), or PBS. Graphed values represent mean gene expression levels relative to the house keeping gene β-actin ± standard error. Statistical significance between treatment groups compared to the infected, PBS treated control group was calculated using an un-paired students t test, and results were considered statistically significant if \( p \leq 0.05 \) (*), \( p \leq 0.01 \) (**), considered statistically significant from the uninfected PBS control group if \( p \leq 0.05 \) (#), and in the case of CpG ODN, statistically significant from the non-CpG ODN group if \( p \leq 0.05 \) (~).
**Figure 5.** Relative gene expression of the cytokines IL-1β, IL-8 and IL-18 in the spleen on days 2, 4 and 7 post-infection as determined by quantitative RT-PCR. Twenty-four hours prior to infection, chickens received intramuscular injections of poly I:C (400 μg), LPS (100 μg), CpG ODN (50 μg), non-CpG ODN (50 μg), or PBS. Graphed values represent mean gene expression levels relative to the housekeeping gene β-actin ± standard error. Statistical significance between treatment groups compared to the infected, PBS treated control group was calculated using an unpaired students t test, and results were considered statistically significant if $p \leq 0.05$ (*) and $p \leq 0.01$ (**), considered statistically significant from the uninfected PBS control group if $p \leq 0.05$ (#), and in the case of CpG ODN, statistically significant from the non-CpG ODN group if $p \leq 0.05$ (~).
Figure 6. Relative gene expression of the interferons and the interferon inducible gene 2'-5'-oligoadenylate synthetase (OAS) in the lungs on days 2, 4 and 7 post-infection as determined by quantitative RT-PCR. Twenty-four hours prior to infection, chickens received intramuscular injections of poly I:C (400 μg), LPS (100 μg), CpG ODN (50 μg), non-CpG ODN (50 μg), or PBS. Graphed values represent mean gene expression levels relative to the house keeping gene β-actin ± standard error. Statistical significance between treatment groups compared to the infected, PBS treated control group was calculated using an un-paired students t test, and results were considered statistically significant if p ≤ 0.05 (*) and p ≤ 0.01 (**), considered statistically significant from the uninfected PBS control group if p ≤ 0.05 (#), and in the case of CpG ODN, statistically significant from the non-CpG ODN group if p ≤ 0.05 (~).
Figure 7. Relative gene expression of the cytokines IL-1β, IL-8 and IL-18 in the lung on days 2, 4 and 7 post-infection as determined by quantitative RT-PCR. Twenty-four hours prior to infection, chickens received intramuscular injections of poly I:C (400 μg), LPS (100 μg), CpG ODN (50 μg), non-CpG ODN (50 μg), or PBS. Graphed values represent mean gene expression levels relative to the housekeeping gene β-actin ± standard error. Statistical significance between treatment groups compared to the infected, PBS treated control group was calculated using an un-paired students t test, and results were considered statistically significant if p ≤ 0.05 (*) and p ≤ 0.01 (**), considered statistically significant from the uninfected PBS control group if p ≤ 0.05 (#), and in the case of CpG ODN, statistically significant from the non-CpG ODN group if p ≤ 0.05 (~).
CHAPTER 4:

General Discussion

Toll-like receptor ligands have the potential to directly activate cells of the immune system thereby enhancing effector functions and the production of cytokines. Here, we report that administration of ligands for TLRs 4 and 21 could up-regulate a variety of immune system genes in the spleen. These genes include the pro-inflammatory cytokines IL-1β and IL-8, as well as those associated with antigen-presentation, namely MHC-II. Importantly, both ligands induced the up-regulation of cytokines associated with anti-viral responses, including IFN-γ and, in the case of LPS, but not CpG ODN, IFN-α. As such, these findings raise the possibility that the TLR-mediated induction of an anti-viral state may confer immunity to a low pathogenic AIV. Indeed, this was the case, as the studies in the present thesis found that the prophylactic administration of poly I:C, LPS and CpG ODN induced a significant reduction in virus shedding, with poly I:C being the most efficacious of the three. Furthermore, analysis of gene expression in the spleen and lungs identified IFN-α and IL-8 as correlates of immunity for poly I:C, and IFN-γ for CpG ODN and LPS.

The administration of TLR ligands prophylactically presents itself as a novel approach to protect chickens against AIV, potentially addressing the several limitations of vaccination. For example, such limitations include a limited efficacy in reducing virus shedding as well as a large time interval (1-2 weeks) between the time of vaccination and the elicitation of protective immune responses. In the present studies, it was shown that TLR ligand prophylaxis may address these limitations, as a significant reduction in virus
shedding was observed when infection occurred as soon as 24 hours post-TLR ligand treatment. This, therefore, raises the possibility that TLR ligand prophylaxis may be effectively employed in different scenarios. For example, if an outbreak were to occur in a farm, all chickens in neighbouring farms within a certain radius may be immediately treated with these ligands to contain the spread. Similarly, if a pen of chickens were to become infected within a farm, TLR ligands may be administered immediately to all of the other pens to limit the spread of the virus. For this to occur, it is imperative that TLR ligands be stockpiled in advance and must be efficacious in conferring immunity to a broad range of influenza virus subtypes. Indeed, considering the fact TLR ligands may be stored for at least a year in a conventional freezer, this is certainly feasible. Importantly, as the immunomodulating effects of these ligands are antigen-independent and non-specific, it is highly likely that they may be efficacious against several AIV subtypes.

What is promising about TLR ligand prophylaxis is that it does not have to be mutually exclusive with existing vaccination campaigns. In fact, it is reasonable to assume that TLR ligand prophylaxis may work synergistically with vaccine induced immunity to further reduce the amount of virus shedding compared to either treatment alone. Furthermore, a complementary effect may potentially be observed if TLR ligands are included in an inactivated whole virus vaccine formulation, as immunity may be conferred within one day post-vaccination by the TLR ligands, thereby protecting chickens until seroconversion to the vaccine antigen occurs. In fact, TLR ligands in this scenario may also act as vaccine adjuvants, thereby inducing even more robust antibody-mediated and cell-mediated responses (Wang et al., 2009; Mallick et al., 2011). If vaccination with vaccines containing TLR ligands were to occur in ovo, not only will
administration of these ligands be facilitated through automation, the protective effects may persist post-hatch as previously demonstrated with respect to other pathogens (Mackinnon et al., 2009; Taghavi et al., 2009).

For the most part, TLR ligand prophylaxis appears to be safe in many different species. For example, in humans several different TLR ligands are either approved for human use or are in phase II/III clinical trials (Kanzler et al., 2007). In chickens, it appears that this is the case as well, as no detectable signs of distress were demonstrated in the present studies. Furthermore, several studies have used TLR ligands in vivo in chickens with no reported signs of toxicity (Dar et al., 2009a; Mallick et al., 2011; Parvizi et al., 2012). Taken together, it is reasonable to assume that TLR ligand prophylaxis may be well tolerated in chickens.

Although TLR ligand prophylaxis shows great promise, there are still a few limitations that need to be addressed. First, it is important to identify ways to enhance the duration of efficacy of these ligands. It has previously been shown in chickens that the protective benefits conferred by the ligands is highest when the challenge occurs within 3 days of treatment, and decreases if this time period is extended to 6 days and is absent at 9 days and onwards (Gomis et al., 2003). One potential reason for this limited duration may be due to the fact that ligands such as CpG ODN and poly I:C may be degraded in vivo by nucleases. As such, these ligands should be formulated in such a way to render them resistant to nucleases, for example with phosphorothioate backbones in the case of CpG ODN (Brown et al., 1994). Similarly, CpG ODN and poly I:C may be encapsulated in protective vehicles, such as nanoparticles, to prevent extracellular degradation and facilitate their delivery to endosomes. As an added bonus, these encapsulated ligands
may subsequently be aerosolized (Evans et al., 2011), thereby addressing the second limitation which is to identify ways to facilitate the administration of these ligands. In the present studies, ligands were administered through the i.m. route, which is laborious and time consuming in a commercial setting, and thereby not practical in the face of an AIV outbreak. However, if these ligands were to be successfully aerosolized while retaining their immunostimulatory properties, large numbers of birds can be treated simultaneously within minutes. Lastly, it is also important to consider the cost-benefit aspect of administering these ligands. For the most part, the costs of these ligands are significantly high, especially in the case of CpG ODN. Therefore, to encourage widespread adoption of TLR ligand prophylaxis, a significant reduction in price of these ligands is required.

An underlying question that remains unaddressed in the thesis is whether or not the protection conferred by these TLR ligands may also extend towards highly pathogenic avian influenza viruses, namely H5N1. Chickens that have been infected with low pathogenic strains like H4N6 tend to have few to no signs of illness, and replication of the virus is typically localized to the cells of the lung and gastrointestinal tract. In contrast, chickens infected with highly pathogenic strains demonstrate significant signs of morbidity which most likely leads to mortality, resulting from rapid dissemination of the virus from the lung and gastrointestinal tract into systemic circulation thereby infecting numerous organs including the brain and kidneys. As such, the difference in pathogenesis and sites of replication may serves as obstacles to reduce the efficacy of TLR ligand treatments against H5N1. Moreover, low pathogenic strains lack many of the virulence factors and immune evasive mechanisms associated with highly pathogenic strains, such as the non-structural protein 1, which acts to suppress the host interferon responses.
Furthermore, it has been demonstrated that highly pathogenic AIV are more resistant to the effects of type I IFNs in chickens (Penski et al., 2011). This presents itself as a potential problem, as immunity conferred by the TLR ligands in the study presented in this thesis was associated with an up-regulation of IFN-α and IFN-γ. Importantly, it is possible that the up-regulation of these cytokines by TLR ligands may in fact exacerbate the signs of disease and increase the incidence of mortality due to the phenomenon of ‘cytokine storm’ and the induction of deleterious immune responses. Despite that, TLR ligands have been shown to protect against both low and highly pathogenic strains in mice, which raises the possibility that TLR ligands may protect chickens against H5N1, and should therefore be the aims of future research to see if this is the case.

Another factor to consider while interpreting some of the results is the fact that the changes in cytokine production and other related molecules was demonstrated at the transcript level and not at the protein level. Although in most cases changes at the transcript level are indicative of similar changes in the protein level, this is not always true. For example, some cytokine genes in certain cell subsets may be translationally, and not transcriptionally regulated, as has been previously shown in murine invariant natural killer T cells (Nagaleekar et al., 2011). On a similar note, it may also be possible that cytokines and other anti-microbial molecules may be stored in pre-formed granules to be released upon TLR stimulation, similar to what has been shown in mammalian platelets and granulocytes (Semple et al., 2011). As such, the results determined by real-time PCR may be slightly different than what would be observed if measuring at the protein level. However, even with these limitations, real-time PCR in chickens is still widely used and
acceptable when analysing cytokine levels, owing in part to the fact that there is a lack of commercially available antibodies against chicken cytokines and surface markers.

Although we speculate that IL-8 and the IFNs may serve as potential correlates of immunity for the TLR ligand treatments, it is presently unclear whether this is indeed the case. It is possible that the changes in gene expression levels observed may not be due to the TLR ligand treatments, but a result of the large differences in virus load amongst the treatment groups. However, to properly address this issue would require blocking the activity of each of these cytokines in vivo, which is possible using the current technology, such as small interfering RNAs or cytokine neutralizing antibodies, and future studies should aim at optimizing these techniques in order to fully assess the roles of each cytokine.

In conclusion, it is clear that TLR ligands may influence the expression of immune system genes in the spleens of chickens as well as confer immunity to AIV. Future studies should focus determining the duration of efficacy of these ligands in this scenario, as well as identifying cost-effective and less labour intensive ways of administering them. Importantly, future studies should determine whether TLR ligand prophylaxis may protect against other subtypes of AIV, especially those which are highly pathogenic.
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