Induction of Immunity against Marek’s Disease using Toll-Like Receptor Ligands

by
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In partial fulfilment of requirements for the degree of Doctor of Philosophy in Pathobiology

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ABSTRACT

INDUCTION OF IMMUNITY AGAINST MAREK’S DISEASE USING TOLL-LIKE RECEPTOR LIGANDS

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University of Guelph, 2018

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Professor Shayan Sharif

Marek’s disease virus (MDV) is a herpesvirus of chickens that causes a lymphoproliferative disease known as Marek’s disease (MD). Vaccines are available to control clinical signs of MD. However, inability of vaccines to prevent replication and transmission of MDV prompts investigation of other possible ways of enhancing host immunity against MD. Innate defense mechanisms may play an essential role in protection against MDV infection in young chicks. Toll-like receptors (TLRs) are an important component of innate defenses and recognize TLR ligands (TLR-Ls) which are primarily microbial constituents. TLR-Ls can be used as prophylactic agents or as adjuvants to enhance immunity against infectious diseases. In this regard, TLR-Ls can be encapsulated in Poly(D, L-lactic-co-glycolic)acid to further enhance host responses by controlled release of TLR-Ls for an extended period. In this thesis, the effect of encapsulated TLR-Ls to improve immunity against MD was investigated.

First, TLR-Ls which inhibit MDV infection were identified as Poly(IC), Pam3CSK4, LPS and CpG. Next, encapsulated CpG and LPS were administered to embryonic day 18 (ED18) chicken embryos for investigating the initiation of innate responses. Cytokine gene expression in the spleen, lung and bursa of Fabricius demonstrated the activation of innate responses. Subsequently, the effect of administration of encapsulated TLR-Ls against MDV infection was
investigated. Significant reduction of tumor incidence was observed in chickens which received encapsulated CpG (ECpG) twice at ED18 via amniotic route and at 14-day post infection via intramuscularly. Association between upregulation of interleukin (IL)-1β and IL-18 and the reduction of tumor formation was observed in response to TLR-L treatments. Finally, the adjuvant effect of ECpG on enhancing the efficacy of herpesvirus of turkey (HVT) vaccine was evaluated. Chickens which were administered ECpG and HVT, exhibited maximum reduction of tumors and significant reduction of MDV load in feathers.

In conclusion, the studies described in this thesis demonstrate that administration of encapsulated TLR-Ls activates innate responses and provides protection against MD in chickens.
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<th>Description</th>
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<tbody>
<tr>
<td>µg</td>
<td>Microgram</td>
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<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
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<td>AIV</td>
<td>Avian influenza virus</td>
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<tr>
<td>BF</td>
<td>Bursa of Fabricius</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BW</td>
<td>Body weight</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CEF</td>
<td>Chicken embryo fibroblasts</td>
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<td>CKC</td>
<td>Chicken kidney cell culture</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CpG</td>
<td>Cytosine-phosphate-Guanine</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post-infection</td>
</tr>
<tr>
<td>ds</td>
<td>Double-stranded</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
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<tr>
<td>ECpG</td>
<td>Encapsulated CpG</td>
</tr>
<tr>
<td>ED18</td>
<td>Embryonic day 18</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ELPS</td>
<td>Encapsulated LPS</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell scanning</td>
</tr>
<tr>
<td>FFE</td>
<td>Feather follicle epithelium</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GaHV-2</td>
<td>Gallid herpesvirus 2</td>
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<td>Gallid herpesvirus 3</td>
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<td>GATA-3</td>
<td>GATA binding protein 3</td>
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<td>gB</td>
<td>Glycoprotein B</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>hps</td>
<td>Hours post-stimulation</td>
</tr>
<tr>
<td>HSV</td>
<td>Human herpes simplex</td>
</tr>
<tr>
<td>HVT</td>
<td>Herpesvirus of turkey</td>
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<td>ICP4</td>
<td>Intra-cellular protein-4</td>
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<td>IFN-α</td>
<td>Interferon α</td>
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<td>IFN-γ</td>
<td>Interferon γ</td>
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<tr>
<td>IFNs</td>
<td>Interferons</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
<td>-----------</td>
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<tr>
<td>ISGs</td>
<td>Interferon-stimulated genes</td>
</tr>
<tr>
<td>Kbp</td>
<td>Kilo base pairs</td>
</tr>
<tr>
<td>LATs</td>
<td>Latency associated transcripts</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MD</td>
<td>Marek’s disease</td>
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<tr>
<td>MD-2</td>
<td>Myeloid differentiation protein-2</td>
</tr>
<tr>
<td>MDV</td>
<td>Marek’s disease virus</td>
</tr>
<tr>
<td>MeHV-1</td>
<td>Meleagrid herpesvirus 1</td>
</tr>
<tr>
<td>meq</td>
<td>Marek’s Disease Virus EcoRI Q</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque-forming units</td>
</tr>
<tr>
<td>Poly(IC)</td>
<td>Polyriboinosinic polyriboctydyl acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly (D, L-lactic-co-glycolic) acid</td>
</tr>
<tr>
<td>pp38</td>
<td>Phosphoprotein 38</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition receptors</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen-free</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin 1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TLR-Ls</td>
<td>Toll-like receptor ligands</td>
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<tr>
<td>TRIF</td>
<td>TIR-domain-containing adaptor protein inducing IFN-β</td>
</tr>
<tr>
<td>v</td>
<td>Virulent</td>
</tr>
<tr>
<td>vv</td>
<td>Very virulent</td>
</tr>
<tr>
<td>vv+</td>
<td>Very virulent plus</td>
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</table>
Introduction

Marek’s disease (MD) is a highly infective, neoplastic viral disease of chickens. MD is caused by Marek’s disease virus (MDV) which belongs to the subfamily *Alphaherpesvirinae* of family *Herpesviridae*. MD causes economic losses to the poultry industry throughout the world. MDV infects chickens via the respiratory route and spreads to lymphoid organs and finally throughout the body of chickens. Pathogenesis of this virus is very complex including replication of MDV in lymphocytes and transformation which results in tumor formation and immunosuppression, and ultimately may lead to death. It is estimated that there is an economic loss around 1-2 billion US dollars related to MD in the poultry industry worldwide (Morrow and Fehler, 2004).

Vaccines are one of the widely used control measures to limit occurrence of MD in chickens. Although live vaccines stimulate the immune system to protect chickens against MD by preventing the appearance of clinical signs in susceptible chickens, the infection, replication and transmission of virulent MDV are not prevented. In addition, the mechanisms of induction of immune responses by vaccines are not well understood. Moreover, replication of virulent MDV in vaccinated chickens facilitates the development of more virulent strains of MDV (Nair, 2005; Schat and Baranowski, 2007). Hence, there is a need for alternative approaches to curtail the problems associated with MD.

Upon viral infection in chickens, both arms of the immune system, including innate defense mechanisms and adaptive immune responses, participate to protect the host from disease. Host responses to MDV are partly dependent on innate responses. Several components of the innate defenses, including germ-line encoded pattern recognition receptors (PRRs), cytokines, cells such as macrophages and natural killer (NK)-like cells, and soluble factors such as nitric
oxide (NO) are involved in the protection against MD in chickens. Toll-like receptors (TLRs) are a class of PRRs which recognize invading pathogens by interacting with appropriate ligands and initiate downstream signaling pathways that lead to production of cytokines and chemokines. Secretion of interferons (IFNs) is one of the initial responses to viral infection and leads to antiviral responses and subsequent adaptive immune responses by differentiation, maturation and proliferation of cells of the immune system. TLR ligands (TLR-Ls) can be used as preventive agents against pathogens or as adjuvants with vaccines. MDV can be recognized by TLRs which leads to the induction of innate responses. In previous studies, the use of TLR-Ls against herpes simplex virus (HSV1 and HSV2) which is similar to MDV, induced protective immunity and suppressed viral replication (Gaajetaan et al., 2012; Gill et al., 2006). Encapsulation of TLR-Ls in Poly (D, L-lactic-co-glycolic) acid (PLGA) may enhance the innate responses by prolonging the availability of TLR-Ls. Particularly, early induction of innate responses may provide protection against MDV during the vulnerable stage in the development of young chicks, immediately after hatching. Hence, studies investigating the role of TLR-Ls encapsulated in PLGA nanoparticles to provide immunity against MD are warranted. The main objective of the studies presented in this thesis was to determine the protective effect of TLR-Ls encapsulated in PLGA nanoparticles against MDV in chickens.
Marek’s disease

Marek’s disease in chickens is caused by a DNA virus and the disease is characterized by paralysis, T cell lymphomas and immunosuppression. Chickens contract the virus via the respiratory route and clinical signs include depression, ruffled feathers, droopy wings, huddling, weight loss, limping and paralysis in susceptible chickens. Tumors can be detected in visceral organs, nervous tissues, lymphoid tissues, muscle and skin upon gross pathological examination. Infiltration of lymphocytes in addition to production of cytokines, NO and edema of the brain are associated with neurological disorders and paralysis which restrict the movements of the birds and lead to starvation and death (Gimeno et al., 2001; Jarosinski et al., 2005). Occurrence of transient paralysis is suggested to be associated with the expression of cytokines, including pro-inflammatory cytokines, due to inflammation of brain tissues (Abdul-Careem et al., 2006b). Morbidity and mortality due to MDV can reach more than 90% in genetically susceptible chickens (Osterrieder et al. 2006). However, genetically resistant chickens do not show clinical signs and are resistant to tumor development caused by mild, virulent and very virulent strains of MDV.

Marek’s disease virus

An infective agent causing MD was first identified as a cell-associated herpes-type virus in chicken kidney cells (Churchill and Biggs, 1967). Later, the infective form of enveloped MDV was detected in feather follicular epithelium (FFE) of chickens (Calnek et al., 1970; Nazerian and Witter, 1970). MDV is currently categorized under family Herpesviridae, subfamily Alphaherpesvirinae, genus Mardivirus, species Gallid herpesvirus (GaHV)-2 (Biggs, 2004;
It is a double-stranded DNA virus with an approximately 178 kbp nucleotide-containing linear genome that encodes approximately 103 proteins (Tulman et al., 2000). All oncogenic MDV classified under GaHV-2, previously known as MDV serotype 1, consist of four pathotypes based on the pathogenicity such as mild, virulent, very virulent and very virulent plus (Witter et al., 2005). Strains of MDV grouped under GaHV-3, earlier considered as MDV serotype 2, are non-oncogenic in chickens. Herpesvirus of turkey (HVT) was categorized as *Meleagrid herpesvirus 1* (MeHV-1), which was considered MDV serotype 3 under the previous classification, is also non-oncogenic in chickens. Increasingly virulent strains of MDV have emerged at different periods of time in the history since it was first identified. MDV is distributed worldwide in varying epidemiological patterns due to its highly contagious nature and general practice of intensive poultry production.

**Pathogenesis of Marek’s disease virus**

MDV exhibits a complex pathogenesis. Depending on the genetic makeup of the host, the outcome of MDV pathogenesis varies. Chickens become infected with cell-free MDV from the dust in the contaminated environment. Transmission of the virus into the respiratory tract of chickens occurs via inhalation. It takes approximately 18-20 hours (h) for replication of the virus (Baigent and Davison, 2004). However, viral replication can take place as early as 12 h after infection with very virulent RB1B MDV which is identified by the expression of glycoprotein B (gB) and viral version of interleukin-8 (vIL-8) (Abdul-Careem et al., 2009a). From the respiratory tract, the virus is transported to other lymphoid organs such as spleen, bursa of Fabricius and thymus. It is believed that macrophages carry the virus to lymphoid tissues (Calnek, 2001; Chakraborty et al., 2017). In addition, infiltration of macrophages into the bursa
of Fabricius indicates the involvement of these cells in the distribution of MDV in chickens (Abdul-Careem et al., 2008b).

Generally, pathogenesis can be categorized into four phases in susceptible chickens from the point of infection to tumor formation (Baigent and Davison, 2004). The first, early cytolytic, phase occurs in the lymphocytes of thymus, bursa of Fabricius and spleen. During this phase, MDV replicates in lymphocytes, mainly in B cells and to a lesser extent in T cells, between two to seven days post-infection (dpi) and causes lysis of these lymphocytes (Calnek, 2001; Baigent and Davison, 2004). A transient immune suppression can occur due to the lysis of cells at this stage. In this phase, MDV antigens such as phosphoprotein (pp) 38 and glycoprotein B (gB) can be identified in these primary and secondary lymphoid organs (Calnek, 2001; Calnek and Schat, 1984).

Host and virus-related factors trigger the commencement of the second phase, i.e. the latent phase, which occurs from 7 to 10 dpi (Buscaglia et al., 1988). In this phase, MDV develops latency mainly in CD4+ T cells (Calnek and Schat, 1984). The majority of the viral genes that are expressed in the cytolytic phase ceases their expression, instead expression of pp14, latency associated transcripts (LATs) and Marek's EcoRI-Q-encoded protein (meq) can be identified in this phase (Parcells et al., 2003). Maintenance of latency is governed by meq, which inhibits apoptosis of latently infected CD4+ T cells (Baigent et al., 2006). During latency, MDV DNA can be integrated into the host genome (Delecluse et al., 1993; McPherson et al., 2016; Robinson et al., 2010) or possibly exists as nonintegrated, closed circular DNA (Tanaka et al., 1978).

The next phase is the late cytolytic and immunosuppressive phase. MDV is re-activated from latency in T cells and causes lysis of more lymphocytes (Baigent and Davison, 2004).
Depending on pathotype of MDV strains, time of reactivation for this phase varies following MDV infection. The stimulus for this re-activation is not known yet. Both cell-mediated and antibody-mediated immune responses are affected, and this may result in permanent immune suppression.  

In the last phase, transformation and proliferation of T cells occur around 19-28 dpi onwards, depending on pathotype and strains of MDV. Some of the latently infected CD4+ T cells transform into tumor cells, proliferate and form tumors which are heterogenous in nature. Meq gene expression is required for initiation and maintenance of the transformation process (Xie et al., 1996; Levy et al., 2005). This transformation occurs in genetically susceptible and unvaccinated chickens but not in resistant and vaccinated birds. When resistant or vaccinated birds become infected with a virulent strain of MDV, their T cells become latently infected, but viral pathogenesis does not progress further. However, these chickens continue to produce infectious cell-free MDV. Genetic susceptibility or resistance is mainly associated with genetic variation of the Major Histocompatibility Complex (MHC), as some genetic lines of chickens are resistant to MD (B21, B2 MHC haplotype), while others are susceptible to the disease (B1, B4, B5, B12, B13, B15, B19 MHC haplotype) (Bumstead and Kaufman, 2004).  

Latently infected and transformed T cells disseminate MDV throughout the body. The virus reaches the epithelial cells of the feathers which is the site of productive replication of MDV (Calnek et al., 1970). T cells which carry MDV become imbedded during the keratinization process in feather follicular epithelium and MDV is transferred to epithelial cells of the feathers (Couteaudier and Denesvre, 2014). The keratinization process preserves the MDV from degradation and enables the continuous shedding of MDV from feathers. The infectious form of enveloped, cell-free MDV is released into the environment via sloughing off of the
keratinized epithelial cells around 10 dpi onward (Nair, 2005). MDV life cycle continues with the spread of infectious MDV to a new susceptible host.

In addition to the genetic background of the host, other factors influence the outcome of the disease, such as pathotype of the virus, status of maternal antibodies, age of the host, environmental factors such as transportation and stress, and presence of other immunosuppressive agents such as Infectious Bursal Disease Virus, Chicken Anemia Virus and Avian Leukosis Virus.

**Host immune responses to Marek’s disease virus**

When a chicken encounters MDV, host responses are initiated. Development of immunity against MD is associated with multiple factors. Several components of the immune system, such as macrophages, NK-like cells, T helper cells (Th cells), cytotoxic T lymphocytes (CTLs), cytokines, antibodies and NO are involved in host immune responses. Both innate and adaptive defense mechanisms are equally important in response to MDV infection.

**Innate immunity to MDV**

The innate responses are the immediate responses which are elicited when a host senses a pathogen through PRR such as TLRs. TLRs are present in different cell types of chickens including cells of the immune system (Iqbal et al., 2005). These cells interact with nucleic acids, viral proteins and lipid-based ligands present in the virus via TLRs. In chickens, 10 TLRs have been identified, including TLR1 type 1 and 2; TLR2 type 1 and 2, and TLRs 3, 4, 5, 7, 15 and 21 (Temperley et al., 2008). Interactions between TLRs and their ligands subsequently trigger the signaling cascades, leading to induction of innate responses. Details of interactions between TLRs and their ligands in the context of MDV infection have not been studied yet. However, MDV might be recognized by TLRs 2, 4, and 21 (the functional ortholog of mammalian TLR9).
as it has been shown in other herpesviruses which have similar genomic organization compared to MDV (Paludan et al., 2011; Villalba et al., 2012). In addition, TLR3, which interacts with double-stranded RNA (dsRNA), might recognize MDV since dsRNA is an interim product during replication of DNA viruses (Akira et al., 2006). In agreement with this notion, the upregulation of TLR3 was observed in the lungs of chickens when they were infected with MDV using an aerosol-based infection model (Abdul-Careem et al., 2009b).

Cytokines play a critical role in orchestrating host responses to MDV. Differential expression of various cytokine genes was observed in several tissues and cells of MDV-infected chickens. In addition, genetic background of chickens, pathotype of MDV strains and stages of MDV lifecycle also influence the expression pattern of cytokines and related genes. The first response of the innate immune system to viral infection is the secretion of IFNs, which exert antiviral activities. For example, IFN-γ inhibits MDV replication directly or indirectly via NO production by macrophages and its expression is usually upregulated in MDV-infected chickens (Djeraba et al., 2000; Xing and Schat, 2000). Jarosinski and colleagues reported that IFN-γ expression was higher in genetically susceptible chickens than in resistant chickens following MDV infection (Jarosinski et al., 2005). In contrast, Kaiser and colleagues demonstrated that there was no difference in the magnitude of IFN-γ upregulation between genetically resistant and susceptible lines of chickens (Kaiser et al., 2003). Consistent with this finding, no difference in the expression of IFN-γ was reported in CD4+ and CD8+ T cells from genetically resistant and susceptible line of chickens (Parvizi et al., 2009). On the other hand, a slight increase of IFN-γ was detected in blood from genetically resistant birds compared to susceptible birds (Quéré et al., 2005). These observations raise the possibility that the association between IFN-γ and genetic resistance/susceptibility to MD may be dependent on host and virus factors. In addition to IFN-γ,
the role of other cytokines in genetic resistance to MD has been studied. For instance, an association between elevated levels of IL-18 and IL-6 gene expression and susceptibility against MDV infection was reported (Kaiser et al., 2003). In agreement with this finding, lower expression of IL-18 and IL-6 was noted with protection against MD in a vaccine-challenge model (Abdul-Careem et al., 2007a). Although IL-18 induces IFN-γ production (Gobel et al., 2003), it also possibly induces IL-10 secretion since IL-18 has been shown to also promote Th2 cytokines (Hoshino et al., 1999; Yoshimoto et al., 2000). Hence, it is feasible that lower expression of IL-18 leads to a decrease in IL-10 secretion which results in enhanced host responses to provide protection against MDV infection. IL-10 is known to curb Th1 responses which are necessary for protection against viral infections (Rothwell et al., 2004). This might be the case with MDV infection, since diminished expression of IL-10 was reported to be related with protection against MDV infection in chickens (Abdul-Careem et al., 2007a; Parvizi et al., 2009). Although several studies investigated the link between cytokine expression and protection against MDV infection, causal association between cytokines and immunity to MD is not well understood.

While Th1 responses are important for the control of MDV infection, there is evidence for the contribution of Th2 cytokines. Heidari and colleagues reported the upregulation of IL-4, IL-13 and IL-10 during the lytic phase of MDV infection (Heidari et al., 2008). In another study, the expression of GATA-3, which is the transcription factor responsible for Th2 differentiation, was observed in spleens of MDV-infected chickens (Sarson et al., 2006). This array of observations indicates that Th2-driven immune responses may also play a role against MDV infection. In fact, the available information indicates a complex association of several factors including cytokines involved in immunity against MD. It is possible to speculate that a fine
balance of cytokines and immune-related components is required in order to enhance host responses to achieve optimal protection against MDV infection.

Virulence of MDV strains influences host responses against the virus. For instance, vv+ strain of MDV dramatically elevated the expression of IL-6, IL-1β, IFN-α, IFN-γ, IL-8 and iNOS in brain of chickens compared to uninfected chickens (Jarosinski et al., 2005) whereas the v strain of MDV induced much lower levels of these cytokines. In another study, vv strain of MDV increased the expression of IL-6, IL-12 and IFN-γ in brain tissues of chickens with transient paralysis (Abdul-Careem et al., 2006b) when compared to uninfected controls. These observations show that more virulent strains of MDV induce a broad spectrum of cytokines and immune-related genes which might be associated with higher replication of more virulent strains than less virulent strains of MDV.

Feathers of chickens are the privileged site for production of infectious cell-free MDV. In order to produce cell-free virus, there should be a unique milieu in feathers. In addition, such a feature might have the ability to dampen the host-induced immune responses in feathers to enable successful replication of the virus. Little is known about replication of MDV and host responses in feathers. Significant upregulation of IFN-γ, IL-18, IL-6 and MHC class I was observed in feathers of MDV-infected chickens (Abdul-Careem et al., 2008c). In addition, increased expression of several immune-related genes, including IFN-γ, IFN-α, IL-1β, IL-6, IL-18, IL-10, iNOS, MHC class I and MHC class II was detected in the skin of MDV-infected chickens (Heidari et al., 2016). Conversely, in the same study, extensive viral replication was apparent in infected skin. This observation indirectly indicates the presence of an immunosuppressive environment in feathers, a notion that is further extended by the observation of decreased expression of IFN-β and CD107a in skin with MDV infection (Heidari et al., 2016).
In addition to soluble mediators of immunity, cells of the immune system should be considered to better understand mechanisms of immunity against MD. Following viral infection, macrophages, which are one of the cell types of the innate immune system, become activated. MDV infection increases the presence of macrophages in the lungs and bursa of Fabricius of chickens (Abdul-Careem et al., 2008b; 2009a). Macrophages have been shown to play a role in the inhibition of MDV via NO production and in phagocytosis of MDV infected cells (Djeraba et al., 2000; Lee, 1979; Xing and Schat, 2000). In addition, macrophage-derived NO suppresses the activation and proliferation of T cells which might reduce the number of T cells available for MDV infection (Xing and Schat, 2000). It has been shown that higher inhibition of MDV replication can be achieved by increasing the concentration of NO (Xing and Schat, 2000).

Another cell type of the innate immune system is NK cells which have antiviral activity against HSV in mammals (Ashkar and Rosenthal, 2003). NK cells control viral replication by activating the apoptosis of virus-infected cells. In chickens, it is not clear whether NK cells similar to those in mammals are present. However, enhanced NK-like cell activity has been observed with MDV infection (Garcia-Camacho et al., 2003) and with MD vaccination (Heller and Schat, 1987). A progressive increase in NK-like cell activity was reported against MD tumor cells, which indicates a probable antitumor role of NK-like cells in chickens (Quere et al., 1988).

Another cell type of interest is γδ T cells which are present at high frequency in chickens compared to other species such as mice and humans. Little is known about these cells in relation to MDV infection. These cells play an important role in immunity against HSV2, another member of the Alphaherpesvirinae subfamily (Nishimura et al., 2004). Recent studies on MDV infection in chickens have shown an increased accumulation of γδ T cells in spleen (Laursen, 2017) and skin tissues (Heidari et al., 2016) of chickens. Furthermore, γδ T cells expressed IFN-γ
and IL-13 at the early stage of MDV infection, but then they switched to increased expression of IL-10 at the later stage of MDV infection (Laursen, 2017). These observations indicate possible involvement of \( \gamma \delta \) T cells against MDV infection in chickens.

**Adaptive immune responses to MDV**

Initial induction of innate responses leads to adaptive immune responses which consist of cell-mediated and antibody-mediated immune responses. The cell-associated nature of MDV prompts mainly the cell-mediated immune responses to be protective rather than antibody-mediated responses. In fact, immunity against MD conferred by antibody-mediated immune responses is not clearly described. However, entry of MDV into target cells seems to be blocked by the neutralizing antibodies produced against gB of MDV (Davison and Kaiser, 2004). In addition, presence of maternal antibodies in chicks has been shown to hinder the appearance of clinical signs, reduce viral replication, tumor formation, morbidity and mortality (Davison and Kaiser, 2004). Despite the presence of neutralizing antibodies produced against MDV glycoproteins, non-neutralizing antibodies are also presumably involved in the prevention of MDV entry into cells (Davison and Kaiser, 2004).

When MDV-derived antigens are presented to CD4+ or CD8+ T cells, cell-mediated immune responses are initiated. Increased MDV titers in CD4+ T cells following removal of CD8+ T cells by monoclonal antibodies in vaccinated chickens indicates the importance of CD8+ T cells in controlling MDV (Morimura et al., 1998). MDV-specific CTLs were identified using cell lines expressing MDV antigens and were shown to be important in elimination of MDV-infected cells (Omar and Schat, 1997). Further, it was reported that CTLs act against various MDV glycoproteins and facilitate cell-mediated immunity (Markowski-grimsrud and Schat, 2002). For example, immunization with recombinant fowlpox virus expressing gB of
MDV induced the expression of gB-specific CTLs in chickens (Omar et al., 1998). Along the same line, increased expression of CD8α and granzyme-A, which are related to CTL activity, was observed in MDV-infected chickens (Sarson et al., 2006, 2008), raising the possibility of involvement of CTLs in immunity. Analysis of cytokine gene profiles of CD4+ and CD8+ T cells highlights the production of an array of cytokines polarizing the response towards a Th1 milieu, which possibly enables CD4+ and CD8+ T cells to perform helper and effector functions, respectively (Parvizi et al., 2009). Infiltration of CD4+ and CD8+ T cells in feather follicles of chickens infected with MDV, as well as accumulation of CD8+ T cells in feathers of vaccinated chickens, suggests that the adaptive immune response actively tries to eliminate MDV-infected cells in feather follicles (Abdul-Careem et al., 2008c; Abdul-Careem et al., 2008). Furthermore, CTL responses appear to play a role in genetic resistance to MDV infection in chickens. MD-resistant chickens exhibited higher CTL responses against intracellular protein 4 (ICP4) compared to susceptible chickens (Omar and Schat, 1996). In feathers, a gradual increase in the numbers of CD4+ and CD8+ T cells in the feather pulp was also demonstrated until 10 dpi (Abdul-Careem et al., 2008c). Vaccination with Rispens-CVI988 and HVT vaccines also triggered increased infiltration of CD8+ T cells and a transient increase in IFN-γ mRNA expression in feather pulp (Abdul-Careem et al., 2008). Although the presence of T cells and expression of cytokines in the feather pulp with MDV infection and vaccination indicates the existence of active host responses, these responses are not effective at controlling MDV replication and shedding. The nature of MDV and the limitation of available reagents limit the investigations of cell-mediated immune responses against MDV. Further studies are needed to dissect the involvement of CD4+ and CD8+ T cells exerting antiviral and antitumor immunity against MDV in chickens.
Despite the understanding of T cell involvement in immunity against MDV, there is a paucity information about the MDV epitopes recognized by T cells. In the process of identifying the epitopes of MDV antigen such as ICP4, pp38, gB and meq, Schat and Xing (Schat and Xing, 2000) determined that some of the CTL epitopes were in the C terminal region of gB. Based on this finding, an attempt to narrow down epitopes, a preliminary study in our laboratory found that T cells from vaccinated chickens were unable to proliferate during re-stimulation with various peptide pools from gB and pp38 antigens. This might be due to the weak binding of peptides with MHC molecules or the mild immunosuppressive nature of commercial vaccines used in this study. As a result, the exact sequence of the epitopes has yet to be determined.

Antitumor immunity

Although immunity against tumor development occurs with vaccination, the mechanism of protection involved in preventing appearance of tumors is not known. In addition, tumor-specific antigens (non-virus coded) have not been identified yet. Further, there is no substantial evidence for the contribution of CTL in antitumor immunity (Schat, 1991). Although there is much supporting information for NK-like cell activity in genetically resistant chickens against MD tumors, controversial observations have also been noted. MDV infection increased NK-like cell activity, however, it was decreased when tumors started to develop (Schat, 1991). Further, NK cell lysis assays suggest that NK-like cells were unable to lyse MD tumor cells in vitro (Schat, 1991). Macrophages may contribute to defend against tumors since an increase in the incidence of tumors was observed when the function of macrophages was inhibited (Schat, 1991). It is possible that antibody-dependent cell-mediated cytotoxicity might play a role in antitumor immunity and lysis of virus-infected cells (Kodama et al., 1979; Schat and Markowski-Grimsrud, 2001). Nonetheless, there is no report of tumor-specific antibodies against
MD tumors. Therefore, more research is needed to understand the antitumor immunity against MD.

**Marek’s disease vaccines and immune responses induced by vaccination**

MD vaccines are the first commercial vaccines that were developed against a neoplastic disease. Vaccines, which can be used alone or in combination, are produced from *GaHV-2*, *GaHV-3* and *MeHV-1* (Morimura et al., 1998). In 1970, the monovalent HVT vaccine (*MeHV-1*) was first used and from the mid-1980s, a bivalent vaccine consisting of HVT plus SB-1 (*GaHV-3*) has been used. Since 1990s, the Rispens-CVI988 (attenuated *GaHV-2*) vaccine has been used by the poultry industry (Baigent et al., 2006). From time to time, different vaccines have been introduced to overcome the evolving virulent field strains of MDV. The use of vaccines has decreased the incidence of MD and associated economic losses to a certain extent.

Efficacy of MD vaccines varies with the virulence of MDV strains. Although the live attenuated Rispens-CVI988 vaccine is more effective compared to other currently available vaccines, vMDV and vvMDV can be protected by HVT and HVT plus SB-1, respectively (Buscaglia et al., 2004). Chickens infected with vvMDV and vv+MDV can be protected by the Rispens-CVI988 vaccine (de Boer et al. 1986, Witter 1992).

MD vaccines are unable to prevent the infection of chickens with virulent strains of MDV, which results in persistent replication and shedding throughout the life of infected chickens. Superinfection with virulent MDV can occur in the presence of vaccine virus after MD vaccination (Baigent et al 2006). Both virulent and vaccine strains of MDV can exist in vaccinated chickens for the rest of their life. Vaccination prolongs the infectious period of virulent strains by extending the lifespan of chickens and leads to the shedding of MDV into the environment for a longer period of time (Read et al., 2015). In order to adapt and replicate in the
vaccinated host, MDV possibly evolves by changing its genetic structure to evade the immune responses induced by vaccination (Nair, 2005). Therefore, it is possible that selection pressure placed on virulent strains by vaccination leads to the evolution of more virulent strains. Continuous evolution of virulent MDV strains has occurred despite the use of various MD vaccines (Nair, 2005). In addition to the use of MD vaccines, which fail to curtail virus shedding, other factors such as intensive farming facilitate the emergence of new, virulent MDV strains which will spread among chickens and overcome the protection provided by current vaccination. Therefore, it is necessary to improve host immune responses to MDV. This can be achieved by the development of a novel vaccine or incorporation of other measures such as the use of Toll-like receptor ligands (TLR-Ls) and cytokines as adjuvant with current MD vaccines.

Induction of immunity by MD vaccines depends on several factors including virulence of MDV strains, genetic background of chickens, presence of maternal antibodies, presence of other infectious agents and the interval between vaccination and infection. Vaccine-induced immunity against MD is not well studied. MD vaccination leads to a reduction in cytolysis of immune system cells during the early stage of pathogenesis of MDV and development of tumors. It has been demonstrated that vaccination with HVT significantly reduces tumor formation and MDV load, which indicate that vaccination exerts both antiviral and antitumor immunity against MD (Witter et al., 1976). Immune responses induced by MD vaccines are presumably similar to those induced by virulent MDV. For instance, a significant upregulation of IFN-γ was observed with MD vaccination (Abdul-Careem et al., 2007a) as well as in MDV infection (Schat and Xing, 2000). In addition, the role of IFN-γ in vaccine-induced immunity was demonstrated by administration of recombinant chicken IFN-γ and knockdown of IFN-γ by small interfering RNA technology (Haq et al., 2011, 2015). On the other hand, decreased expression of IL-18 and IL-6
is reported to be associated with vaccine-induced protection against MD (Abdul-Careem et al., 2007a). However, the underlying role of these cytokines in immunity induced by MD vaccination is not clearly understood.

With respect to adaptive immune responses, although virus-neutralizing antibodies appear 1-2 weeks after vaccination, protection provided by antibody-mediated immune responses is not well documented (Morimura et al., 1998). It seems that T cell responses are central to the vaccine-induced immune responses (Schat and Calnek, 1978; Gupta et al., 1982). Administration of Rispens-CVI988 was shown to reduce replication of a virulent strain of MDV in feathers (Haq et al., 2012) as well as to significantly increase infiltration of CD8+ T cells in feathers (Abdul-Careem et al., 2008c). However, MD vaccines are unable to inhibit shedding of MDV from feathers. It has been postulated that apoptosis of CD4+ T cells, and contributions of γδ T cells and NK-like cells might be crucial for vaccine-induced antitumor immunity (Morimura et al., 1998). In support of this view, the observation of enhanced NK-like cell activity with vaccination of HVT plus SB-1 suggests the contribution of NK-like cell function in vaccine-induced immunity (Heller and Schat, 1987).

MD vaccines can be administered at embryonic day (ED) 18 via in ovo (amniotic) route or on the day of hatch via the subcutaneous route. The first MD vaccine which was administered to ED18 embryos was the HVT vaccine (Sharma and Burmester, 1982). In ovo administration of vaccines provides greater protection against challenge compared to post-hatch vaccination (Schijns et al., 2013). Enhanced NK-like cell activity was reported with in ovo HVT vaccination compared to at-hatch HVT vaccination (Sharma et al., 1984). This may be due to the accessibility of wider mucosal surface for vaccine virus to enter and initiate host responses with in ovo vaccination. In contrast to subcutaneous administration after hatch, vaccines administered
into the amniotic fluid possibly enter the embryos by ingestion via the oral cavity and by the sucking reflex via cloaca. Localization of substances administered \textit{in ovo} was investigated in several tissues by histological methods at different time points and it was identified that substances first localized in the lungs and bursa of Fabricius of late-stage embryos (Jochemsen and Jeurissen, 2002). Additionally, it was reported that soluble substances which enter the cloacal cleft are imbibed into the lumen of the bursa of Fabricius by unknown mechanisms (Oláh et al., 2013). It is suggested that negative pressure within the lumen of the bursa of Fabricius might cause the suction effect. Further, amniotic drinking in late-stage embryos and absorption from the intestines might possibly lead to the dissemination of \textit{in ovo}-administered substances to systemic organs, including the spleen.

**Development of the chicken immune system**

The immune system of chickens consists of primary lymphoid tissues, including the thymus, bursa of Fabricius and bone marrow, and secondary lymphoid tissues including the spleen, mucosal-associated lymphoid tissues and diffused lymphoid aggregates instead of lymph nodes (Oláh et al., 2013). The thymus starts to develop as a rudimentary structure at ED3 and its well-demarcated internal structures can be identified at ED12 (Fellah et al., 2008). T cells can first be detected in the thymus at ED15. Similar to the thymus, splenic primordium starts to appear at ED3 and specialized immunoreactive reticular fibers, which are characteristics of lymphoid tissues, can be identified at ED15 (Yassine et al., 1989). A week after hatching, the adult spleen structure can be detected (Jeurissen, 1991). T cells migrate to the spleen in three separate waves at different stages of the development (Dunon et al., 1997). The first migration occurs from ED15 to ED20 and the second movement occurs from ED21 to day (D) 6 post-hatch followed by the third wave which takes place between D7 and D9 (Dunon et al., 1997).
Constitutive expression of cytokines IFN-γ, IL-18, IL-4 and IL-10 is detectable from ED12 in the spleen and the expression of all these cytokines increases at D7 (Abdul-Careem et al., 2007b). The expression pattern of these cytokines coincides with the homing pattern of T cells in the spleen. The other primary lymphoid structure of chickens, i.e. the bursa of Fabricius, develops between ED4.5 and ED10 and becomes colonized with pre-B cells between ED11 and ED14. In chickens, development of the immune system begins at an early stage of embryonation (i.e ED3) and it becomes fully developed after hatching (around 2 weeks).

Maturation of the immune system is a gradual process which occurs until a few weeks post-hatch. There are differences between embryos and adults in structural and physiological components of the immune system. Although the development of the immune system is not complete in ED18 embryos, it is able to trigger host responses against antigenic stimulation. This is supported by the fact that, in ovo administration of immunostimulants or vaccines induces host responses in chickens against microorganisms (Sharma and Burmester, 1982; Taghavi et al., 2009; Dar et al., 2014). Both arms of the immune system, innate and adaptive, are necessary to provide immunity against infectious diseases. Generally, induction of innate responses plays a vital role in the first few days post-hatch until the fully mature adaptive immune system offers a major role in protection. Involvement of cytokines, macrophages and NK-like cells in the protection against pathogens in young chicks indicates the important role of innate responses (Haq et al., 2013; Thapa et al., 2015; Abdul-Cader et al., 2018b). However, the adaptive immune system manifests suboptimal responses to antigenic exposure in early life. It has been shown that D12 chicks mounted significant antibody responses compared to early post-hatch chicks and embryos (Mast and Goddeeris, 1999). In addition, higher titers of antibodies were detected when chickens were vaccinated against infectious bronchitis virus at two weeks of age compared to
one-day-old and one-week-old chicks (Van Ginkel et al., 2015). Further, it has been reported that although T cells from day-old chicks were phenotypically mature, they were unable to proliferate and produce cytokines compared to T cells from week-old chicks (Lowenthal et al., 1994). All these observations indicate that the adaptive immune system becomes functionally competent around two weeks of age in chickens and innate responses are crucial in the first few days of post-hatch life. However, exposure to antigens at an early age possibly accelerates the maturation of the immune system (Hegde et al., 1982; Gimeno et al., 2015). It has been suggested that the development of bronchial-associated lymphoid tissues depends on antigenic stimulation and it becomes mature a few weeks after hatching (Reese et al., 2006). Further, maturation of lymphoid tissues in germ-free chickens has been shown to be enhanced by antigenic stimulation from gut microbes (Hegde et al., 1982). Importantly, in ovo stimulation of chick embryos may have a profound effect on the development of their immune system. For instance, in ovo administration of HVT vaccine resulted in the expansion of various immune system cell types and the vaccinated chickens were more responsive to distinct antigenic exposure (Gimeno et al., 2015).

Modulating immune responses using TLR ligands

Toll-like receptors and their ligands

Pattern recognition receptors are a part of the innate immune system. PRRs recognize and respond to PAMPs and are classified into different categories, such as TLRs, nucleotide-binding oligomerization domain like receptors (NLR) and retinoic acid inducible gene-1 like receptors (RLR) (Kawai and Akira, 2010).

TLRs are transmembrane glycoproteins which are conserved throughout the evolution from low-order worms to high-order mammals (Kawai and Akira, 2010). TLRs are made up of three distinct parts, such as the N-terminal leucine-rich repeats, the transmembrane domain and
the Toll/IL-1 Receptor (TIR) homology domain. PAMPs interact with the extracellular domain of TLRs, which contains a number of leucine-rich repeats (Kawai and Akira, 2010). Following that interaction, the conformational change of the TLRs allows communication with adapter molecules via the intracellular domain (Kawai and Akira, 2010). These communications initiate downstream signaling pathways for gene expression via activation of transcription factors that lead to expression of several molecules, including cytokines, chemokines, adhesion molecules, and upregulation of MHC molecules (Mogensen, 2009). Although 13 TLRs have been identified in mammals, there are 10 TLRs present in chickens, namely TLR1 type 1 and 2; TLR2 type 1 and 2, TLR3, TLR4, TLR5, TLR7, TLR15 and TLR21 (Temperley et al., 2008). Despite the presence of several evolutionarily similar TLR orthologs in mammals and chickens, TLR21 is identified as a unique TLR to chickens. Among these TLRs, TLR1, 2, 4 and 5 are present on the cell surface and recognize molecules from extracellular pathogens (Mogensen, 2009). TLR3, 7 and 21 are present in the endosomal compartments and recognize microbial components present in the cytosol of the cell, such as DNA and RNA (Mogensen, 2009). In chickens, TLRs have been identified in many tissues, including the thymus, spleen, bursa, lungs, liver, kidney, brain and muscles and in various types of cells, such as macrophages, heterophils, B cells, T cells, kidney cells and embryo fibroblasts (Iqbal et al., 2005). Each TLR responds to a different type of PAMP. TLRs that recognize microbial nucleic acids are TLR3, 7 and 21. TLR3 and 7 detect viral double-stranded RNA (dsRNA) along with synthetic dsRNA-like polynosinic:polycytidylic acid (Poly (IC)) and single-stranded RNA, respectively. TLR21, similar to mammalian TLR9, detects unmethylated CpG DNA (Brownlie et al., 2009). TLRs that sense lipid-based structures are TLR2 and 4. Peptidoglycan, lipopeptide and lipoproteins of bacteria, including synthetic lipoprotein such as Pam3CSK4 are recognized by TLR2, and lipopolysaccharide (LPS) of gram-
negative bacteria is recognized by TLR4. TLR5 is a protein-based receptor which recognizes flagellin of bacteria. In regard to MDV, similar to other herpesviruses, glycoproteins and double-stranded DNA of the virus may be recognized by TLR2 and 21, respectively (Paludan et al., 2011). In addition, TLR4 might become activated directly through viral components of MDV or indirectly via MDV-induced danger signals, as has been shown in other herpesviruses (Villalba et al., 2012). TLR3 recognizes dsRNA which is a transitional product in the formation of DNA viral progeny (Akira et al., 2006). Therefore, it is possible for the chicken innate immune system to recognize dsRNA by TLR3 during MDV infection.

As mentioned above, assembly of PRRs and appropriate ligands leads to subsequent signaling pathways. Depending on the type of TLR, myeloid differentiation factor 88 (MyD88) or toll interleukin receptor 1 (TIR) domain-containing adaptor protein (TIRAP) inducing interferon (TRIF) are involved in the signaling pathways (Kawai and Akira, 2010). Involvement of MyD88 directs the signaling pathways to the secretion of pro-inflammatory cytokines such as IL-1β and IL-6 and also type I IFNs via activation of NF-κB and mitogen-activated protein kinases (MAPKs). In this regard, interaction of MyD88 with TLR2 and 4 leads to the production of pro-inflammatory cytokines whereas interaction with TLR7 and 21 leads to the secretion of type I IFNs (Kawai and Akira, 2010). Association of TRIF leads to the secretion of type I IFNs via IFN regulatory factors (IFRs) (Kawai and Akira, 2010). All TLRs recruit MyD88 as part of the downstream signaling cascade with the exception of TLR3 (Kawai and Akira, 2010). TLR3 recruits TRIF and induces interferon production. In the case of TLR4, which interacts with both MyD88 and TRIF, interactions with its ligands culminate in the production of proinflammatory cytokines and type I IFNs (Lester and Li, 2014).
Use of TLR ligands

Interaction of TLRs and their ligands initiates innate defense mechanisms and leads to adaptive immune responses against pathogens. TLR-Ls have been used against viral and bacterial diseases, allergic reactions and as anti-cancer agents. TLR-Ls contribute to induction of host immune responses in several ways such as increasing the expression of MHC and co-stimulatory molecules and enhancing cross-presentation by antigen-presenting cells (APCs), improving T cell memory; and also improving phagocytosis (Gupta et al., 2014). In addition, TLR-Ls induce maturation of DCs and activation of T cells which are essential for activation of adaptive immune responses (Michelsen et al., 2001; Liang et al., 2013;). Generally, Th1 responses rather than Th2 responses are induced by most TLR-L stimulations (Akira et al., 2006) which is important to elicit antiviral responses. However, targeted activation of TLRs can be achieved to provoke a desired immune response to act against a pathogen. Various TLR-Ls favor the induction of Th1 or Th2 responses when used as an adjuvant with vaccines (Toussi and Massari, 2014).

There is evidence for the enhancement of immune responses by TLR-Ls in chickens. Various types of cells have been shown to respond to treatment with different TLR-Ls. Stimulation of chicken splenocytes with TLR2 ligands upregulated IFN-γ, IL-12, IL-4 and IL-13 expression, which indicates the induction of both Th1 and Th2 responses (St. Paul et al., 2013b). The expression of IFN-γ, which is involved in antiviral immunity was increased in bursal cells in response to treatment with LPS or Poly(IC) (St. Paul et al., 2012c). In addition, stimulation of splenocytes with TLR3 ligands increased the expression of IFN-α, IFN-β, and IFN-γ (Villanueva et al., 2011). Similarly, upregulation of IFN-β and TLR3 was observed when leukocytes from chickens were stimulated with Poly(IC) (Karpala et al., 2008). In vivo administration of TLR-Ls
also induced innate responses in chickens. Increased expression of IFN-γ and IL-1β was noticed in the spleens of chickens after stimulation with LPS or CpG (St. Paul et al., 2011) and in neonatal chicks (Patel et al., 2008), respectively.

TLR-Ls have been used as prophylactic agents and as adjuvants in order to provide protective immunity to the host. Administration of CpG has been shown to protect against pathogens such as *Escherichia coli* (*E. coli*) (Gomis et al., 2003), *Salmonella* (Taghavi et al., 2008), infectious bronchitis virus (Dar et al., 2009, 2014), infectious laryngotracheitis virus (ILTV) (Thapa et al., 2015) and influenza virus (Mallick et al., 2012; Barjesteh et al., 2015b). Further, administration of LPS or CpG has been shown to delay MD progression in chickens (Parvizi et al., 2014). In addition, the administration of Poly(IC) with the HVT vaccine decreased tumor formation in MDV-infected chickens (Parvizi et al., 2012a). These lines of evidence indicate the effects of TLR-Ls in induction of immune responses against invading pathogens of chickens.

*In ovo* administration of various TLR-Ls also has been shown to induce host responses against infectious organisms. *In ovo* inoculation of CpG controlled replication of infectious bronchitis virus in embryos and post-hatch chickens (Dar et al., 2009, 2014). Furthermore, the use of CpG in ED18 embryos reduced ILTV-related mortality and recruited innate and adaptive immune system cells in tissues (Thapa et al., 2015; Abdul-Cader et al., 2018a). In another study, *in ovo* administration of the TLR2 ligand lipoteichoic acid induced antiviral responses against ILTV and increased macrophage numbers in lung in response to infection (Haddadi et al., 2015). In the context of avian influenza virus, replication of this virus was reduced in chorioallantoic membranes following *in ovo* inoculation of TLR 2, 4, 7 and 21 ligands via activation of IFN-γ and interferon stimulatory genes (ISGs) (Barjesteh et al., 2015a). The evidence from previous
studies indicates that early administration of TLR-Ls to chicken embryos can induce host responses to act against potential pathogens.

*Use of encapsulated TLR ligands*

Recent developments in pharmaceutical and delivery systems have paved the way to delivery of immune potentiators such as TLR-Ls to boost host immunity against infectious diseases. Efficacy of administered TLR-Ls can be reduced because of their short half-life and rapid removal from the body (Engel et al., 2011). To overcome these limitations, TLR-Ls can be encapsulated into PLGA as nanoparticles (Panyam and Labhasetwar, 2003). These encapsulated TLR-Ls can be slowly released from PLGA nanoparticles upon hydrolysis in tissues or within cells following uptake. In addition, PLGA nanoparticles enable targeted delivery which improves the uptake of the encapsulated substance. For example, encapsulation of nucleic acids into PLGA nanoparticles modified the negative charge of nucleic acids and facilitated their uptake by cells (Hanagata, 2017). Moreover, the ability of PLGA nanoparticles to release TLR-Ls slowly over time reduces the need for frequent administration and reduces the number of doses of TLR-Ls. It has been demonstrated that administration of formulated CpG ODN in a polyphosphazene delivery system reduced mortality of neonatal chickens when challenged with *E. coli* (Taghavi et al., 2009). Further, administration of nanoparticles containing TLR4 and TLR7/8 ligands along with simian immunodeficiency virus (SIV)-derived antigens stimulated immune responses which protected macaques from challenge with SIV (Kasturi et al., 2017). Vaccination of nanoparticles containing an antigen with TLR4 and TLR7 ligands protected mice against avian and swine influenza virus infections (Kasturi et al., 2011). These studies provide evidence for enhancement of host immune responses to infectious diseases with methods that use TLR-Ls in nanoparticles.
Statement of rationale

MD is a major concern for the poultry industry because of the potential for substantial economic losses if the disease is not controlled properly. Despite the availability of different vaccines and vaccination strategies, MDV still infects and efficiently replicates in tissues of vaccinated chickens, therefore, viral shedding is inevitable. Evolution of MDV to more virulent strains of MDV occurs partly due to the incomplete protection provided by MD vaccines. Administration of TLR-Ls may improve immunity against MD in chickens as it has been demonstrated for other viral infections in chickens or in other species. The efficacy of vaccines can be improved by integrating various substances into vaccines such as TLR-Ls and cytokines.

Immunity to MD is provided by both innate and adaptive defense mechanisms. Enhancement of innate responses in neonatal chicks may provide improved immune protection during this vulnerable period. It is thought that young chicks are exposed to MDV in the first few days of life, therefore, it is important to confer early protection prior to maturation of adaptive immunity. Activating host responses at an early stage of life enables the newly hatched chicks to mount immunity against MD. There has been no study investigating in ovo administration of TLR-Ls conferring protective immunity against MD in young chicks. In addition, bioavailability of administered TLR-Ls can be extended by encapsulating them into PLGA nanoparticles to further enhance the immune responses against MDV in chickens.
Hypotheses

1. Pre-treatment of cells with TLR-Ls can inhibit replication of MDV \textit{in vitro}

2. Administration of encapsulated TLR-Ls can induce innate responses in chicken embryos

3. \textit{In ovo} administration of encapsulated TLR-Ls can enhance immunity to MD in chickens

4. \textit{In ovo} administration of encapsulated TLR-Ls in conjunction with an HVT vaccine can enhance the protective effect of the vaccine
Experimental approach

Objective 1

Investigation of the ability of TLR-Ls to reduce replication of MDV in chicken embryo fibroblast cells (CEFs).

Key steps involved

- Propagation of enhanced green fluorescent protein (EGFP) tagged MDV in CEFs
- Determination of the expression of TLRs in CEFs
- Pre-treatment of CEFs with TLR-Ls
- Infecting CEFs with MDV at 24 h post-stimulation (hps)
- Harvesting CEFs at 96 and 120 hours post-infection (hpi) and performing flow cytometry to determine MDV infected CEFs
- Harvesting CEFs at 6, 12, 24, 48 and 72 hps and determining antiviral genes and ISGs expression

Objective 2

Characterization of innate responses induced by encapsulated and free forms of LPS and CpG ODN following in ovo administration to chickens at ED18.

Key steps involved

- Preparation of encapsulated TLR-Ls
- Administration of encapsulated and free forms of TLR-Ls to ED18 chicken embryos
- Collection of samples from the spleen, lungs and bursa of Fabricius at 6, 18 and 48 hps
- Determination of cytokine gene expression in collected tissues
Objective 3

Determination of the effectiveness of administration of encapsulated TLR ligands *in ovo* in improving immunity against MD

**Key steps involved**

- Conducting an experiment to identify the suitable form of TLR-Ls (free or encapsulated) and the day of *in ovo* administration (ED18 or ED19) of TLR-Ls which can provide protection against MDV infection
- Performing a second experiment to determine whether a single or a double dose of encapsulated TLR-Ls could provide protective immunity against MDV
- Conducting a third experiment to address the potential immunological mechanisms involved in the protective effect of encapsulated TLR-L treatments against MDV infection
- Recording tumor incidence and collecting samples from the spleen, bursa of Fabricius and feathers
- Determination of MDV genome load in feathers and cytokine gene expression in spleens and bursas of Fabricius

Objective 4

To determine whether the immunization of chickens with HVT vaccine, along with encapsulated CpG, could enhance vaccine-mediated protection

**Key steps involved**

- Preparation of CpG-encapsulated PLGA nanoparticles (ECpG)
- Immunization of ED18 embryos with either ECpG or HVT or both
- Challenging five-day-old chicks with MDV
• Administration of ECpG to chickens at 14 dpi
• Recording spleen, bursa of Fabricius and body weights and collecting samples from spleens, bursas of Fabricius and feathers at 4, 10 and 21 dpi
• Recording tumor incidence at 21 dpi
• Determination of MDV genome load in feathers and cytokine gene expression in spleens and bursas of Fabricius
Chapter 2

Reduction of Marek’s disease virus infection by Toll-like receptor ligands in chicken embryo fibroblast cells

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Abstract

Evolutionarily conserved pattern recognition receptors, including Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) that are present in microbes. PAMPs induce several pathways downstream of TLRs which lead to induction of anti-viral responses. The objective of the present study was to investigate the stimulatory effect of various PAMPs (in the form of TLR ligands) in reducing Marek’s disease virus (MDV) infection in chicken embryo fibroblast cells (CEFs). To this end, CEFs were pre-treated with Pam3CSK4, Poly(IC), lipopolysaccharide (LPS) and CpG ODN as TLR2, TLR3, TLR4 and TLR21 ligands, respectively for 24 h followed by infection with MDV. The results indicated that pre-treatment with Poly(IC) resulted in a robust reduction (by about 81%) of MDV infection in CEFs at 96-hour post-infection while a moderate reduction was observed with treatment of Pam3CSK4 (35%), LPS (26%) and CpG ODN (23%) PAMPs. Transcriptional analysis of gene expression in CEFs demonstrated that all TLR ligand treatments and MDV infection significantly increased the expression of type I interferons, interleukin (IL)-1β, interferon regulatory factor 7 (IRF7), interferon induced protein with tetratricopeptide repeats 5 (IFIT5) and myxoma-resistance protein (Mx). Further studies are needed to explore the mechanism by which PAMPs, particularly the TLR3 ligands could reduce MDV infection in CEFs which may play an important role in controlling the replication of MDV in chickens.

Keywords: TLR3 ligands, Marek’s disease virus, Chicken embryo fibroblast cells
Introduction

Marek’s disease (MD) is a neoplastic disease of chickens caused by Marek’s disease virus (MDV). MDV causes tumors in visceral organs and skin. MDV is classified as Gallid herpesvirus 2 which belongs to the Alphaherpesvirinae subfamily (Osterrieder and Vautherot, 2004). According to a recent study, out of 116 countries surveyed, 48% of the countries reported that MD incidence increased in the last 10 years (Dunn and Gimeno, 2013). Although there are vaccines available to control clinical signs of MD (Bublot and Sharma, 2004), MDV replication and shedding are not prevented by any of these vaccines (Haq et al., 2013). The inability to control MDV replication and transmission of MDV by current immunization strategies may be one of the reasons for the appearance of more virulent strains of MDV.

Evolutionarily conserved pattern recognition receptors, including Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) that are present in microbes. Several studies including those conducted by our group have explored the use of TLR ligands (TLR-Ls) as prophylactic agents or as adjuvants to provide protective immunity against various infectious diseases in several species, including chickens (St. Paul et al., 2013a, 2012a). Several TLRs such as TLR1 type 1 and 2; TLR2 type 1 and 2, TLR3, TLR4, TLR5, TLR7, TLR15 and TLR21 are reported to be present in various tissues of chickens (Kogut et al., 2005; Temperley et al., 2008). Among them, TLR3 and TLR21 detect nucleic acid based TLR-Ls. Viral double-stranded RNA (dsRNA) as well as synthetic dsRNA (polyinosinic:polycytidylic acid known as Poly(IC)) are recognized by TLR3. TLR21 detects unmethylated CpG motifs present in bacterial and viral DNA. TLR2 and TLR4 detect lipid-based ligands. Peptidoglycan, lipopeptide and lipoproteins of bacteria including synthetic lipoproteins such as PAM3CSK4 are recognized by
TLR2, whereas bacterial lipopolysaccharide (LPS) is recognized by TLR4 and bacterial flagellin by TLR5.

In regard to MDV, similar to other herpes viruses, glycoproteins and double-stranded DNA of the virus may be recognized by TLR2 and TLR21, respectively, with the latter being the homolog of TLR9 in mammals (Paludan et al., 2011). In addition, TLR4 might become activated directly through viral components of MDV or indirectly via MDV induced danger signals as it happens in other herpes viruses (Villalba et al., 2012). TLR3 recognizes double stranded RNA (dsRNA) which is a transitional product in the formation of DNA viral progeny (Akira et al., 2006). Therefore, it is possible for the chicken innate immune system to recognize dsRNA by TLR3 during MDV infection. Importantly, we have previously demonstrated that the synthetic TLR3 ligand, Poly(IC), can reduce tumor formation in MDV-infected chickens (Parvizi et al., 2012a).

The interaction of TLRs and TLR-Ls initiates downstream signaling pathways via activation of transcription factors that lead to the expression of several immune system genes, including cytokines, chemokines, adhesion molecules and receptors. Interactions of TLR2, TLR4 and TLR5 with their ligands leads to the production of pro-inflammatory cytokines whereas interaction of TLR7 and TLR9 with their ligands leads to the secretion of type 1 interferons (IFNs) (Mogensen, 2009). Interactions of type 1 IFNs with their receptors result in the induction of interferon stimulated genes (ISGs), many of which have antiviral activities, such as 2’, 5’-oligoadenylatesynthase (OAS), dsRNA-dependent protein kinase (PKR), interferon induced protein with tetratricopeptide repeats 5 (IFIT5 – also known as ISG58) and myxoma-resistance protein (Mx). These ISGs play a critical role in the inhibition of virus replication at different levels of the virus life cycle.
The aim of this study was to investigate the ability of TLR-Ls to reduce replication of MDV in cultured cells. To this end, we used a fluorescent tagged MDV in chicken embryo fibroblast cells (CEFs) and various TLR-Ls were screened to determine their efficacy in reducing virus replication. In addition, to shed light on some of the underlying mechanisms of TLR-Ls effects on MDV replication, expression of several immune system genes was examined in treated and infected CEFs.

Materials and methods

Cells and virus

Specific pathogen free (SPF) eggs were obtained from the Animal Disease Research Institute, Canadian Food Inspection Agency (Ottawa, Ontario, Canada). SPF eggs were incubated at the recommended temperature and humidity conditions. Chicken embryo fibroblast cells were prepared from 11-day old chicken embryos. Enhanced green fluorescent tagged MDV, recombinant RB1B strain (vUL47-EGFP), originated in Dr. Benedikt Kaufer’s lab (Freie Universität, Berlin, Germany) was obtained from Dr. Masahiro Niikura (Simon Fraser University, Burnaby, Canada) and used in this study to infect CEFs. All experiments involving chickens were approved by the Animal Care Committee, University of Guelph and complied with institutional regulations.

TLR ligands

Synthetic triacylated lipoprotein, Pam3CSK4, was purchased from InvivoGen (San Diego, CA). LPS from Escherichia coli 0111: B4, Poly(IC) and synthetic class B CpG ODN 2007 [5’-TCGTCGTTGTCGTTTGTGTT-3’] with phosphorothioate backbone and non-CpG ODN (NCpG) [5’-TGCTGCTTGTGCTTTTGTGCTT-3’] (Brownlie et al., 2009) were purchased from
Sigma–Aldrich Canada (Oakville, ON). All ligands were re-suspended in endotoxin free sterile water.

**Experimental design**

First, uninfected CEFs were cultured and harvested for RNA extraction to determine the presence of TLR2, TLR3, TLR4, TLR5, TLR7 and TLR21. Next, CEFs were cultured and seeded on 96 well plate at $1 \times 10^5$/ml in RPMI-1640 medium (Invitrogen, Burlington, ON) supplemented with 10% heat-inactivated fetal bovine serum, 2% chicken serum, 200 U/mL penicillin, 80 µg/mL streptomycin, and 5% tryptose phosphate buffer. Monolayers of CEFs were stimulated with Pam3CSK4 0.1 µg/ml as a low dose and 1 µg/ml as a high dose, Poly(IC) 2.5 µg/ml as a low dose and 25 µg/ml as a high dose, LPS 0.1 µg/ml as a low dose and 1 µg/ml as a high dose, CpG ODN 0.5 µg/ml as a low dose and 5 µg/ml as a high dose; and NCpG 5 µg/ml for 24 hours or left untreated. Stimulated cells were washed and infected with vUL47-EGFP MDV at MOI 0.1. CEFs were collected at 96 and 120-hour post-infection (hpi) for flow cytometry analysis to determine the MDV infected cells. In the subsequent experiment, selected TLR -Ls were used to treat the CEFs before MDV infection and cells were collected for RNA extraction to determine gene expression at 6, 12 and 24-hour post-stimulation (hps); and 24 (48 hps) and 48 (72 hps) -hour post-infection.

**Flow cytometry**

CEF were harvested at 96 and 120 hpi and re-suspended in FACS buffer (PBS with 1% BSA). Cells were stained with Live/dead fixable near-IR dead cell stain kit (Life technology, Eugene, OR, USA) for exclusion of dead cells and incubated for 30 minutes on ice. At the end of the incubation, CEFs were washed and re-suspended in FACS buffer and the data were acquired using a FACSCanto flow cytometer (BD Biosciences, Mississauga, ON, Canada).
All samples within this experiment were analyzed individually using the FlowJo software (Tree Star, Ashland, USA). Initial gating involved all live cells acquired within an SSC-A versus FSC-A plot. Then through a two-step process involving FSC-W versus FSC-H followed by SSC-W versus SSC-H, all doublets were eliminated in order to avoid false positive stains. Final percentages were subsequently determined by gating on the cells that showed positive signals in the emission spectrum range of 530/30.

**RNA extraction and cDNA synthesis**

CEF s from two wells were pooled for extraction of RNA and considered as one replicate. There were 6 replicates. RNA was extracted using Trizol reagent (Life Technologies, Burlington, Canada) according to manufacturer’s protocol. RNA quantity and quality were determined using the NanoDrop® ND-1000 spectrophotometry (NanoDrop Technologies, Wilmington, DE). Ten micrograms of extracted RNA were treated with DNase enzyme (Ambion, Austin, TX) according to the manufacturer’s protocol. Then cDNA was synthesized from 1µg of DNase treated RNA using Superscript II (Life Technologies, Burlington, Canada). Synthesized cDNA was diluted at 1:10 ratio with nuclease free water.

**Real-time polymerase chain reaction**

Real-time polymerase chain reaction (PCR) was performed using SYBR green dye in LightCycler 480 II (Roche Diagnostics, Laval, Canada) to quantify the relative expression of target genes. Chicken β actin gene was used as the reference gene to calculate the relative expression of all selected genes in this study. Primer sequences of target and reference genes are listed in Table 1. The primers were synthesized by Sigma–Aldrich Canada (Oakville, ON).
**Statistical analysis**

To determine the statistical significant difference in MDV infected CEFs, data were analyzed using one-way ANOVA followed by Dunnett’s multiple comparison test to compare the pre-treated group with infection only control group in GraphPad Prism version 6.04. For gene expression analysis, REST (Relative Expression Software Tool) software v2009 (Qiagen Valencia, CA) was used to calculate fold changes and standard errors. Data are presented as the mean fold change ($\pm$ standard error of the mean) of the relative gene expression in the TLR-Ls treated groups and MDV infection only control group compared to PBS control group. If $p$ value was $\leq 0.05$, it was considered statistically significant.

**Results**

*Expression of TLR transcripts in CEFs*

The results of this study indicated that TLRs 2, 3, 4, 5, 7 and 21 were expressed by CEFs (Fig.1). Constitutive expression of TLRs from highly expressed to minimally expressed was as follows: TLR2, TLR4, TLR21, TLR3, TLR7 and TLR5 respectively.

*Reduction of MDV infection by TLR-Ls in CEFs*

TLR-Ls pre-treated CEFs were infected with vUL-47-EGFP MDV at 24 hps. The percentage of MDV infected CEFs was determined by flow cytometry at 96 and 120 hpi (Fig 2). Pre-treatment with TLR-Ls significantly reduced the percentage of MDV-infected CEFs at one or both time points (Fig 3A and 3B). Pre-treatment with low and high doses of Poly(IC) (Fig 2 and Fig 3A) and low dose of Pam3CSK4 significantly reduced the percentage of MDV infected CEFs compared to the untreated group at both 96 and 120 hpi (Fig 3A). In addition, pre-treatment with high dose of Pam3CSK4 significantly reduced the percentage of MDV infected CEFs at 120 hpi (Fig 3A). Pre-treatment with high dose of CpG ODN, low and high doses of
LPS significantly reduced the percentage of MDV-infected CEFs at 96 hpi compared to the untreated group (Fig 3B). However, at 120 hpi, pre-treatment with a high dose of CpG ODN and NCpG unexpectedly significantly increased MDV-infected CEFs compared to the untreated group. There was no significant difference in MDV infection in NCpG treated CEFs compared to that of CpG treated CEFs.

Based on the significant percentage of reduction of MDV infected CEFs (Table 2), high dose of Poly(IC) (81% and 69%), low dose of Pam3CSK4 (35% and 31%) and low dose of LPS (26%) were selected for further experiment to investigate the involvement of related cytokine genes and ISGs.

*Induction of cytokine genes and ISGs expression*

CEF were pre-treated with high dose of Poly(IC), low dose of Pam3CSK4 and low dose of LPS for 24 h and infected with vUL47-EGFP MDV. Expression of cytokine genes and ISGs was analyzed at 6, 12, 24, 48 (24 hpi) and 72 hps (48 hpi) with TLR-Ls. Stimulation of CEFs with Poly(IC) significantly upregulated the expression of IFN-α gene at all time points except at 6 hps (Fig 4A). In addition, Pam3CSK4 and LPS significantly upregulated the expression of IFN-α gene at 48 and 72 hps (Fig 4A). Further, MDV infection alone significantly upregulated IFN-α gene expression at 48 hps (Fig 4A). The expression of IFN-β gene was significantly upregulated by Poly(IC) pre-treatment at all time points except 24 hps (Fig 4B). All TLR-Ls pre-treatment and MDV infection only control significantly upregulated IFN-β gene expression at 48 and 72 hps (Fig 4B). In addition, Pam3CSK4 pre-treatment significantly upregulated IFN-β gene expression at 12 hps (Fig 4B).

Pre-treatment with TRL-Ls significantly upregulated the expression of IL-1β gene at all time points except 24 hps at which LPS pre-treatment only upregulated IL-1β gene (Fig 5A). In
addition, MDV infection also significantly upregulated IL-1β gene expression at 48 and 72 hps. Poly(IC) pre-treatment significantly upregulated IRF7 gene expression in all time points except 72 hps (Fig 5B). Further, all TLR-Ls pre-treatment and MDV infection only control significantly upregulated IRF7 gene expression at 48 hps (Fig 5B).

Poly(IC) pre-treatment significantly upregulated IFIT5 gene expression at all time points (Fig 6A). Pam3CSK4 pre-treatment significantly upregulated IFIT5 gene at 6, 24 and 48 hps (Fig 6A). LPS pre-treatment and MDV infection only control significantly upregulated IFIT5 gene expression at 48 and 72 hps (Fig 6A). Mx gene expression was significantly upregulated by Poly(IC) pre-treatment at all time points except 6 hps (Fig 6B). Pam3CSK4 and LPS pre-treatment significantly upregulated Mx gene expression at 48 and 72 hps (Fig 6B). Furthermore, Mx gene expression was significantly upregulated by MDV infection at 48 hps (Fig 6B).

Discussion

In the current study, initially the constitutive expression of TLRs, such as TLR 2, 3, 4, 5, 7 and 21 was investigated in CEFs. Next, various TLR-Ls, including Poly(IC), Pam3CSK4, LPS and CpG ODN, were evaluated for their ability to inhibit MDV infection in CEFs. Following that, TLR-L treatments causing significant reduction of MDV infection were selected for further study, in which the expression of selected cytokine genes and ISGs was investigated.

We demonstrated that all TLRs examined in the current study were expressed in CEFs. The relative expression of these TLRs in CEFs, from the highest to the lowest, was TLR2, TLR4, TLR21, TLR3, TLR7 and TLR5. In comparison to this observation, another study reported that high expression of TLR3 was followed by TLR7 and TLR4 in CEFs from MD resistant and susceptible chicken lines (Haunshi and Cheng, 2014). In addition, the expression of TLR3, TLR4 and TLR7 in uninfected CEFs was higher in MD resistant chicken lines than that of
susceptible chicken lines (Haunshi and Cheng, 2014). This difference suggests that expression pattern of TLRs in CEFs might vary depending on the genotype of chickens.

The current study illustrated that it is possible to reduce MDV infection by pre-treatment of cells with TLR-Ls. Although pre-treatment of cells with all TLR-Ls caused significant reduction of MDV infected CEFs, pre-treatment with Poly(IC) had a more potent effect compared to other TLR-Ls. Similarly, in chickens, Poly(IC) treatment significantly reduced the oropharyngeal and cloacal shedding of avian influenza virus (St. Paul et al., 2012a). MDV and herpes simplex viruses (HSV) are categorized under the subfamily Alphaherpesvirinae because of their structural similarities. In addition, MDV has some physiological properties comparable to HSV. Recent reports have also shown that Poly(IC) pre-treatment could effectively reduce HSV type 1 and 2 (HSV1 and HSV2) infection in vitro (Ashkar et al., 2004; Gaajetaan et al., 2012). Additionally, strong antiviral effect of Poly(IC) against HSV2 was demonstrated when vaginal delivery of Poly(IC) completely inhibited the viral replication in mice (Gill et al., 2006).

The potent effect of Poly(IC) pre-treatment was observed even with low level expression of TLR3 in CEFs. In this case, Poly(IC) might have induced the expression of additional TLR3 in CEFs leading to more effective recognition of this dsRNA mimic to trigger the downstream signaling pathway. Changes in the expression of TLR3 due to TLR-L treatment in CEFs were not examined in the present study, however, other researchers have demonstrated the upregulation of TLR3 following Poly(IC) treatment (Rivieccio et al., 2006; Karpala et al., 2008) where the upregulation of TLR3 occurred due to the secretion of type 1 IFN in cell lines of chickens and mammals (Tissari et al., 2005; Karpala et al., 2008). It is well known that Poly(IC) recognition by TLR3 leads to the induction of type 1 IFN. Therefore, it is plausible that the positive feedback mechanism of type 1 IFN leads to the increased expression of TLR3 by
Poly(IC) pre-treatment which might have contributed to the strong antiviral response against MDV infection in this study. Moreover, recognition of Poly(IC) is not solely dependent on TLR3. Other cytoplasmic receptor like melanoma-differentiation-associated gene 5 (MDA5) can act as a receptor for Poly(IC) (Kato et al., 2006; Kawai and Akira, 2010). Since MDA5 is present in most cells including DF-1 cells (Hui and Leung, 2015), it is likely that MDA5 might have compensated for the low constitutive expression of TLR3 by CEFs. The assessment of transcriptional profiles of cytokines and ISGs in the present study revealed that all target genes were upregulated after MDV infection in all experimental groups. This raises the possibility that induction of these genes by TLR-Ls was responsible for the reduction in MDV replication in TLR-L pre-treated cells. The significant upregulation of both IFN-β and IFN-α genes by Poly(IC) pre-treatment occurred at early time points. In contrast, a study by Gill and colleagues (Gill et al., 2006) indicated that IFN-β, but not IFN-α, stimulated by Poly(IC) played a major role in protection against HSV2 infection in mice. In addition to type 1 IFN, IRF7, IFT5 and Mx genes were significantly upregulated by Poly(IC) pre-treatment at early time points in the current study. Type 1 IFN, induced by the TLR3 signaling cascade, can act on cells in an autocrine and/or paracrine fashion and induce the subsequent signaling events to activate ISGs.

The ISGs studied here, such as IFIT5 and Mx, act at different steps of viral replication and halt the production of new generation of viruses (Schneider et al., 2014). Further, IRF7 which is an IFN regulator, can directly trigger the expression of certain ISGs like IFIT5, independent of IFN secretion (Schmid et al., 2010; Schneider et al., 2014). All these events might be attributed to the potent antiviral response conferred by Poly(IC) pre-treatment.

In general, two scenarios for the reduction of MDV infected cells can be envisaged. First, this result could be due to the interference in replication of MDV in infected CEFs. This could
have happened due to the presence of several ISGs including IRF7 and IFIT5 which generate a hostile environment for virus replication. Second, even after successful replication in CEFs, reduction could be due to the ineffective spreading of MDV to adjacent cells, possibly due to the activation of an antiviral state in bystander cells by Poly(IC). Type 1 IFN secreted by infected cells may have acted on neighboring cells and induced the activation of ISGs to prevent viral entry. The mechanism of MDV entry into the cells has yet to be elucidated, however, similar to other viruses, Mx expression in CEFs might have contributed to resistance of the cells to virus infection (Schneider et al., 2014). Hence, the anti-viral state induced by Poly(IC) pre-treatment prior to MDV infection may be the reason for the effective reduction in the MDV infected CEFs by Poly(IC). Pam3CSK4 and LPS failed to induce a functional antiviral state equal to that triggered by Poly(IC). Poly(IC) significantly upregulated all genes examined in the present study, however, Pam3CSK4 significantly upregulated only IFN-β, IL-1β and IFIT5 genes while LPS significantly upregulated only IL-1β and IRF7. This insufficient production of antiviral components for the preparation of CEFs by Pam3CSK4 and LPS might be the reason for the lower reduction of MDV infected CEFs compared to Poly(IC) pre-treatment.

Surprisingly, high dose of CpG and NCpG pre-treatment significantly increased MDV infected CEFs at 120 hps when compared to MDV infection only control. Similar to this observation, pro-viral activity of CpG was reported with human cytomegalovirus (HCMV) infection in neonatal human dermal fibroblasts when CpG was used as post viral treatment (Iversen et al., 2009). The reason for this pro-viral activity is assumed to be the involvement of the PI3K/Akt pathway, which is activated in a TLR9 dependent manner (Sester et al., 2006) and HCMV uses the same pathway for its replication (Johnson et al., 2001). However, since cellular activation and signaling pathways induced by MDV are not well studied, it is difficult to
speculate the possible mechanism of enhancement of MDV infection in CpG treated cells. Further, enhanced viral replication is not uncommon in cells treated with CpG. CpG pre-treatment increased the replication of Friend retrovirus and led to erythroleukemia in resistant mice (Olbrich et al., 2003). In another study, splenocytes from human immunodeficiency virus (HIV) transgenic mice were stimulated ex-vivo with CpG and enhanced HIV replication was observed (Equils et al., 2003).

In conclusion, TLR2, TLR3 and TLR4 ligands (Pam3CSK4, Poly(IC) and LPS respectively), significantly limited MDV infection in CEFs. This effect was mediated by upregulation of cytokines related to antiviral responses and ISGs. Additional studies are needed to explore the mechanisms by which PAMPs reduce MDV infection in CEFs with the aim of developing methods to control MDV replication in chickens. Moreover, further investigation of the effect of Poly(IC) on reducing MDV infection in chickens, particularly in feather follicular epithelial cells (FFEs) is necessary. This might reveal the possibility of interrupting transmission of MDV to susceptible chickens.

Acknowledgements

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Table 2.1: Target genes, primer sequences and references used for real-time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>References</th>
</tr>
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</table>
| β-actin    | F: 5’-CAACACAGTGCTGTCTGGTGGTA-3’  
R: 5’-ATCGTACTCCTGCTTGCTGATCC-3’ | (Brisbin et al., 2008)               |
| IFN-α      | F: 5’-ATCCTGCTGTCACGCTCCTTTCT-3’  
R: 5’-GGTGTGGCTGGTGTCAGGATG-3’ | (Brisbin et al., 2008)               |
| IFN-β      | F: 5’-GCCTCCAGCTCCTTTCAGAATAC G-3’  
R: 5’-CTGGATCTGGTGAGGAGGCTGT-3’ | (Abdul-Careem et al., 2008b)         |
| IL-1β      | F: 5’-GTGAGGCTCAACATTGCGCTGTA-3’  
R: 5’-TGTCAGGGCGGTAGAAGATG-3’ | (Abdul-Careem et al., 2009a)         |
| IRF7       | F: 5’-CTCCCCCTCTCCTCAGAGCTG-3’  
R: 5’-CTGGGAGCGAAGGAGGAATG-3’ | (Alkie et al., 2015)                 |
| IFIT5      | F: 5’-CAGAATTTAATGCNGCTATGC-3’  
R: 5’-TGCAAGTAAAGCAAAAGGCTGT-3’ | (Barber et al., 2013)                |
| Mx         | F: 5’-GGACTTCTGCAACGAATTG-3’  
F: 5’-TCCCAAGTCATTACGTAAG-3’ | (Barber et al., 2013)                |
| TLR2       | F: 5’-ATCCTGCTGAGCCCCATTACGGAGAGGAGCCACTC-3’  
R: 5’-TTGCTCTCATCAGAGGAGCCACTC-3’ | (St. Paul et al., 2012b)             |
| TLR3       | F: 5’-TGCACTTGGTCACCAACCTACCCGCGG-3’  
R: 5’-GGCGCTGATAATCAACACTCCG-3’ | (Abdul-Careem et al., 2009a)         |
| TLR4       | F: 5’-TGCCATCCCAACCCACACACAG-3’  
R: 5’-ACACCCACGTGACGACCAACAA-3’ | (St. Paul et al., 2012b)             |
| TLR5       | F: 5’-TTCTGGCAACCCCTACAGGTTTCCG-3’  
R: 5’-CAGTTCCAGACACCAAGATT-3’ | (St. Paul et al., 2012b)             |
| TLR7       | F: 5’-TTCTGGCCACAGATGTGAC-3’  
R: 5’-CCTCAACTTGGCAGTGCA-3’ | (Abdul-Careem et al., 2009a)         |
| TLR21      | F: 5’-CCTGCGCAAGGTGCCGCTCA-3’  
R: 5’-GCCCCAGGGTCCAGGAAGCAG-3’ | (St. Paul et al., 2012b)             |
Table 2.2: Percentage reduction of MDV infected CEFs following pre-treatment with TLR-Ls

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<tr>
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<th>Cpg low</th>
<th>Cpg high</th>
<th>LPS low</th>
<th>LPS high</th>
<th>Poly(IC) low</th>
<th>Poly(IC) high</th>
<th>Pam3CSK4 low</th>
<th>Pam3CSK4 high</th>
</tr>
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<tbody>
<tr>
<td>96 hpi</td>
<td>20.82</td>
<td>22.59</td>
<td>26.26</td>
<td>21.22</td>
<td>58.91</td>
<td>80.71</td>
<td>34.59</td>
<td>17.82</td>
</tr>
<tr>
<td>120 hpi</td>
<td>-4.88</td>
<td>-18.86</td>
<td>7.32</td>
<td>4.07</td>
<td>57.41</td>
<td>68.52</td>
<td>30.56</td>
<td>27.78</td>
</tr>
</tbody>
</table>
Figure 2.1: Relative gene expression of TLR transcripts in CEFs

Gene expression of TLRs 2, 3, 4, 5, 7 and 21 transcripts in CEFs relative to β-actin.
Figure 2.2: EGFP MDV infected CEFs following pre-treatment with TLR-Ls

Illustrative flow cytometry plot shows the percentage of MDV infected CEFs at 96 and 120 hpi following pre-treatment with Poly(IC) for 24 h and infection with EGFP MDV along with the MDV infection only (Untreated CEFs but infected) control group.
Figure 2.3: Reduction of MDV infected CEFs by TLR-Ls pre-treatment.

CEF s were pre-treated with (A) Pam3CSK4 and Poly(IC) (B) CpG ODN and LPS for 24 h and infected with EGFP MDV. There were also MDV infection only (Untreated CEFs but infected) and medium control groups. Percentage of MDV infected CEFs was determined by flow cytometry at 96 and 120 hpi. Data were statistically analyzed by one-way ANOVA. For the post hoc comparison, Dunnett’s test was used to compare the treated groups with infection only control group. Error bars indicate the standard error of the mean. $p \leq 0.05$ (*) was considered statistically significant.
Figure 2.4: Relative expression of IFN-α and IFN-β in TLR-Ls pre-treated, MDV infected CEFs. CEFs were pre-treated with Pam3CSK4, Poly(IC) and LPS, and infected with EGFP MDV. There were MDV infection only (Untreated CEFs but infected) and medium control groups. (A) IFN-α and (B) IFN-β gene expression were determined at 6, 12, 24, 48 and 72 hps. Data were analyzed by REST software and presented as the mean fold change (± standard error of the mean) of the relative gene expression in the TLR-Ls pre-treated groups and MDV infection only control group compared to PBS control group. $p \leq 0.05$ (*) was considered statistically significant.
Figure 2.2: Relative expression of IL-1β and IRF7 in TLR-Ls pre-treated, MDV infected CEFs. CEFs were pre-treated with Pam3CSK4, Poly(IC) and LPS, and infected with EGFP MDV. There were MDV infection only (Untreated CEFs but infected) and medium control groups. (A) IL-1β and (B) IRF7 gene expression were determined at 6, 12, 24, 48 and 72 hps. Data were analyzed by REST software and presented as the mean fold change (± standard error of the mean) of the relative gene expression in the TLR-Ls pre-treated groups and MDV infection only control group compared to PBS control group. \( p \leq 0.05 \) (*) was considered statistically significant.
Figure 2.3: Relative expression of IFIT5 and Mx in TLR-Ls pre-treated, MDV infected CEFs. CEFs were pre-treated with Pam3CSK4, Poly(IC) and LPS, and infected with EGFP MDV. There were MDV infection only (Untreated CEFs but infected) and medium control groups. (A) IFIT5 and (B) Mx gene expression were determined at 6, 12, 24, 48 and 72 hps. Data were analyzed by REST software and presented as the mean fold change (± standard error of the mean) of the relative gene expression in the TLR-Ls pre-treated groups and MDV infection only control group compared to PBS control group. $p \leq 0.05$ (*) is considered statistically significant.
Chapter 3

Characterization of innate responses induced by *in ovo* administration of encapsulated and free forms of ligands of Toll-like receptor 4 and 21 in chicken embryos

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Abstract

Toll-like receptors (TLRs) are a family of innate receptors that recognize pathogen-associated molecular patterns, including double-stranded RNA, CpG DNA and lipopolysaccharide (LPS). After interaction with their ligands, TLRs initiate innate responses that are manifested by activating cells and inducing expression of cytokines that help mediate adaptive immune responses. TLR ligands (TLR-Ls) have the potential to be used prophylactically (alone) or as vaccine adjuvants to promote host immunity. Encapsulating TLR-Ls in nanoparticles, such as Poly (D, L-lactic-co-glycolic acid), may prolong responses through sustained release of the ligands. PLGA nanoparticles protect encapsulated TLR-Ls from degradation and extend the half-life of these ligands reduce their rapid removal from the body.

In this study, encapsulated and free forms of LPS and CpG ODN were administered to embryonation day 18 (ED18) chicken embryos. Spleen, lungs and bursa of Fabricius were collected at 6, 18 and 48 hours post-stimulation (hps) and cytokine gene expression were evaluated using quantitative real-time PCR. Results indicate that both the free and encapsulated forms of LPS and CpG ODN induced innate immune responses in ED18 chicken embryos. Innate responses induced in embryos seem similar to those reported in mature chickens. Significant upregulation of cytokine genes generally occurred by 48 hps. Further studies are needed to evaluate long term immunomodulatory effects of encapsulated TLR-Ls and their ability to mediate protection against pathogens of young chicks.

Key words: Cytokine genes, Chicken embryos, In ovo, PLGA nanoparticles, TLR ligands
Introduction

Toll-like receptors (TLRs) are a family of innate receptors that recognize pathogen-associated molecular patterns (PAMPs). For example, lipopolysaccharide (LPS) from the outer membrane of gram-negative bacteria is recognized by TLR4 whereas CpG DNA motifs found in bacterial and viral nucleic acids, are recognized by TLR9 (Chow et al., 1999). In chickens, TLR21, which recognizes CpG DNA, is the functional orthologue of mammalian TLR9 (Brownlie et al., 2009). TLRs are conserved throughout evolution (Medzhitov, 2001). Thirteen TLRs have been identified in mammals, whereas only 10 TLRs have so far been described in chickens (Temperley et al., 2008). Generally, TLRs are expressed both in lymphoid and non-lymphoid tissues (Iqbal et al., 2005). After recognizing TLR ligands (TLR-Ls), TLRs trigger signaling pathways that induce the expression of cytokines to help mediate both innate and adaptive immune responses. Depending on the type of TLRs involved in PAMPs recognition, either the myeloid differentiation factor 88 (MyD88), or toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP) inducing interferon (TRIF) are involved in downstream signaling pathways (Kawai and Akira, 2010). Involvement of MyD88 leads to the secretion of pro-inflammatory cytokines such as interleukin (IL) 1β and IL-6 and also type 1 interferons (IFNs) via activation of mitogen-activated protein kinases (MAPKs) and NF-κB pathway signaling via TRIF leads to the secretion of type 1 IFNs through IFN regulatory factors (IFRs) (Kawai and Akira, 2010). Interaction of MyD88 with TLR4 leads to the production of pro-inflammatory cytokines, and interactions with TLR21 leads to the secretion of type 1 IFNs (Kawai and Akira, 2010; Kogut et al., 2012).
TLR-Ls have been used as prophylactic agents and adjuvants to enhance protective immunity against mammalian pathogens (Dupont et al., 2006; Klinman et al., 2010; Hickey et al., 2013). There are evidences to support the stimulation of immune response by TLR-Ls in chickens. Gomis and colleagues (Gomis et al., 2003) showed the protective effect of CpG oligodeoxynucleotides (ODN) as a prophylactic agent against *Escherichia coli* (*E. coli*) infection in chickens. Furthermore, administration of CpG ODN to embryonation day 18 (ED18) chicken embryos controlled infectious bronchitis virus (IBV) replication in various embryonic tissues (Dar et al., 2009). CpG ODN also reduced IBV replication in spleen and lungs of chickens when administered through the parenteral routes (Dar et al., 2014). Recent studies further demonstrated the possibility of using CpG ODN as a prophylactic agent or as a vaccine adjuvant for the control of avian influenza virus (AIV) in chickens (Mallick et al., 2012; Barjesteh et al., 2015b; Singh et al., 2016). *In vitro* and *in vivo* studies involving humans and mice subjects revealed the responsiveness of constituent cells of the innate and adaptive immune systems to stimulation with TLR-Ls. Similar to mammalian cells, various chicken cell types, including splenocytes, cecal tonsil cells, bursal cells, T cells and thrombocytes have been examined for their responses to TLR-Ls (St. Paul et al., 2011, 2012b; Alkie et al., 2015; Taha-abdelaziz et al., 2016). These studies have shown that depending on the cell subsets and types of TLR-Ls, a variety of cytokines and other effector molecules may be induced, including T helper 1 (Th1) cytokines, such as IFN-γ and IL-12 as well as Th2 cytokines, such as IL-4 and IL-13.

Recent advances in pharmaceuticals and vaccine delivery systems, including nanoparticles, provide a promising approach to deliver immunostimulating molecules such as TLR-Ls for inducing optimal immune responses. PLGA nanoparticles enhance the bioavailability and increase the half-life of encapsulated compound and reduce the rapid
clearance from the body. In addition, nanoparticle delivery facilitates the targeted delivery and enhances the uptake of encapsulated compound. For instance, strong negative charge of nucleic acids may affect their uptake by target cells (Hanagata, 2017). This can be overcome by encapsulation of nucleic acids into nanoparticles which modify the negative charge of nucleic acids and facilitate their uptake by cells. Further, slow releasing characteristics of PLGA nanoparticles can reduce the need for repeated administration and the required dose of the free form of TLR-Ls.

An antigen or ligands for pattern recognition receptors (PRR) can be encapsulated into nanoparticles and released slowly over time (Panyam and Labhasetwar, 2003; Pavot et al., 2013). A study by Pavot and colleagues (Pavot et al., 2013) showed that antibody responses to human immunodeficiency virus are improved by administration of nucleotide-binding oligomerization domain-like receptors (NLR) ligand encapsulated in biodegradable nanoparticles compared to un-encapsulated NLR ligand. Additionally, two recent studies in mice and macaques showed enhanced adjuvant property of ligands specific for TLR4 and TLR7/8 encapsulated in poly(D,L, lactic-co-glycolic) acid (PLGA)-based nanoparticles (NPs) for inducing robust and durable immune responses to different immunogens (Kasturi et al., 2011, 2017). Another study revealed that vaccination of mice with CpG ODN encapsulated into liposomes induced IFN-γ secretion and protected mice against *Leishmania major* infection (Jaafari et al., 2007). In chickens, administration of CpG ODN formulated in a polyphosphazene delivery system reduced mortality of neonatal chickens challenged with *E. coli*. (Taghavi et al., 2009). These studies provide evidence for the improvement of immune stimulatory effects of range of ligands for PRRs by a nanoparticle-based delivery method.
Innate responses to various TLR-Ls have been demonstrated *in vitro* and *in vivo* in chickens; however, studies in chicken embryos are generally limited in scope. Administration of the encapsulated form of TLR-Ls in biodegradable nanoparticles evaluated in several animal species maintain the innate responses for a prolonged period. Recently, we have demonstrated the ability of TLR ligands encapsulated in polymeric nanoparticles to induce prolonged innate responses when administered to chickens, which indicates controlled release system for TLR-Ls (Alkie et al., 2017). The induction of strong innate responses in newly hatched chicks may confer resistance against early life exposure to pathogens, including *Salmonella* and *Escherichia coli*. Stimulating innate immune responses in newly hatched chicks can be achieved by administering the encapsulated TLR-Ls to ED18 chicken embryos. This strategy helps chicks to mount strong immune responses when they encounter pathogens immediately after hatching. The objective of this study was to characterize innate responses induced by the encapsulated (in nanoparticles) and the free forms of TLR 4 and 21 ligands, LPS and CpG ODN respectively, following *in ovo* administration to chicken embryos.

**Materials and methods**

*Chicken eggs and incubation*

White Leghorn layer chicken eggs were received from Arkell Poultry Research Station (Guelph, Ontario) and incubated at recommended temperature and relative humidity conditions. The studies conducted here were approved by the University of Guelph’s Animal Care Committee. On ED18, the eggs were candled and eggs with live embryos were disinfected with 70% ethanol. After the air space was marked, a hole was drilled at the blunt end using egg hole puncture. Free and encapsulated forms of LPS and CpG ODN were administrated into the eggs via hole into the amniotic sac using a 23-gauge 2.5 cm needle as recommended. The holes were
sealed with Permoun solution. The eggs were further incubated until we collect tissues from embryos.

**TLR ligands**

LPS from *E. coli* O111:B4 and synthetic Class B CpG ODN 2007 [5’-TCGTCGTTGTCGTTTTGTCGTT-3’] with phosphorothioate backbone was purchased from Sigma-Aldrich (Oakville, ON, Canada). All ligands were re-suspended in endotoxin-free sterile water.

**Encapsulation of TLR ligands and their characterization**

LPS and CpG ODN were encapsulated in PLGA, (Resomer® RG 503H, free carboxylic acid, MW 24-38 kD, Sigma-Aldrich) using the modified double emulsion solvent evaporation method to generate PLGA NPs (Alkie et al., 2017; Kazzaz et al., 2006). Polyethalenimine (linear, MW 2.5 kD, Sigma-Aldrich) was used to form complexes with highly water soluble CpG ODN at a defined ratio to reduce diffusion of the ligand to the watery phase during the encapsulation process (Boussif et al., 1995). The TLR-Ls (LPS, and CpG ODN complexes) were layered onto 4.5% PLGA dissolved in dicholoromethane (DCM, Sigma-Aldrich) and sonicated for one minute at 40% amplitude using an Ultrasonic Processor with 3 mm diameter probe (Fisher Scientific). The resulting emulsion was sonicated with 2% polyvinyl alcohol (PVA) and 1% poloxamer solution (PVA, MW 30-70 kD, 87-90% hydrolyzed, Sigma Aldrich) for two minutes at 60% amplitude. The resulting PLGA NPs were allowed to harden by mixing with 50 mL of 2% PVA and 1% poloxamer solution and left for two hours with a magnetic stirrer for evaporation of DCM. The nanoparticles were harvested by centrifugation at 20,000 x g for 20 minutes at 4°C. The pellet was washed with nuclease-free water at least three to five times. PLGA NP suspension (0.5 mL) was frozen in 2 mL Eppendorf tubes at -80°C for 1 hour. The
pellet was then lyophilized for 18-22 hours (FreeZone®18 Liter Freeze Dry Systems, Labconco Corporation, Kansas City, Missouri).

Dynamic light scattering (Zetasizer Nano, Malvern Instruments, Worcestershire, UK) was used to measure the size and surface charge (zeta potential) of prepared PLGA NPs. Briefly, 1mg PLGA NPs encapsulating CpG ODN or LPS was dissolved in 1 mL particle free deionized water (pH5.5). From that 1:1000 dilution of the particle suspension was placed in a cuvette and subjected to Zetasizer Nano analyze for the determination of both size and surface charges. Lyophilized PLGA NPs encapsulating CpG ODN (1 mg/mL in TE buffer) was dissolved in 1 mL DCM and shaken for 1 hour at room temperature. The suspension was further centrifuged at 9,000 x g for 5 minutes. Then the encapsulation efficiency of CpG ODN was determined. The Quant-iT™ OliGreen® ssDNA reagent and kit system (Life Technology, Burlington, Ontario) and a GloMax®-Multi Detection System-Fluorometer (Promega, Madison, WI) were used to determine the quantity of CpG ODN that was released into the aqueous phase (top layer). A standard curve was generated and plotted as a function of oligonucleotide concentration to determine the exact encapsulation efficiency. The percentage encapsulation efficiency was used to calculate the dose required for the experiment. LPS encapsulation efficiency was determined by encapsulating an equivalent amount of Rhodamine-labeled LPS and the procedure remained identical to LPS encapsulation. The fluorescence intensity of Rhodamine-LPS extracted from a determined quantity of PLGA nanoparticles using hot phenol-water treatment (Boussif et al., 1995) was measured using a GloMax®-Multi Detection System-Fluorometer. Next, a standard curve was generated and plotted as a function of known Rhodamine-LPS concentration to determine the exact encapsulation efficiency.
Experimental design

At ED18, fertile eggs were allocated into five experimental groups per experiment and each group had 21-24 eggs. Two experiments (LPS and CpG ODN) were performed separately. Both the encapsulated and the free forms of CpG ODN at two doses (5 µg/egg as low dose and 25 µg/egg as high dose) and LPS also at two doses and, two forms (2 µg/egg as low dose and 20 µg/egg as high dose) were administered to ED18 chicken eggs. Doses of LPS and CpG ODN were chosen based on previous studies in embryonated eggs or in day-old chicks (Barjesteh et al., 2015a; Parvizi et al., 2014; St. Paul et al., 2011). Phosphate buffered saline (PBS) was used as control preparation. At each time points, 7 – 8 chicken embryos were sacrificed. Spleen, lungs and bursa of Fabricius were collected from embryos at 6, 18 and 48 hps and stored in RNAlater at -80°C until further processing.

RNA extraction and cDNA synthesis

Each sample collected in this study was processed individually. Approximately 50 mg of each tissue was used to extract RNA using Trizol reagent (Life Technologies, Burlington, Ontario) according to the manufacturer’s protocol. RNA Quantity and quality was determined using the NanoDrop® ND-1000 spectrophotometry (NanoDrop Technologies, Wilmington, DE). Ten micrograms of extracted RNA were treated with DNase enzyme (Ambion, Austin, Texas). cDNA was synthesized from 1 µg of DNase treated RNA using Oligo(dT)12–18 primers and the Super-Script™ First-Strand Synthesis System kit (Life Technologies, Burlington, Canada) according to manufacturer’s instructions. Synthesized cDNA was diluted at a 1:10 ratio with diethylpyrocarbonate-treated water.
Real-time PCR

Real-time PCR was performed using SYBR green dye in a LightCycler 480 II (Roche Diagnostics, Laval, Quebec) (St. Paul et al., 2011) to quantify the expression of selected cytokine genes. Target gene expression levels were normalized to chicken β-actin. Target genes and their respective primer sequences and accession numbers are listed in Table 1. The primers were synthesized by Sigma–Aldrich Canada (Oakville, Ontario).

Statistical analysis

The housekeeping gene β-actin was used as the reference gene to calculate the relative expression of all genes used in this study. Relative expression was calculated using LightCycler 480 II advanced relative quantification software. Data were logarithmically transformed and analyzed using general linear model (Proc GLM) in Statistical Analysis Software version 9.3 (SAS, Cary, NC). When there was significant difference among the groups, Duncan’s multiple range test was performed to determine which group was significantly different. The Kruskal-Wallis test was used when data were not normally distributed. Results were considered significant if the p value was <0.05. Results were presented as geometric mean of relative expression ± standard error of mean.

Results

The physical properties of PLGA NPs encapsulating LPS and CpG ODN were comparable to our previous report (Alkie et al., 2017). The size of encapsulated nanoparticles for CpG ODN and LPS varied between 640 nm to 670 nm in diameter and zeta potential varied from -5 mV to -2 mV. Furthermore, the encapsulation efficiency calculated for LPS was 72% and for CpG ODN was 69%. From the surface charges, we anticipated the adsorption of some levels of TLR-Ls on the surface of the nanoparticles, which may have cell priming effects. Therefore, it is plausible
that TLR4 on the cell surface can recognize the surface adsorbed LPS. Furthermore, none of the
doses of encapsulated or free form of TLR-Ls caused lethality in chicken embryos in this study
compared to the PBS inoculated group.

Expression of cytokine genes following LPS treatment

In spleen, high dose of the free form of LPS significantly downregulated IFN-γ at 6 hps
compared to all other groups (Fig. 1A). At 18 hps, both low and high doses of the free form of
LPS significantly downregulated IFN-γ compared to the PBS group, but both doses of the
encapsulated LPS induced significantly higher IFN-γ expression compared to free form of LPS
(Fig. 1A). However, the high dose of encapsulated form of LPS significantly upregulated IFN-γ
in spleen at 48 hps, indicated a typical slow release and stimulation of splenocytes (Fig. 1A).

There were no significant changes in the expression of IFN-α (Fig.1B). Administration of
high dose of the encapsulated form of LPS led to significant upregulation of IL-1β in spleen at 6
and 48 hps compared to all other groups (Fig. 1C). IL-10 gene expression was significantly
increased at 48 hps in high dose of the free and the encapsulated forms of LPS groups (Fig. 1D).
There were no significant changes in the expression IL-13 in spleen in all groups of LPS
treatment compared to the PBS control group (Fig.1E).

The expression of IFN-γ in lungs was not significantly different from the PBS control
group at any of the time points except high dose of free form of LPS at 6 hps (Fig. 2A). High
dose of the free form of LPS downregulated IFN-α expression at 48 hps in lungs (Fig. 2B). The
expression of IL-1β was significantly upregulated by the high dose of (free form) LPS compared
to the low dose of (free form) LPS at 48 hps and both groups were significantly higher than the
PBS group at 48 hps (Fig. 2C). There were no significant changes in the expression of IL-10 and
IL-13 in lungs at any time point (Fig. 2D and 2E).
In the bursa of Fabricius, the expression of IFN-γ was significantly increased by the low dose of the free form of LPS compared to the PBS control group at 48 hps (Fig. 3A). The expression of IFN-α, IL-10 and IL-13 in LPS groups was not significantly different from the PBS group at any of the time points (Fig. 3B, 3D and 3E). The expression of IL-1β was significantly upregulated with the high dose of the free form of LPS group at 48 hps compared to the PBS group (Fig. 3C).

There was tissue dependent cytokine gene expression in response to free and encapsulated forms of LPS. In general, cytokine gene expression was induced by the encapsulated form of LPS in spleen and by the free form of LPS in lungs and bursa of Fabricius. Significant upregulation of cytokine genes generally occurred at 48 hps.

*Expression of cytokine genes following CpG ODN treatment*

Administration of the free and the encapsulated forms of CpG ODN induced different cytokines in different tissues. In spleen at 48 hps, IFN-γ gene expression was upregulated significantly by the high dose of the free form of CpG ODN compared to all other treatments (Fig. 4A). IFN-α gene expression was significantly upregulated in high dose of the encapsulated form of CpG ODN compared to PBS group (Fig. 4B). IL-1β expression in spleen at 18 hps was significantly increased with high dose of the free form of CpG ODN, compared to all other groups (Fig. 4C). At 6 hps in spleen, IL-10 expression was significantly downregulated in all CpG ODN groups, compared to the PBS control group (Fig. 4D). There was no significant change in the expression of IL-13 in spleen at any time point (Fig. 4E).

In the lung tissue, IFN-γ and IFN-α expression was significantly upregulated in both low and high doses of the free form of CpG ODN groups at 48 hps compared to the PBS group (Fig. 5A and 5B). Low dose of the encapsulated form of CpG ODN also significantly induced IFN-α.
expression at 48 hps compared to the PBS group (Fig. 5B). The low dose of the free form of CpG ODN significantly downregulated IL-1β gene expression at all time points compared to all other groups, but the high dose of the free and the encapsulated forms of CpG ODN significantly increased the expression of IL-1β at 18 hps compared to the PBS group in lung tissue (Fig. 5C). There was no significant change in the expression of IL-10 in lungs at any time point (Fig. 5D).

In the bursa of Fabricius, mean IFN-γ expression was high for high dose free and encapsulated forms of CpG ODN at 48 hps, but it was not statistically different from the PBS control group (Fig. 6A). IFN-α gene expression in all four treatment groups of CpG ODN at 6 and 18 hps was significantly downregulated compared to the PBS group (Fig. 6B). IL-1β gene expression was significantly downregulated at 18 hps in all four treatment groups receiving CpG ODN, and at 6 hps for the high dose of free form CpG ODN compared to all other treatments (Fig. 6C). There was no significant change in the expression of IL-10 in the bursa of Fabricius at any time point (Fig 6D). In all four treatment groups receiving CpG ODN, IL-13 expression was significantly downregulated at 6 and 18 hps except for low dose free form CpG ODN at 6 hps (Fig. 6E).

In general, cytokine gene expression was induced by the free form of CpG ODN in spleen, and by both the free and encapsulated forms of CpG ODN in lungs. None of the forms of CpG ODN upregulated any of the cytokine genes in bursa of Fabricius.

Discussion

Innate responses to various TLR-Ls have been demonstrated in vitro and in vivo in chickens, but they have not been systematically studied in chicken embryos. A variety of nanoparticle-based delivery methods are available to administer several compounds, including TLR-Ls, into an animal for induction of immune responses. One of these delivery methods
involves encapsulating active compounds into nanoparticles of different physical and chemical properties. In the present study, an in ovo model was used, whereby the free and encapsulated forms of TLR-Ls were administered into the amnion of ED18 chicken embryos to evaluate the effectiveness and duration of innate responses induced in several embryonic tissues. Previous studies suggested that the rhythmic movement of respiration which starts between ED17 and ED18 in chick embryos (Windle and J. Barcroft, 1938) causes the uptake of amniotic fluid and its contents into the mouth, which will then reach the lungs and intestines (Jochemsen and Jeurissen, 2002). As a proof of principle, Jochemsen and Jeurissen (Jochemsen and Jeurissen, 2002) demonstrated the localization of carbon particles in the lungs and bursa of Fabricius of late stage chicken embryos following amniotic injections of carbon colloids. Furthermore, earlier studies reported that active transport of particles in the bursa of Fabricius, a primary lymphoid organ in chickens, began at ED18 (Ekino et al., 1985). It was speculated that abdominal movements of late stage chick embryos may have a suction effect of amniotic contents into the cloaca, which finally reach the bursa of Fabricius. Therefore, it is possible that in a similar manner, the free and encapsulated forms of TLR-Ls may enter chick embryos following amniotic administration for subsequent deposition in the lungs, intestine and bursa of Fabricius. Further absorption from the intestine may result in particle dissemination in systemic organs, such as spleen.

In the present study, administration of TLR-Ls to ED18 chicken embryos was shown to induce innate responses. These responses included the induction of IFN-α, IFN-γ and IL-1β expression in spleen and lungs following administration of CpG ODN. In addition, LPS induced expression of IFN-γ, IL-1β and IL-10 in spleen, IL-1β in lungs, and IFN-γ and IL-1β in the bursa of Fabricius. Spleen and bursa of Fabricius are major organs of the immune system. In chickens,
immune system development in the spleen begins at ED15 with formation of immunoreactive reticular fibers (Yassine et al., 1989). Following that, a fully formed spleen can be seen a week after hatching (Jeurissen, 1991). Bursal vesicle formation starts to form between ED4.5 to ED10 and colonization of bursal follicles with pre-B cells occurs between ED 10-15 (Fellah et al., 2008). Therefore, the development of the chicken immune system starts at an early stage of embryonation. The immune system develops rapidly in the late stage of embryonation and is fully mature a first few weeks after hatching. Although the development of the immune system is not complete in ED18 embryos, we and others have shown that chicken embryos can respond to TLR-Ls.

In the present study, TLR-Ls induced the expression of genes whose products play a role in mounting immunity against viruses, including IFN-α and IFN-γ. In addition, TLR-Ls also induced the expression of IL-1β, which is a pro-inflammatory cytokine. Significant upregulation of cytokine genes indicates that ED18 embryos are immunologically competent to mount innate responses to TLR-Ls stimulation. This is further supported by the fact that in ovo vaccination of chicken embryos provides protection against infectious disease in early life. Therefore, the chicken embryo must be capable of inducing a balance of innate and adaptive responses, although there are structural and physiological differences between the embryo’s immune system compared to that of adults.

Innate responses induced by LPS and CpG ODN in embryos have similarities to those induced in mature chickens, in terms of the type of cytokines that are induced. In this regard, several immune system genes were induced following TLR-Ls stimulation including IFN-γ, IFN-α, IL-1β and IL-10 in spleen, lungs and bursa of Fabricius of chicken embryos. This observation is comparable to observations made by St Paul and colleagues (St. Paul et al., 2011, 2012c) in
post-hatch chickens. LPS administration to 14-day old chickens significantly increased the expression of IFN-γ, IL-1β and IL-10 in spleen. (St. Paul et al., 2011). Moreover, LPS stimulation of bursal cells from post-hatch chickens significantly upregulated IFN-γ expression (St. Paul et al., 2012c). However, differences in the number and type of cells in embryonic tissues and mature chickens may result in differences either in the magnitude or quality of innate responses. Notwithstanding these differences, the observation reported here clearly show that ED18 chick embryos are able to respond to TLR-Ls.

Administration of TLR-Ls to chicken embryos might induce the maturation of cells of the immune system following recognition of TLR-Ls and initiation of downstream signaling pathways within these cells. A study (Hegde et al., 1982) performed in germ-free chickens indicates that antigenic stimulation from gut microflora enhances the maturation process of lymphoid tissues. In addition, Reese and colleagues (Reese et al., 2006) proposed that exposure to antigens can prompt the development and maturation of bronchus associated lymphoid tissues based on their study in newly hatched chicks. Furthermore, in sheep, it has been shown that intra-amniotic administration of LPS induced the maturation of monocytes to macrophage in lungs of preterm fetuses (Kramer et al., 2007). Therefore, it is possible that the functional competency of immune system cells can be accelerated by exposure of late stage embryos to TLR-Ls.

Administration of the encapsulated form of TLR-Ls in the current study influenced the induction of innate responses in embryos similar to that of the free form of TLR-Ls. Encapsulation with PLGA NPs protects TLR-Ls from enzymatic degradation (Panyam and Labhasetwar, 2003) and enhances the bioavailability of TLR-Ls in the microenvironment. This leads to the slow release of TLR-Ls over a period of time and sustained responses to these
ligands. Sustained responses may have beneficial effects for the host, especially for protection against pathogens. In the present study, significant upregulation of antiviral and pro-inflammatory cytokine genes by the encapsulated form of TLR-Ls indicates that encapsulation influenced the induction of innate responses in chicken embryos. In the current study, a significant upregulation of IFN-α gene was observed at 48 hps both in spleen and lungs. However, Dar and colleagues (Dar et al., 2009) showed that there was no difference in the expression of the IFN-α gene in spleen compared to that of the control group when the ED18 chicken eggs were treated with the free form of CpG ODN. This difference is most likely due to the effect of encapsulation which led to the sustained release of the encapsulated CpG ODN in the embryos.

There was tissue-dependent expression of cytokine genes in response to the free and encapsulated forms of TLR-Ls. These differences may be due to the type of cells which are exposed to different forms of TLR-Ls and the various uptake mechanisms used with different forms of TLR-Ls by the cells. It is well known that the free forms of LPS and CpG ODN initiate downstream signaling pathways to induce the expression of cytokine genes via the interaction with TLR4 present on the cell surface and TLR21 present in the endosome, respectively. On the other hand, it has been reported that various cell types can take up PLGA NPs including dendritic cells, macrophages, endothelial cells and different types of epithelial cells (Lutsiak et al., 2002; Cartiera et al., 2009; Voigt et al., 2014). Therefore, it is apparent that spleen, lungs and bursa of Fabricius might have handled the encapsulated LPS and CpG ODN differently since the cell composition of these organs varies. Furthermore, a number of internalization mechanisms, such as phagocytosis, endocytosis and micropinocytosis are involved in the uptake of PLGA NPs into the cells for further processing (Lutsiak et al., 2002; Gamucci et al., 2014; Voigt et al.,
Hence, these diverse mechanisms might have played a role in the processing of the encapsulated LPS and CpG ODN by cells of spleen, lungs and bursa of Fabricius. Several studies indicate that endocytosed PLGA nanoparticles are processed not only by lysosomal degradation but also by endosomal escape which exposes PLGA nanoparticles to the cytoplasm of the cells (Panyam et al., 2002; Shen et al., 2006; Cartiera et al., 2009; Song et al., 2016). In the case of encapsulated CpG ODN, it is possible that it might have encountered TLR21 and cytosolic DNA sensors like DNA-dependent activator of IRFs (DAI) and cyclic GMP-AMP synthase (cGAS) which activate interferon production by stimulator of interferon gene (STING) (Unterholzner, 2013). In addition to intracellular uptake of PLGA NPs, a study by Xu and colleagues (Xu et al., 2009) demonstrated that PLGA NPs are not internalized by cells, instead the contents of the particles are released into the extracellular milieu or transferred to contacting cells. Hence, a detailed cellular study to determine how and by which cells PLGA-encapsulated TLR-Ls are taken up and processed, is warranted.

In conclusion, induction of innate responses in ED18 chicken embryos by administration of free and encapsulated forms of TLR-Ls was demonstrated in the present study. Importantly, encapsulated TLR-Ls can activate expression of cytokine genes without causing adverse effects on the viability of embryos. The innate responses induced in embryos appear to be similar to those induced in mature chickens. Further investigation into the mode of action and the long-term effects of encapsulated TLR-Ls will facilitate the development of in ovo strategies for accelerating immune system development and maturation in chickens.

Acknowledgement

We would like to thank Dr. Sharif’s lab members for technical assistance in this study.
Table 3.1: Primer sequences and accession numbers used for real-time PCR

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer sequences</th>
<th>Accession numbers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α</td>
<td>F: 5’-ATCCTGCTGCTCACGCTCCTTCT-3’&lt;br&gt;R: 5’-GGTGTTGCTGCTGTTCCAGGATG-3’</td>
<td>AB021154</td>
<td>(Brisbin et al., 2008)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>F: 5’-ACACTGACAAGTCAAAGCCGCACA-3’&lt;br&gt;R: 5’-AGTCGGTTCATCGGGAGCTTGGC-3’</td>
<td>X99774</td>
<td>(Brisbin et al., 2010)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>F: 5’-GTGAGGCTCAACATTGCCTGTA-3’&lt;br&gt;R: 5’-TGTCCAGGCGTTAGAAGATGAAG-3’</td>
<td>Y15006</td>
<td>(Abdul-Careem et al., 2009a)</td>
</tr>
<tr>
<td>IL-10</td>
<td>F: 5’-AGCAGATCAAGGAGACGTTC-3’&lt;br&gt;R: 5’-ATCAGCAGGTACTCCTCGAT-3’</td>
<td>AJ621614</td>
<td>(Abdul-Careem et al., 2007a)</td>
</tr>
<tr>
<td>IL-13</td>
<td>F: 5’-ACTTGTCCAAGCTGAAGCTTC-3’&lt;br&gt;R: 5’-TCTTGCAGTCCGTGGTCATGTTGTC-3’</td>
<td>AJ621250</td>
<td>(Abdul-Careem et al., 2008a)</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5’-CAACACAGTGCTTGCTGTTGGTGTA-3’&lt;br&gt;R: 5’-ATCGTACTCCTGTGGCTGATCC-3’</td>
<td>X00182</td>
<td>(Brisbin et al., 2008)</td>
</tr>
</tbody>
</table>
Figure 3.1: Relative expression of cytokine genes in spleen after LPS administration.

Gene expression relative to the housekeeping gene β-actin was calculated. Relative gene expression of cytokines IFN-γ (A), IFN-α (B), IL-1β (C), IL-10 (D) and IL-13 (E) in spleen at 6, 18 and 48 hps induced by 2 µg and 20 µg of free form (LPS-2 µg, LPS-20 µg) and encapsulated LPS (ELPS-2µg, ELPS-20µg), and PBS. Y axis indicates geometric mean of relative expression. Error bars represent standard error of the mean. Data were analyzed statistically using general linear models and Kruskal-Wallis test. Significant differences between a treatment group and PBS group were considered statistically significant for p values <0.05 (*). Significant differences among the groups were considered statistically significant for p values <0.05 (#).
Figure 3.2: Relative expression of cytokine genes in lungs after LPS administration.

Gene expression relative to the housekeeping gene β-actin was calculated. Relative gene expression of cytokines IFN-γ (A), IFN-α (B), IL-1β (C), IL-10 (D) and IL-13 (E) in lungs at 6, 18 and 48 hps induced by 2 µg and 20 µg of free form (LPS-2 µg, LPS-20 µg) and encapsulated LPS (ELPS-2µg, ELPS- 20µg), and PBS. Y axis indicates geometric mean of relative expression. Error bars represent standard error of the mean. Data were analyzed statistically using general linear models and Kruskal-Wallis test. Significant differences between a treatment group and PBS group were considered statistically significant for p values <0.05 (*). Significant differences among the groups were considered statistically significant for p values <0.05 (#).
Figure 3.3: Relative expression of cytokine genes in bursa of Fabricius after LPS administration

Gene expression relative to the housekeeping gene β-actin was calculated. Relative gene expression of cytokines IFN-γ (A), IFN-α (B), IL-1β (C), IL-10 (D) and IL-13 € in bursa of Fabricius at 6, 18 and 48 hps induced by 2 µg and 20 µg of free form (LPS-2 µg, LPS-20 µg) and encapsulated LPS (ELPS-2µg, ELPS- 20µg), and PBS. Y axis indicates geometric mean of relative expression. Error bars represent standard error of the mean. Data were analyzed statistically using general linear models and Kruskal-Wallis test. Comparisons were considered statistically significant for p values <0.05 (*).
Figure 3.4: Relative expression of cytokine genes in spleen after CpG ODN administration. Gene expression relative to the housekeeping gene β-actin was calculated. Relative gene expression of cytokines IFN-γ (A), IFN-α (B), IL-1β (C), IL-10 (D) and IL-13 (E) in spleen at 6, 18 and 48 hrs induced by 5 µg and 25 µg of free form (CpG-5 µg, CpG-25 µg) and encapsulated CpG ODN (EcpG-5 µg, EcpG-25 µg), and PBS. Y axis indicates geometric mean of relative expression. Error bars represent standard errors of the mean. Data were analyzed statistically using general linear models and Kruskal-Wallis test. Comparisons were considered statistically significant for p values <0.05 (*).
Figure 3.5: Relative expression of cytokine genes in lungs after CpG ODN administration.

Gene expression relative to the housekeeping gene β-actin was calculated. Relative gene expression of cytokines IFN-γ (A), IFN-α (B), IL-1β (C) and IL-10 (D) in lung at 6, 18 and 48 hps induced by 5 µg and 25 µg of free form (CpG-5 µg, CpG-25 µg) and encapsulated CpG ODN (EcpG-5 µg, EcpG-25 µg), and PBS. Y axis indicates geometric mean of relative expression. Error bars represent standard errors of the mean. Data were analyzed statistically using general linear models and Kruskal-Wallis test. Comparisons were considered statistically significant for p values <0.05 (*).
Figure 3.6: Relative expression of cytokine genes in bursa of Fabricius after CpG ODN administration.

Gene expression relative to the housekeeping gene β-actin was calculated. Relative gene expression of cytokines IFN-γ (A), IFN-α (B), IL-1β (C), IL-10 (D) and IL-13 (E) in bursa of Fabricius (A-E) at 6, 18 and 48 hps induced by 5 µg and 25 µg of free form (CpG-5 µg, CpG-25 µg) and encapsulated CpG ODN (EcpG-5 µg, EcpG-25 µg), and PBS. Y axis indicates geometric mean of relative expression. Error bars represent standard errors of the mean. Data were analyzed statistically using general linear models and Kruskal-Wallis test. Comparisons were considered statistically significant for p values <0.05 (*).
Chapter 4

*In ovo* administration of Toll-like receptor ligands encapsulated in PLGA nanoparticles impede tumor development in chickens infected with Marek’s disease virus

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Abstract

One of the economically important diseases in the poultry industry is Marek’s disease (MD) which is caused by Marek’s disease virus (MDV). The use of current vaccines provides protection against clinical signs of MD in chickens. However, these vaccines do not prevent the transmission of MDV to susceptible hosts, hence they may promote the development of new virulent strains of MDV. This issue persuaded us to explore alternative approaches to control MD in chickens. Induction of innate responses at the early stage of life in the chicken may help to prevent or reduce MDV infection. Further, prophylactic use of Toll-like receptor ligands (TLR-Ls) has been shown to generate host immunity against infectious diseases. In this regard, encapsulation of TLR-Ls in Poly (D, L-lactic-co-glycolic acid) (PLGA) may further enhance host responses by controlled release of TLR-Ls for an extended period. Hence, in the current study, protective effects of encapsulated TLR4 and TLR21 ligands, LPS and CpG, respectively, were investigated against MD. Results indicated that administration of encapsulated CpG and LPS first at embryonic day (ED)18, followed by post-hatch at 14 days-post infection (dpi) intramuscularly, diminished tumor incidence and MDV genome copy numbers in feathers at 21 dpi. In addition, analysis of cytokine gene profiles of interferon (IFN)-α, IFN-β, IFN-γ, inducible nitric oxide synthase (iNOS), interleukin (IL)-1β, IL-18 and IL-10 in spleen and bursa of Fabricius at different time points suggests that TLR-Ls possibly triggered host responses through the expression of IL-1β and IL-18 to reduce tumor formation. However, further studies are needed to explore the role of these pro-inflammatory cytokines and other influencing elements like lymphocytes in the hindrance of tumor development by TLR-Ls treatment in chickens.

Key words: Marek’s disease virus, TLR ligands, Chicken embryos, In ovo administration, cytokine genes, PLGA encapsulation
Introduction

Marek’s disease virus (MDV) infects chickens via the respiratory route and causes Marek’s disease (MD) which is characterized by T cell lymphoma and immunosuppression. Following infection, MDV spreads to lymphoid organs and causes lysis of B and T cells. This initial cytolytic phase, which takes place around 2-7 days post-infection (dpi), is followed by the latent phase which occurs around 7-10 dpi (Baigent and Davison, 2004). In the next phase, which is the late cytolytic and immunosuppressive phase, MDV is reactivated from latency and continues to the transformation and proliferation phase which leads to tumor formation in internal organs and skin. Further, the infectious form of MDV produced in the feather follicular epithelium is shed into the environment with feather dander and transmitted by inhalation of dust particles.

Marek’s disease is currently controlled by vaccination. However, vaccines are not able to control MDV shedding. Both innate and adaptive immune mechanisms are involved in control of MDV infection. The innate components of the immune system promptly act against MDV infection and activate adaptive immune responses. Toll-like receptors (TLRs) are an indispensable part of the innate mechanisms. Similar to other herpesviruses (Paludan et al., 2011), MDV may also be detected by TLR2, TLR3, TLR7 and TLR21 (the avian counterpart of TLR9). An increased expression of TLR3 and TLR7 in the lungs of MDV-infected chickens supports this notion (Abdul-Careem et al., 2009a). Similar to mammalian models, TLR-Ls such as CpG and lipopolysaccharide (LPS), which is detected by TLR4, can trigger innate defense mechanisms in vitro, in ovo and in vivo in chickens (Alkie et al., 2017; Bavananthasivam et al., 2017; St. Paul et al., 2011) and elicit host responses against MDV in chickens (Parvizi et al., 2014, 2012a).
Enhancement of innate responses during the neonatal period may provide improved immune protection in chickens. Young chicks are exposed to MDV in the first few days of life, therefore, it is important to confer early protection prior to maturation of adaptive immunity. In this regard, previous studies have revealed that in ovo administration of TLR-Ls provide protective immunity and control replication of microbial pathogens such as infectious laryngotracheitis virus (Thapa et al., 2015), infectious bronchitis virus (Dar et al., 2014) and Escherichia coli (Taghavi et al., 2009). However, no study has investigated the in ovo administration of TLR-Ls conferring protective immunity against MDV in young chicks.

There are limitations to the use of TLR-Ls as antimicrobial agents or adjuvants, such as their short half-life and rapid elimination from the body which can reduce their efficacy (Engel et al., 2011). To overcome these limitations, TLR-Ls can be encapsulated into poly(D,L, lactic-co-glycolic) acid (PLGA) as nanoparticles (Panyam and Labhasetwar, 2003). This facilitates slow release of encapsulated TLR-Ls from PLGA nanoparticles upon hydrolysis in tissues or within cells following uptake. Therefore, in this study, we evaluated the efficacy of TLR-Ls encapsulated PLGA nanoparticles against MD when these ligands were administered in ovo and post-hatch.

Materials and methods

Chicken eggs

Specific pathogen free eggs were obtained from the Canadian Food Inspection Agency (Ottawa, Canada) and incubated at recommended temperature and relative humidity at Arkell Poultry Research Station, University of Guelph. All experiments were conducted in accordance with the guidelines of the University of Guelph's Animal Care Committee. At ED18, TLR-Ls
were administered into the eggs and they were set for hatching. Day old chicks were transported to the animal isolation facility of Ontario Veterinary College, University of Guelph.

**TLR-Ls and Virus**

LPS from *E. coli* O111:B4 and synthetic Class B CpG ODN 2007 with phosphorothioate backbone were purchased from Sigma-Aldrich (Oakville, ON, Canada). *In vivo* propagated, very virulent strain of MDV (RB1B), which was provided by Dr. Schat (Schat et al., 1982), was used to infect chicks.

**Encapsulation of TLR ligands**

LPS and CpG were encapsulated in PLGA, (Resomer® RG 503H, Sigma-Aldrich) using the modified double emulsion solvent evaporation method to generate PLGA nanoparticles as described in our previous studies (Alkie et al., 2017; Bavananthasivam et al., 2017). The encapsulation efficiency of the PLGA nanoparticles for both TLR ligands were determined as in our previous studies (Alkie et al., 2017; Bavananthasivam et al., 2017)

**Experimental design**

Three independent experiments were performed to examine the protective effect of encapsulated TLR-Ls (LPS and CpG) against MDV infection. The purpose of the first experiment was to identify the suitable form of TLR-Ls (free or encapsulated) and the day of *in ovo* administration (ED18 or ED19) of TLR-Ls that can provide protection against MDV infection. In the first experiment, four groups of ED18 embryos were injected via the amniotic route with 20µg soluble or encapsulated LPS (ELPS), or 25µg soluble or encapsulated of CpG (ECpG) and were designated as 18LPS, 18ELPS, 18CpG, 18ECpG groups. ED19 embryos were similarly inoculated with the above formulations and were designated as 19LPS, 19ELPS, 19CpG, 19ECpG groups. Mock PLGA nanoparticles were administered to ED18 embryos
(18PLGA). Each group had 11-12 embryonated eggs. All day-old chicks, except the PBS group were infected with 250 plaque-forming units of very virulent RB1B MDV/chick via intra-abdominal route. TLR-Ls sham treated, but MDV-infected group (MDV only) was used as a positive control group. The experiment was terminated at 21 dpi. Based on the results of the tumor incidence, encapsulated TLR-Ls were selected for subsequent experiments.

The second experiment was designed to determine whether a single or a double dose of encapsulated TLR-Ls could provide protective immunity against MDV. In this experiment, the first dose of ELPS or ECpG or both (ELPS+ECpG) TLR-Ls or PLGA were administered to ED18 embryos. All day-old chicks were infected with MDV as indicated above. The groups that received the second dose of ELPS or ECpG or combination of above, or PLGA were injected at 14 dpi through the intramuscular route. The experimental groups were designated as ECpG, ELPS, ECpG+ELPS, 2ECpG, 2ELPS, 2(ECpG+ELPS), PLGA, 2PLGA, MDV only and PBS groups and each group had 15 ED18 embryos. The dose and volume of TLR-Ls remained identical to the first experiment. The experiment was terminated at 21 dpi and tumor incidence was recorded. Based on the results, double doses of encapsulated TLR-Ls were selected to use in the subsequent experiment.

The third experiment was designed to address the potential immunological mechanisms involved in the protective effect of encapsulated TLR-L treatments against MDV infection. ELPS or ECpG or PLGA was initially administered to ED18 embryos. Day-old chicks were infected with MDV as mentioned above. The second dose of ELPS or ECpG or PLGA was administered at 14 dpi. The groups were designated as 2ECpG, 2ELPS, 2(ECpG+ELPS), 2PLGA, MDV only and PBS groups and each group had 32-33 ED18 embryos. At 4, 10 and 21
dpi, spleen and bursa of Fabricius (BF) and body weights (BW) were recorded and samples from spleen, BF and feathers were collected. Tumor incidence in chickens was recorded at 21 dpi.

**DNA and RNA extraction**

Genomic DNA was extracted from feather tips as described previously (Abdul-Careem et al., 2006a). One hundred nanograms of DNA was used in real-time PCR. RNA was extracted as described previously (Bavananthasivam et al., 2017). The quantity and quality of DNA and RNA were determined using the NanoDrop® ND-1000 spectrophotometry (NanoDrop Technologies, Wilmington, DE).

**Real-time PCR**

Real-time PCR was performed using SYBR green dye in a LightCycler 480 II to quantify MDV genome copy numbers and cytokine gene expression (Roche Diagnostics, Laval, Quebec) as described previously (Abdul-Careem et al., 2006a; St. Paul et al., 2011). Primer sequences of target and reference genes are listed in Table 1. The primers were synthesized by Sigma–Aldrich Canada (Oakville, ON).

**Statistical analysis**

Relative expression was calculated using LightCycler 480 II advanced relative quantification software in relation to chicken β-actin. For gene expression analysis, data were logarithmically transformed and analyzed using general linear model (Proc GLM) or Kruskal-Wallis test in Statistical Analysis Software version 9.3 (SAS, Cary, NC). Duncan’s or Dunn’s multiple range test was performed to determine significantly different groups. The Kruskal-Wallis test was used to analyze data of MDV load. Gene expression results are presented as geometric mean of relative expression ± standard error of mean. Fisher’s exact test was used to analyze the tumor incidence data using GraphPad Prism 6.04. Data of BF:BW spleen:BW ratios
were analyzed using one-way ANOVA followed by Dunnett’s multiple comparison test in GraphPad Prism 6.04. Results were considered significant if $p$ value was $<0.05$.

Results

*Incidence of Marek’s disease in treatment groups*

In the first experiment, at 21 dpi, lower tumor incidence was observed in the 18LPS, 18ELPS, 18PLGA and 19CpG groups ($P<0.05$, Fig 1A) suggesting that the administration of TLR-Ls to ED18 or ED19 embryos, prior to exposure to MDV, can inhibit MDV-induced tumor formation in chickens. In the second experiment, at 21 dpi, only 2ECpG and 2(ECpG+ELPS) groups showed a significant reduction in tumor formation ($P<0.01$, Fig 1B) suggesting that encapsulation and second administration of TLR-Ls, after exposure to MDV, improves the protective role of the treatment. In the third experiment, tumor incidence was significantly reduced in all TLR-L treatment groups ($P<0.05$, Fig 1C). However, the lowest tumor incidence was observed in chickens that had received 2ECpG treatment. 2ECpG treatment reduced tumor incidence in the second and third experiment by 54.5% and 60% respectively. In all the experiments, the tumor formation in the treatment groups was compared to that in the MDV only group, in which 100% of birds developed lymphoma (Fig 1).

*MDV load in feathers*

MDV genome copy numbers were not significantly reduced by TLR-L treatments in all three experiments (Fig 2). However, at 21 dpi, all groups which received TLR-L treatments at ED18 in the first experiments (Fig. 2B) and, all TLR-L treatment groups in the second and third experiments (Fig 2B and 2C) showed reduction in MDV load when compared to the MDV only group (Fig 2A).
**Bursa of Fabricius:BW and spleen:BW ratios**

In all groups which received MDV, BF:BW ratios were the same as the PBS group at 4 dpi (Fig 3A). However, at 10 and 21 dpi, BF:BW ratios were significantly lower in all groups which received MDV when compared to PBS group ($P < 0.0001$, Fig 3A). Spleen:BW ratios were significantly higher than PBS group at 10 dpi and 21d pi in all groups which received MDV and, at 4 dpi in 2ECpG and 2(ECpG+ELPS) groups ($P < 0.001$, Fig 3B).

**Cytokine gene expression**

In the spleen, there was no significant change in interferon (IFN)-α and inducible nitric-oxide synthase (iNOS) expression at any time points (Fig 4A and 4E). However, at 10 dpi, IFN-β was significantly upregulated in all treatment groups and MDV only group when compared to PBS ($P < 0.0001$, Fig 4B). Further, at all time points, IFN-γ and interleukin (IL)-10 were significantly upregulated in all TLR-L treatments and MDV only groups when compared to the PBS group ($P < 0.002$, Fig 4C and $P < 0.001$, Fig 4D). In addition, at 21 dpi, IFN-γ was significantly upregulated in 2ELPS treatment when compared to MDV only group ($P < 0.0001$, Fig 4C). IL-1β and IL-18 were significantly upregulated at 10 dpi and 4 dpi, respectively in all TLR-L treatment groups compared to MDV only group ($P < 0.0001$, Fig 4F and 4G). At 21 dpi, all groups which received MDV showed upregulation of IL-18 when compared to PBS group ($P < 0.0002$, Fig 4G).

In the bursa of Fabricius, at 10 dpi, IFN-α was upregulated in 2ECpG and MDV only groups ($P < 0.0001$, Fig 5A) and, IFN-β was upregulated in all groups which received MDV when compared to the PBS group ($P < 0.0001$, Fig 5B). IFN-γ was upregulated at 4 dpi in 2(ECpG+ELPS) group ($P < 0.02$) and at 10 dpi in all groups ($P < 0.0001$) which received MDV when compared to PBS group (Fig 5C). IL-10 was upregulated in all groups which received MDV at 10dpi ($P < 0.0009$) and 21 dpi ($P < 0.002$) except the MDV only at 10dpi and 2ECpG
groups at 21 dpi (Fig 5D). All groups which received MDV at 10 dpi and 21 dpi exhibited upregulation of iNOS ($P<0.0001$, Fig 5E). At 10 dpi, IL-1β was upregulated in 2ECpG group when compared to MDV only group ($P<0.01$, Fig 5F). IL-18 was upregulated only in 2(ECpG+ELPS) group at 4 dpi ($P=0.05$, Fig 5G).

**Discussion**

The present study was conducted to determine the optimal time of *in ovo* administration of TLR-Ls, the effective form of TLR-Ls and the frequency of administration of TLR-Ls to induce protective immunity against MDV in chickens. Based on the results, encapsulated TLR-Ls administered as a double dose reduced tumor formation and MDV load in feathers by 21 dpi.

The organ weight indices for spleen and BF were determined to evaluate any changes caused by the administration of TLR-Ls in these lymphoid organs. Bursal atrophy and spleen enlargement are common characteristics of MDV infection. Lower ratio of BF:BW indicates the occurrence of bursal atrophy at 10 and 21 dpi and suggests that TLR-L treatments did not inhibit B cells depletion which leads to bursal atrophy. Higher ratio of spleen:BW at 4, 10 and 21 could be due to the infiltration of lymphocytes and/or tumor formation.

In all three experiments, treatment with TLR-Ls reduced tumor formation. However, in the third experiment, the percentage reduction of tumor incidence in 2ECpG group was remarkably higher than that in the other treatment groups. Activation of CD8 T cells, natural killer cells and other effector cells and/or inhibition of T regulatory cell function by TLR-Ls might be involved in their inhibitory effects on tumor formation (Komai-Koma et al., 2009; Voo et al., 2014). The important role of regulatory T cells in MDV induced immunosuppression has been recently described (Gurung et al., 2017). Other contributing anti-tumor effects of TLR-Ls
are their anti-proliferative, anti-metastatic, anti-angiogenic and pro-apoptotic properties (Damiano et al., 2006; Brignole et al., 2010; Kabilova et al., 2016).

In the current study, the observation of significant upregulation of IL-18 and IL-1β in TLR-L treatment groups compared to MDV only group suggests that expression of these cytokines might be involved in the protective role of TLR-Ls against tumor development. There is evidence for the paradoxical role of these pro-inflammatory cytokines exerting pro-tumor and anti-tumor effects in neoplastic conditions (Yip et al., 1995; Palma et al., 2013; Kolb et al., 2014; Fabbì et al., 2015). Depending on the specific circumstances, these cytokines may favor or oppose tumor progression. It has been shown that IL-18 and IL-1β can be induced by administration of CpG in mice (Roman et al., 1997; Knuefermann et al., 2007) which is proven to be the case in chickens, as demonstrated in the present study. In addition, IL-18 and IL-1β secretion causes pyroptosis, a form of inflammatory cell death, of virus infected cells to control infection (Jacobs and Damania, 2012). In mice, CpG induced IL-18 in the mucosal surface of the genital tract was identified to be associated with protection against herpes simplex virus type 2 (Harandi et al., 2003). This observation supports that IL-18 in our study potentially played a role in conferring protective immunity to MD. Furthermore, IL-18 can induce ICAM-1 expression which supports the infiltration of lymphocytes to the target tissues (Kohka et al., 1998).

Moreover, suppression of murine gamma herpesvirus 68 reactivation from latency via activation of NF-kB was noticed with CpG administration (Haas et al., 2014). Involvement of NF-kB in the expression of IL-1β raises the possibility that ECpG and ELPS triggered upregulation of IL-1β in the spleen at 10 dpi might have paused the reactivation of MDV from latency. However, a comprehensive study is needed to evaluate the role of IL-1β in repression of MDV reactivation. Further, induction of IL-1β helps to mature dendritic cells (Conroy et al., 2008) which can
enhance the presentation of tumor antigens. Therefore, the reduction of MDV induced tumor formation may be mediated by anti-tumor effects of IL-18 and IL-1β.

Another reason for the reduction of tumors observed in the current study could be due to the induction of lymphocytopenia which leads to a reduction of target cells for MDV infection, thereby limiting transformation of CD4 T cells. Similar to MDV, the target cells for HIV are CD4 T cells. These cells have been reported to be depleted due to the secretion of type 1 IFN in HIV infection and the use of neutralizing antibodies against type 1 IFN recovered depletion of CD4 T cells (Cheng et al., 2017; Sivaraman et al., 2011). In the current study, similar mechanisms might have been in operation, however, further experiments are needed to confirm this notion.

Another possible explanation for tumor reduction by TLR-L treatments is that it might have enhanced the function of CD8 T cells which eliminated MDV-infected cells. CpG is known to induce the expression of costimulatory molecules like CD69, CD80 and CD86 (Sun et al., 2000). In addition, indirect activation of antigen presenting cells via secretion of cytokines by CpG contributes to improving the function of CD8 T cells (Sun et al., 1998; Vollmer and Krieg, 2009). Further, it was observed that peri-tumoral administration of CpG in murine colon carcinoma model caused obliteration of established tumors locally and distantly, mainly via CD8 T cells (Heckelsmiller et al., 2002). Based on these lines of evidence, it appears that effector functions of CD8 T cells can be enhanced by indirect effects of CpG. Similar mechanisms might have occurred in the present study to eliminate tumor cells in ECpG administered chickens.

The importance of natural killer (NK) cells in reduction of tumors in the current study may warrant attention. NK cells have anti-tumor effects in humans and mice, however, presence of these cells in chickens is not well established. Nevertheless, NK like cells which show
cytolytic functions (Dinarello et al., 2013) might eliminate transformed tumor cells in MDV-infected chickens. Importantly, in human administration of CpG can induce the NK cell activity (Voo et al., 2014).

In summary, administration of encapsulated TLR-Ls at ED18 and 14 dpi was effective for control of MD tumors in chickens. Significant upregulation of IL-18 and IL-1β in TLR-L treatment groups suggests a role of these cytokines in immunity against MDV infection. Further research is required to gain more insight into the importance of CD8 T and NK cells in immunity against MDV induced by TLR-Ls.

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Author Disclosure Statement

There is no conflict of interest presence regarding the publication of this paper.
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Figure 4.1: Presence of tumor in percentage following TLR-L treatments.

(A) Free form (CpG and LPS) or encapsulated form (ECpG and ELPS) of TLR-Ls or PLGA were administered to chicken embryos at ED18 or ED19. (B) ECpG or ELPS or PLGA were administered to ED18 embryos and/or to 14 dpi chickens. (C) ECpG or ELPS or PLGA were administered to both ED18 embryos and 14 dpi chickens. PBS group and MDV only (untreated, MDV-infected) groups served as control groups. Tumor incidence were recorded during necropsy at 21 dpi. Data were statistically analyzed by Fisher’s exact test in GraphPad Prism 6.04. $p \leq 0.05$ (*) was considered statistically significant.
Figure 4.2: MDV genome copy numbers in feathers following TLR-L treatments. (A) Free form (CpG and LPS) or encapsulated form (ECpG and ELPS) of TLR-Ls or PLGA were administered to chicken embryos at ED18 or ED19 and feathers collected at 21 dpi (B) ECpG or ELPS or PLGA were administered to ED18 embryos and /or to 14 dpi chickens and feathers collected at 21 dpi. (C) ECpG or ELPS or PLGA were administered to both ED18 embryos and 14 dpi chickens and feathers collected at 4, 10 and 21 dpi. PBS group and MDV only (untreated, MDV-infected) groups served as control groups. MDV genome copy numbers per 100ng of DNA were calculated. Data were statistically analyzed by Kruskal-Wallis test in GraphPad Prism 6.04. $p \leq 0.05$ was considered statistically significant. Error bars indicate the standard error of the mean.
ECpG or ELPS or PLGA was administered to ED18 embryos and 14 dpi chickens. PBS and MDV only (untreated, MDV-infected) groups served as control groups. Bursa of Fabricius weight, spleen weight and body weight were recorded at 4, 10 and 21 dpi and, BF:BW (A) and S:BW (B) ratios were calculated. Data were statistically analyzed by one-way ANOVA and followed by Dunnett’s post comparison test in GraphPad Prism 6.04. $p \leq 0.05$ (*) was considered statistically significant. Error bars indicate the standard error of the mean.
Figure 4.5: Relative expression of genes in the spleen following TLR-L treatments. ECpG or ELPS or PLGA were administered to ED18 embryos and 14 dpi chickens. PBS and MDV only (untreated, MDV-infected) groups served as control groups. Target gene expression were determined relative to β-actin at 4, 10 and 21 dpi in spleen. Data were logarithmically transformed and analyzed using general linear model (Proc GLM) and followed by Duncan’s multiple range test in Statistical Analysis Software version 9.3 (SAS, Cary, NC). The Kruskal-Wallis test was used when data were not normally distributed. Gene expression results were presented as geometric mean of relative expression ± standard error of mean. $p \leq 0.05$ was considered statistically significant when compared to PBS control (*) or MDV only control (#).
Figure 4.6: Relative expression of genes in the bursa of Fabricius following TLR-L treatments. ECpG or ELPS or PLGA were administered to ED18 embryos and 14 dpi chickens. PBS and MDV only (untreated, MDV-infected) groups served as control groups. Target gene expression were determined relative to β-actin at 4, 10 and 21 dpi in the BF. Data were logarithmically transformed and analyzed using general linear model (Proc GLM) and followed by Duncan’s multiple range test in Statistical Analysis Software version 9.3 (SAS, Cary, NC). The Kruskal-Wallis test was used when data were not normally distributed. Gene expression results were presented as geometric mean of relative expression ± standard error of mean. $p \leq 0.05$ was considered statistically significant when compared to PBS control (*) or MDV only control (#).
Chapter 5

The effects of in ovo administration of encapsulated Toll-like receptor 21 ligand as an adjuvant with Marek’s disease vaccine

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Abstract

Marek’s Disease Virus (MDV) is the causative agent of a lymphoproliferative disease, Marek’s disease (MD) in chickens. MD is only controlled by mass vaccination; however, immunity induced by MD vaccines is unable to prevent MDV replication and transmission. The herpesvirus of turkey (HVT) vaccine is one of the most widely used MD vaccines in poultry industry. Vaccines can be adjuvanted with Toll-like receptor ligands (TLR-Ls) to enhance their efficacy. In this study, we examined whether combining TLR-Ls with HVT can boost host immunity against MD and improve its efficacy. Results demonstrated that HVT alone or HVT combined with encapsulated CpG-ODN partially protected chickens from tumor incidence and reduced virus replication compared to the control group. However, encapsulated CpG-ODN only moderately, but not significantly, improved HVT efficacy and reduced tumor incidence from 53% to 33%. Further investigation of cytokine gene profiles in spleen and bursa of Fabricius revealed an inverse association between interleukin (IL)-10 and IL-18 expression and protection conferred by different treatments. In addition, the results of this study raise the possibility that interferon (IFN)-β and IFN-γ induced by the treatments may exert anti-viral responses against MDV replication in the bursa of Fabricius at early stage of MDV infection in chickens.
Introduction

Marek’s disease (MD) in chickens is caused by an alpha herpesvirus, Marek’s Disease Virus (MDV), which is currently controlled in chicken farms by vaccination and biosecurity measures. Depending on the host and virus virulence, MDV can cause more than 90% morbidity and mortality in susceptible chickens (Osterrieder et al., 2006). Chickens become infected by MDV following inhalation of infected dust via the respiratory route. MDV primarily infects macrophages, B and T cells but it can only transform CD4+ T cells (Baigent and Davison, 2004). Generally, MDV life cycle can be divided into the cytolytic phase (2-7 days post-infection - dpi), latent phase (7-10 dpi), late cytolytic phase, and transformation phase (Baigent and Davison, 2004). Lysis of lymphocytes and activation of TGF-beta + regulatory T cells induce immunosuppression during MDV infection (Gurung et al., 2017). Furthermore, transformed T cells proliferate and form lymphoma which can lead to immunosuppression and clinical and pathological signs of disease. In the course of infection, MDV replicates in feather follicular epithelium and sheds into the environment throughout the lifespan of an infected chicken.

Since MDV was first identified more than 50 years ago, several vaccines have been developed to control clinical signs of the disease, although none of them can fully prevent replication or transmission of MDV. Because of the inability of vaccines to control MDV transmission, it is believed that vaccines have prompted the emergence of virulent strains of MDV (Nair, 2005). Herpesvirus of turkey (HVT) is one of the MD vaccines which is extensively used worldwide either alone or in combination with other MD vaccine (Bublot and Sharma, 2004). HVT can be administered in ovo at embryonic day 18 (ED18) to provide protection against MDV in young chicks. However, protection offered by HVT is inadequate especially against very virulent and very virulent plus pathotypes of MDV (Witter, 1997).
Moreover, this vaccine will not be protective against emerging highly virulent strains of MDV in the future. Therefore, it would be of interest to determine whether efficacy of HVT can be enhanced by addition of immune stimulants.

Innate and adaptive defense mechanisms are necessary to control MDV infection in chickens. In young chicks, it is likely that in ovo administered vaccines initially confer protection mainly via innate immune system cells including Natural Killer (NK) like cells and macrophages rather than adaptive immune responses (Negash et al., 2004; Haq et al., 2013; Schijns et al., 2013; Abdul-Cader et al., 2018b). In general, the activation of innate responses orchestrates the induction of adaptive responses which in turn require several days to provide protective immunity against an infection. However, this is not well understood in regard to MDV vaccination. The immune system in young chicks undergoes maturation and becomes fully mature around 1-2 weeks of age. Therefore, the induction of adaptive immune responses might be limited in the early stage of life and immediately after hatching. It has been reported that chicken T cells exhibit poor responsiveness to mitogen stimulation and are functionally immature during the first 2 weeks of age (Lowenthal et al., 1994). In addition, although it has been shown that chickens start to mount antibody-mediated immune responses at 1 week of age, they usually display higher levels of antibody production when they are immunized around 2 weeks of age (Mast and Goddeeris, 1999; Van Ginkel et al., 2015). There is a paucity of information on how MD vaccine modulates innate responses in young chickens. The constitutive expression of IFN-γ and IL-18 that is measurable from ED12 and higher expression of these cytokines by day 7 post-hatch (Abdul-Careem et al., 2007b) indicates that innate responses may be functional prior to hatching. As a result, these responses can be exploited to accelerate the maturation of innate and adaptive immune responses. Increased IFN-γ expression observed with
in ovo HVT vaccination in chickens (Abdul-Careem et al., 2008a) as well as increased NK like activity observed with HVT vaccination at hatch and post-hatch (Heller and Schat, 1987; Quere et al., 1988) revealed that innate responses can be elevated to significant level with appropriate immune modulations (Bavananthasivam et al., 2017).

TLR-Ls have been used experimentally as vaccine adjuvants or as stand-alone anti-viral agents (Mallick et al., 2012; St. Paul et al., 2012a, 2014; Barjesteh et al., 2015b). For instance, TLR3 ligand, polyinosinic:polycytidylic acid (Poly(IC)), has been shown to induce innate responses in chickens (Parvizi et al., 2012b) and its administration to chickens combined with HVT could lead to reduction of tumor incidence after challenge with a very virulent strain of MDV (Parvizi et al., 2012a). In addition, TLR4 ligand, lipopolysaccharide (LPS), and CpG-ODN, the ligand for TLR21 have been shown to stimulate innate responses mainly via the induction of interferons (IFNs) and interleukin (IL)1-β and, which may lead to a reduction in MD progression in chickens (Parvizi et al., 2014). Prolonged availability of TLR-Ls in a controlled-release fashion which can be achieved by encapsulation in poly (D, L-lactic-co-glycolic) acid (PLGA) nanoparticles has been shown to induce innate responses in vitro, in ovo and in vivo (Alkie et al., 2017; Bavananthasivam et al., 2017). Administration of encapsulated CpG (ECpG) as a prophylactic agent demonstrated considerable effects on the outcome of MD in our previous study (manuscript submitted), which prompted further investigation of the adjuvant effect of ECpG with MDV vaccine in chickens.

The present study was designed to study the adjuvant effect of ECpG in conjunction with HVT vaccine administered either in ovo to ED18 embryos or to chickens 14 days after an experimental infection with MDV or at both time points to improve immunity against MDV infection in chickens.
Materials and methods

Chicken eggs, incubation and housing

Specific pathogen free (SPF) eggs that were obtained from the Animal Disease Research Institute, Canadian Food Inspection Agency (Ottawa, Ontario, Canada) were incubated at recommended temperature and relative humidity at the Arkell Research Station, University of Guelph. All experiments were conducted according to the guidelines of the Animal Care Committee of the University of Guelph. At ED18, live embryos were administrated with ECpG or HVT or both into the amniotic sac using a 23-gauge, 2.5 cm needle as recommended. Once the chicks hatched, they were transported to the animal isolation facility at the Ontario Veterinary College, University of Guelph.

TLR ligand, Vaccine and Virus

Synthetic Class B CpG ODN 2007 [5’-TCGTCGTTGTCGTTTTGTCGTT-3’] with phosphorothioate backbone was purchased from Sigma-Aldrich (Oakville, ON, Canada). FC-126 strain of HVT vaccine was received from Merial Canada Inc (Boehringer-Ingelheim Canada Ltd, Quebec, Canada). In vivo propagated, very virulent strain of MDV (RB1B) titrated in chicken kidney cells was used to infect chicks (Schat et al., 1982).

Encapsulation of TLR ligand

CpG ODN was encapsulated in PLGA, (Resomer® RG 503H, free carboxylic acid, MW 24-38 kD, Sigma-Aldrich) using the modified double emulsion solvent evaporation method to generate PLGA nanoparticles as described in our previous studies (Alkie et al., 2017; Bavananthasivam et al., 2017). Briefly, polyethalenimine (linear, MW 2.5 kD, Sigma-Aldrich) was complexed with CpG at a defined ratio (Boussif et al., 1995). This complex was layered onto 4.5% PLGA dissolved in dichloromethane (DCM, Sigma-Aldrich) and sonicated for one
minute at 40% amplitude using an Ultrasonic Processor (Fisher Scientific). The resulting emulsion was sonicated with 2% polyvinyl alcohol (PVA)/1% poloxamer (PVA, MW 30-70kDa, 87-90% hydrolyzed, Sigma Aldrich) for two minutes at 60% amplitude and the resulting suspension was hardened by mixing with 50mL of 2%PVA/1% poloxamer solution. The PLGA nanoparticles were harvested by centrifugation at 20,000g for 50 min at 4°C and washed three times with ultrapure nuclease free water. The PLGA nanoparticle suspension was frozen at -80°C for 1-2 hours, then lyophilized for 18-22 hours (FreeZone®18 Liter Freeze Dry Systems, Labconco Corporation, Kansas City, Missouri) and sterilized as indicated (Alkie et al., 2017).

Encapsulation efficiency was determined as in our previous studies (Alkie et al., 2017; Bavananthasivam et al., 2017)

**Experimental design**

Seven groups of ED18 chicken embryos (38-45 embryos per group) were injected via the amniotic route with approximately 25µg ECpG and a quarter (1/4) of the recommended dose of HVT or HVT alone or phosphate buffered saline (PBS). Once the chicks hatched, at 5 days of age (5d), all chicks, except the PBS group were infected with 250 plaque-forming unit (PFU) of very virulent RB1B MDV per chick via intra-abdominal route. The untreated, but MDV-infected group was used as a positive control group. At 14 dpi, chickens were given a second dose of ECpG or PBS. Experimental groups in this study were designated as follows. Chickens in group 1 (G1) received ECpG and HVT at ED18, infected with MDV at day 5 (d5) and received a second dose of ECpG at 14 dpi. Group 2 (G2) chickens were administered with ECpG and HVT at ED18 and infected with MDV at d5. Chickens in group 3 (G3) were injected with HVT at ED18, infected with MDV at d5 and administered ECpG at 14 dpi. Group 4 (G4) chickens were administered with HVT at ED18 and infected with MDV at d5. Group 5 (G5) chickens received
MDV at d5 and ECpG at 14 dpi. Group 6 (G6) chickens received MDV at d5. Chickens in group 7 (G7) received PBS. At 4, 10 and 21 dpi, spleen, and bursa of Fabricius weight (BF), and body weight (BW) were recorded and organ weight indices were calculated relative to the body weight of chickens. Samples from spleen, BF and feathers were collected in RNAlater and stored at -80°C until further processing. Tumor incidence following infection with RB1B MDV was documented at 21 dpi.

**DNA and RNA extraction and cDNA synthesis**

Genomic DNA was extracted from feather tips as described previously (Abdul-Careem et al., 2006a). Feather tips were cut into small pieces and incubated with 500µl cell lysis buffer (10mM Tris, pH 7.5, 10mM NaCl, 1mM EDTA, pH 8 with 0.5% (w/v) Sarkosyl) containing 100µl of proteinase K (10mg/ml) overnight at 65°C water bath. Extracted DNA was precipitated using 25µl 5M NaCl and 2.3ml 95% ethanol. DNA concentration was measured and dilutions at a concentration of 50ng/µl of DNA samples were prepared. A hundred nanograms of diluted DNA was used for MDV genome copy numbers quantification using quantitative real-time PCR.

RNA was extracted from spleen and BF using Trizol (Life Technologies, Burlington, Ontario) according to the manufacturer’s protocol. cDNA was synthesized from 1µg of DNase treated RNA using Oligo(dT)12–18 primers and the Super-Script™ First-Strand Synthesis System kit (Life Technologies, Burlington, Canada) according to manufacturer’s instructions. Synthesized cDNA was diluted at 1:10 in nuclease free water for evaluating the expression of cytokine genes. The quantity and quality of DNA and RNA were determined using the NanoDrop® ND-1000 spectrophotometry (NanoDrop Technologies, Wilmington, DE).
**Real-time PCR**

Real-time PCR was performed using SYBR green dye in a LightCycler 480 II to quantify the MDV genome copy numbers and cytokine gene expression (Roche Diagnostics, Laval, Quebec) as described previously (Abdul-Careem et al., 2006a; St. Paul et al., 2011). Primer sequences of target and reference genes are listed in Table 1. The primers were synthesized by Sigma–Aldrich Canada (Oakville, ON).

**Statistical analysis**

Relative target gene expression was calculated using chicken β-actin in LightCycler 480 II advanced relative quantification software. Data was logarithmically transformed and analyzed using general linear model (Proc GLM) in Statistical Analysis Software version 9.3 (SAS, Cary, NC). Duncan’s multiple range test was performed to determine significantly different groups. Gene expression results were presented as the geometric mean of relative expression ± standard error of mean. Tumor incidence data was analyzed by Fisher’s exact test (GraphPad Prism version 6.04). One-way ANOVA followed by Dunnett’s multiple comparison test was used to analyze BF:BW and spleen:BW ratios, and MDV genome copy numbers. When data were not normally distributed Kruskal-Wallis test was used. Results were considered significant if \( p \) value was <0.05.

**Results**

**MDV tumor incidence**

Treatment groups were compared to the positive control group (G6), in which all chickens developed tumors (Fig 1). Chickens in group 1 that received ECpG and HVT at ED18, and ECpG again at 14 dpi showed 40% tumors which was statistically significant compared to positive control chickens \( (p=0.002, \text{ Fig 1}) \). Chickens in group 2 which received ECpG and HVT
at ED18 developed the lowest tumor incidence compared to positive control chickens (33.33%, $p=0.001$). In HVT only group (G4), 53.33% of the chickens exhibited tumors, which was significantly different compared to positive control chickens ($p=0.01$, Fig 1). In G3 and G5, 72.72% and 90% of the chickens developed tumors respectively (Fig 1). Although there was 20% difference in tumor incidence between the chickens in G2 and G4, the difference was not statistically significant (Fig 1). Therefore, it seems that ECpG partially improved the HVT induced protection against MD.

**MDV genome copy numbers in feathers**

At 4 dpi, there was no genome detected in feather DNA. MDV load gradually increased throughout the experiment and was at the highest level at 21 dpi (Fig 2). Chickens in group 2, 3 and 4 had significantly lower MDV load in feathers at 21 dpi when compared to that of G6 ($p<0.0001$, Fig 2). In addition, significantly lower MDV load was detected in G4 chickens when compared to G1 and G5 chickens ($p<0.0001$, Fig 2). Unfortunately, administration of ECpG was unable to enhance the HVT induced reduction of MDV load in feathers.

**Organ weight indices**

At 4 dpi, bursa of Fabricius weight to body weight (BF:BW) ratios were significantly higher in G4, G5 and G6 chickens when compared to the chickens in the PBS control group (G7) ($p<0.0001$, Fig 3A). Although at 4 dpi BF:BW ratios in G1, G2 and G3 chickens were almost similar when compared to G7 chickens, they were significantly different from that of G6 chickens ($p<0.0001$, Fig 3A). At 10 dpi, G2, G5 and G6 chickens showed significantly lower BF:BW ratios when compared to G7 chickens ($p=0.0001$). However, G3 chickens exhibited significantly higher ratios when compared to G6 chickens ($p=0.0001$, Fig 3A). At 21 dpi, chickens in all MDV-infected groups showed significantly lower BF:BW ratios when compared
to G7 chickens (p<0.0001, Fig 3A). However, the ratio was significantly higher in G4 when compared to G6 (p<0.0001, Fig 3A). This indicates that HVT vaccine can partially inhibit bursal atrophy induced by MDV.

At 4 dpi, spleen:BW ratios were significantly higher in G4, G5 and G6 chickens when compared to G7 chickens and ratios were significantly lower in G1, G2 and G3 chickens when compared to G6 chickens (p<0.0001, Fig 3B). At 10 dpi, chickens in all groups which received MDV except G3 had significantly higher spleen:BW ratios when compared to G7, and G3 chickens had significantly lower spleen:BW ratios when compared to G6 chickens (p<0.0001, Fig 3B). At 21 dpi, G5 chickens showed significantly higher spleen:BW ratio when compared to G7 chickens (p<0.0015, Fig 3B). However, G4 chickens showed significantly lower ratio when compared to G6 chickens (p<0.0015, Fig 3B).

**Cytokine gene expression**

In spleen, IFN-β was significantly upregulated at all time points in all MDV-infected groups except G2 and G4 at 4 dpi when compared to G7 (p≤0.001, Fig 4A). Similarly, IFN-γ was significantly upregulated at 4, 10 and 21 dpi in all MDV-infected groups, except G4 at 4 dpi, compared to G7 (p≤0.001, Fig 4B). IL-18 was significantly upregulated in G2, G3, G5 and G6 at 4 dpi, whereas this cytokine was downregulated in G1, G2 and G4 at 10 dpi. Again, at 21 dpi, IL-18 was upregulated in G2, G3, G4, G5 and G6 when compared to G7 (p≤0.025, Fig 4C). Although there was no significant upregulation of IL-1β at any time point, slightly higher expression of IL-1β was observed in G1, G3 and G4 chickens at 21 dpi when compared to G6 (Fig 4D). IL-10 expression was significantly upregulated in the chickens in all the groups that received HVT at 4 dpi (P=0.007) as well as in MD-infected groups at 10 and 21 dpi (p<0.0001) when compared to G7 chickens (Fig 4E). Noticeably, IL-10 expression was significantly lower
in G3 chickens at 10 dpi and G1, G2 and G4 chickens at 21 dpi when compared to the chickens in G6 (p<0.0001, Fig 4E).

In the bursa of Fabricius, at 4 dpi, IFN-β was significantly upregulated in G1, G3, G4 and G5 chickens when compared to G6 and G7 chickens (p<0.0001, Fig 5A). At 10 dpi, IFN-β was significantly upregulated in G1 chickens when compared to G6 and G7 chickens, and in G2 when compared to G7 (p=0.002, Fig 5A). At 21 dpi, IFN-β was upregulated in all groups that were challenged with MDV (p<0.0001, Fig 5A). Further, IFN-γ was upregulated in chickens at all time points and in all the groups which received MDV when compared to chickens in G7 except the chickens in G2, G5 and G6 at 4 dpi (p≤0.0002, Fig 5B). Expression of IFN-γ was significantly upregulated in G1 and G4 chickens at 4 dpi when compared to G6 chickens (p≤0.0002, Fig 5B). Downregulation of IL-18 was observed in G3, G4 and G5 chickens at 4 dpi and also at 10 dpi in chicken in all the groups that received HVT when compared to G6 chickens (p≤0.009, Fig 5C). However, at 21 dpi, IL-18 was upregulated in chickens in all MDV-infected groups, except in G5 chickens, compared to G7 chickens (p=0.001, Fig 5C). Expression of IL-1β was upregulated in G2 chickens compared to G6 chickens at 4 dpi (Fig 5D). IL-1β was upregulated at 10 dpi in G1 chickens and at 21 dpi in chickens in all the groups that received MDV when compared to G7 chickens (p≤0.04, Fig 5D). Expression of IL-10 was upregulated in the chickens in all MDV-infected group at 4, 10 and 21 dpi compared to G7 chickens except G3 and G6 at 4 dpi and, G3 at 10 dpi (p≤0.0005, Fig 5E). IL-10 was significantly higher at 4 dpi and lower at 21 dpi in G4 chickens compared to G6 chickens (p≤0.0005, Fig 5E).

**Discussion**

To enhance host responses against MDV, administration of ECpG along with HVT was investigated in the current study. Among the HVT-administered groups, a significant reduction
in both tumor incidence and MDV load in feathers was observed in G2 and G4 chickens. Chickens in G1 showed significant reduction only in tumors whereas chickens in G3 showed significant reduction only in MDV load in feathers. Although there was no statistically significant difference in tumor incidence between chickens in G4 and groups receiving ECpG (G1, G2 and G3), administration of ECpG along with HVT at ED18 seems to provide effective protection against MDV as noticed by the lowest tumor incidence in G2 chickens.

The observed reduction of MDV load in feathers of chickens treated with ECpG and HVT or HVT alone is similar to the reduction reported in a previous study using Rispens-CVI988 vaccine (Haq et al., 2012), which is known to be the most effective vaccine against very virulent and very virulent plus MDV (De Boer et al., 1986). Since the substantial reduction of MDV load in feathers was observed in the current study, further investigation of MDV transmission pattern in the environment and chickens will answer whether this reduction interrupts the spread of MDV. Particularly, the use of resistant and susceptible genotypes of chickens may validate the use of ECpG with HVT as the virulence of MDV can be masked in resistant line of chickens (Hunt and Dunn, 2015).

Commonly, MDV infection results in bursal atrophy due to B cell cytolysis and spleen enlargement is due to inflammation as well as tumor formation. Hence, organ weight indices were recorded to evaluate the changes occurring in BF and spleen following different treatments and MDV challenge in chickens. Administration of either ECpG and HVT or HVT alone possibly inhibited the initial cytolysis of B cells in BF as indicated by the ratios similar to the control group. HVT administration with or without ECpG might have resolved the physiological changes which occurred in spleen at the initial stage of MDV infection since there was no
difference in the ratios of spleen:BW between the chickens in HVT-administered group and uninfected group at 21 dpi. However, further investigation is needed to test this possibility.

The significant upregulation of the cytokines, IFN-β and IFN-γ, in BF at 4 dpi raises the possibility that the anti-viral activity of these cytokines might have repressed MDV replication at the initial stage of MDV pathogenesis and led to the reduction in tumor incidence and MDV load in feathers. Previous study indicated that involvement of IFN-β and IFN-γ in preventing DNA and virion synthesis in the case of herpes simplex virus type 1 (HSV1) infection (Pierce et al., 2005). Since MDV and HSV belong to the subfamily Alphaherpesvirinae, we can speculate that IFN-β and IFN-γ may have similar functions for preventing the replication of MDV in infected cells. Another study also revealed that TLR-Ls induced IFN-β expression provide protection against HSV2 infection (Gill et al., 2006). These lines of evidence and our findings support the role played by IFN-β induced by ECpG and HVT in immunity against MD in chickens. IFN-γ possibly exerts anti-viral effects on MDV-infected cells directly or indirectly via activation of macrophages (Djeraba et al., 2000). Significant upregulation of IFN-γ at 4 dpi in BF in some treatment groups compared to G6 indicates initial anti-viral effects which might have contributed to the subsequent reduction of tumors in chickens. Overall, the expression profile of IFN-γ in spleen and BF shows the association of IFN-γ with immunity to MDV infection. This is in agreement with previous studies showing the importance of IFN-γ in HVT conferred immunity in chickens (Haq et al., 2011, 2015).

IL-10 expression in chickens is associated with MD progression. It has been suggested that IL-10 curtails T helper (Th)1 immune responses, which are critical for protection, by deviating responses towards Th2 responses (Rothwell et al., 2004). This could also be the case for MDV infection as an association between IL-10 expression and the disease was reported
IL-10 affects dendritic cell (DC) maturation by inhibition of costimulatory molecules, DC induced T cell activation, antigen-specific T cell proliferation and function (Steinbrink et al., 1997, 1999); and it promotes the expansion of inducible T regulatory cells (Hsu et al., 2015). Notably, the experimental groups in the current study that exhibited significant reduction in tumor incidence displayed lower expression of IL-10 in spleen at 21 dpi. In addition, chickens that had tumors, expressed elevated levels of IL-10 in spleen and BF compared to those chickens that did not have tumors in each treatment group (data not shown). A similar pattern of IL-10 expression was recorded in previous studies in a different context, for example when chickens were vaccinated with bivalent or Rispens-CVI988 vaccine followed by MDV infection (Abdul-Careem et al., 2007a; Haq et al., 2012). Therefore, previous reports were further confirmed and extended in the current study as the suppression of IL-10 was inversely associated with protection.

The other interesting finding in the current study was the downregulation of proinflammatory cytokines, particularly IL-18 expression in chickens in the treatment groups compared to G6 chickens. It has been noted previously that expression of IL-18 diminished in MD resistant lines of chickens (Kaiser et al., 2003) compared to that of susceptible lines and also in vaccine protected chickens compared to unvaccinated-infected controls (Abdul-Careem et al., 2007a; Haq et al., 2011). Similar observations were confirmed in the current study as indicated by the association between decreased expression of IL-18 and protection against MDV as shown by the reduction of tumor incidence and MDV load in feathers. Moreover, IL-18 supports the metastasis of melanoma cells via elevated expression of vascular cell adhesion molecule-1 (VCAM-1) (Vidal-Vanaclocha et al., 2000). Therefore, it is probable that decreased expression
of IL-18 in the current study may have abrogated the expression of VCAM-1, which possibly contributed to the reduction of tumor incidence.

In conclusion, administration of ECpG with HVT provided protection against MDV in chickens by reducing tumor incidence and MDV loads in feathers. The anti-viral ability of IFN-β and IFN-γ might have contributed to the outcome of the treatments against MDV infection. In addition, downregulation of IL-10 and IL-18 by HVT and/or ECpG treatment appears to be linked to defense against MDV infection as demonstrated by the significant reduction of tumor formation and MDV load in feathers.

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Author contributions

Conceived and designed the experiments: JB SS. Performed the experiments: JB LR JA AY. Analyzed the data: JB. Contributed to writing and reviewing of the manuscript: JB LR JA AY TNA MFA SKW SB SS.

Competing interests

Authors declare no competing interest regarding the publication of this work.
Table 5.1: Target genes, primer sequences and references used for real-time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>β-actin</td>
<td>F: 5’-CAACACAGTGCTGTCTGGTGGA-3’&lt;br&gt;R: 5’-ATCGTACTCCTGCTTGATGC-3’</td>
<td>(Brisbin et al., 2008)</td>
</tr>
<tr>
<td>meq</td>
<td>F: 5’-GTCCCCCCTCGATCTTTC-3’&lt;br&gt;R: 5’-CGTCTGCTTCCTGCTGTC-3’</td>
<td>(Abdul-Careem et al., 2006)</td>
</tr>
<tr>
<td>IFN-β</td>
<td>F: 5’-GCCTCCCAAGCTCCTTCAGAATA-3’&lt;br&gt;R: 5’-CTGGATCTGGAGGAGGCTGT-3’</td>
<td>(Abdul-Careem et al., 2008b)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>F: 5’-ACAAGGCAAGTCAAGCGGCACA-3’&lt;br&gt;R: 5’-AGTCGTTTCACTGGAGTGGC-3’</td>
<td>(Brisbin et al., 2010)</td>
</tr>
<tr>
<td>IL-18</td>
<td>F: 5’-GAAACGTAAAGCAGTTTC-3’&lt;br&gt;R: 5’-TCCCATTGCTTTTCTCAACA-3’</td>
<td>(Abdul-Careem et al., 2006)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>F: 5’-GTGAGGCTCAACATTGCGCTG-3’&lt;br&gt;R: 5’-TGTCCAGGCGGATGAGAAGATG-3’</td>
<td>(Abdul-Careem et al., 2009)</td>
</tr>
<tr>
<td>IL-10</td>
<td>F: 5’-AGCAGATCAAGGAGACGTT-3’&lt;br&gt;R: 5’-ATCAGCAGTTACTCCTCGAT-3’</td>
<td>(Abdul-Careem et al., 2007a)</td>
</tr>
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Figure 5.1: Presence of tumors in different treatment groups.

Presence of tumors was recorded at 21 dpi. Experimental groups were as follows: G1-ECpG and HVT were administered at ED18 and the second dose of ECpG was injected at 14 dpi. G2-ECpG and HVT was administered at ED18. G3-HVT was administered at ED18 and ECpG was given at 14 dpi. G4-HVT was administered at ED18. G5-ECpG was injected at 14 dpi. G6-Untreated, MDV-infected group. G7-PBS control group. Data were statistically analyzed by Fisher’s exact test in GraphPad Prism 6.04. $p \leq 0.05 (*)$ was considered statistically significant.
Figure 5.2: MDV genome copy numbers in feathers in different treatment groups.

MDV genome copy numbers per 100ng of DNA were calculated from feathers collected at 4, 10 and 21 dpi. The different experimental groups were: G1-ECpG and HVT was administered at ED18 and second dose of ECpG was injected at 14 dpi. G2-ECpG and HVT was administered at ED18. G3-HVT was administered at ED18 and ECpG was given at 14 dpi. G4-HVT was administered at ED18. G5-ECpG was injected at 14 dpi. G6-Untreated, MDV-infected group. G7-PBS control group. Data were statistically analyzed by Kruskal-Wallis test in GraphPad Prism 6.04. \( p \leq 0.05 \) was considered statistically significant (*). Comparisons were made between groups as indicated or with positive control group G6. Error bars indicate the standard errors of the mean.
Figure 5.6: BF:BW and spleen:BW ratios in different treatment groups.

BF, spleen and body weight were recorded at 4, 10 and 21 dpi, and (A) BF:BW and (B) S:BW ratios were calculated. The different experimental groups were: G1-ECpG and HVT was administered at ED18 and second dose of ECpG was injected at 14 dpi. G2-ECpG and HVT was administered at ED18. G3-HVT was administered at ED18 and ECpG was given at 14 dpi. G4-HVT was administered at ED18. G5-ECpG was injected at 14 dpi. G6-Uprotected, MDV-infected group. G7-PBS control group. Data were statistically analyzed by one-way ANOVA and followed by Tukey’s multiple comparison test in GraphPad Prism 6.04. *p ≤0.05 (*) was considered statistically significant when compared to G7 (*) or G6 (#). Error bars indicate the standard errors of the mean.
Figure 5.7: Relative expression of genes in spleen in different treatment groups.

(A) IFN-β (B) IFN-γ (C) IL-18 (D) IL-1β (E) IL-10 expression were determined relative to β-actin at 4, 10 and 21 dpi in spleen. The different experimental groups were: G1-ECpG and HVT was administered at ED18 and second dose of ECpG was injected at 14 dpi. G2-ECpG and HVT was administered at ED18. G3-HVT was administered at ED18 and ECpG was given at 14 dpi. G4-HVT was administered at ED18. G5-ECpG was injected at 14 dpi. G6-Untreated, MDV-infected group. Data were logarithmically transformed and analyzed using general linear model (Proc GLM) and followed by Duncan’s multiple range test in Statistical Analysis Software version 9.3 (SAS, Cary, NC). The Kruskal-Wallis test was used when data were not normally distributed. Gene expression results were presented as geometric mean of relative expression ± standard error of mean. $p \leq 0.05$ was considered statistically significant when compared to G7 (*) or G6 (#).
Figure 5.8: Relative expression of genes in the bursa of Fabricius in different treatment groups. (A) IFN-β (B) IFN-γ (C) IL-18 (D) IL-1β (E) IL-10 expression were determined relative to β-actin at 4, 10 and 21 dpi in BF. The different experimental groups were: G1-ECpG and HVT was administered at ED18 and second dose of ECpG was injected at 14 dpi. G2-ECpG and HVT was administered at ED18. G3-HVT was administered at ED18 and ECpG was given at 14 dpi. G4-HVT was administered at ED18. G5-ECpG was injected at 14 dpi. G6-Untreated, MDV-infected group. Data were logarithmically transformed and analyzed using general linear model (Proc GLM) and followed by Duncan’s multiple range test in Statistical Analysis Software version 9.3 (SAS, Cary, NC). The Kruskal-Wallis test was used when data were not normally distributed. Gene expression results were presented as geometric mean of relative expression ± standard error of mean. $p \leq 0.05$ was considered statistically significant when compared to G7 (*) or G6 (#).
Chapter 6

General discussion

Currently, vaccination is central to the control of MD in the poultry industry. However, immunity provided by vaccination is incomplete and virulent strains of MDV can infect vaccinated chickens. Vaccinated chickens are protected from the development of clinical signs and tumor formation, but they are not protected from infection, replication and transmission of virulent MDV. Hence, lifetime shedding of infectious MDV from vaccinated chickens, which can serve as a source of infection to uninfected chickens, is of real concern to the poultry industry. In addition, it is believed that the incomplete immunity by vaccination and co-infection of virulent strains in vaccinated chickens may drive the emergence of highly virulent strains of MDV (Read et al., 2015). The pattern of appearance of pathogenic strains of MDV following the beginning of vaccine usage suggests that virulent MDV has evolved over the years (Nair, 2005). These drawbacks of current vaccines demand the development of alternative ways to boost immunity against MD in chickens and to prevent the shedding of MDV into the environment.

The first responses to MDV infection through innate defense mechanisms play a key role in chickens. Both by providing some level of protection to infectious diseases and by directing the immune system towards adaptive immune responses, innate responses are crucial in neonatal chicks. Priming the innate responses in neonates can be achieved by \textit{in ovo} administration of either immunostimulants or vaccines adjuvanted with immunostimulants such as TLR-Ls. In addition, advanced delivery methods such as PLGA nanoparticles can be used to dispense the substances for an extended period of time, which may enhance host responses.

To investigate the possibility of using TLR-Ls to enhance the host responses against MDV infection, first, several TLR-Ls were tested for their ability to inhibit MDV infection \textit{in}
The results of this study revealed that Poly(IC) and Pam3CSK4 reduced MDV infection in CEFs at 96 and 120 hpi whereas CpG and LPS reduced MDV infection in cells at 96 hpi only. In addition, induction of expression of type I IFN, IRF7, IFIT5 and Mx genes by TLR-L pre-treatments correlated with inhibition of MDV infection. Results of this study demonstrate that TLR-Ls are able to stimulate antiviral responses which can lead to a reduction in the percentage of cells infected with MDV. Depending on the type of TLR-L, the percentage of reduction of MDV infected cells varied. This may be due to variation in the level of expression of TLRs and the involvement of other possible signaling pathways for the induction of antiviral responses. Pre-treatment with Poly(IC) caused the upregulation of antiviral cytokines and ISGs in most of the time points investigated when compared to Pam3CSK4 and LPS. This might have led to the higher percentage of reduction in MDV-infected cells after treatment with Poly(IC) compared to the other TLR-Ls. This observation indicates the potency of TLR-Ls in controlling MDV infection through induction of antiviral responses. However, there are a few limitations in this study. Although it is useful to evaluate the kinetics of MDV inhibition at several time points, the nature of MDV infection in cells and practical issues in keeping infected cells for a long period of time, limited the selection of time points to 96 and 120 hpi.

Following identification of TLR-Ls that can limit MDV infection, the use of TLR-Ls to elicit innate responses in ED18 chicken embryos was investigated (Chapter 3). To enhance the delivery and availability of TLR-Ls, they were encapsulated in PLGA nanoparticles. Both free and encapsulated TLR-Ls were used in this study to compare the effect on induction of innate responses. It was demonstrated that TLR-Ls can induce innate responses not only in a cell culture system but also in the late stage of embryogenesis. Although the development of the immune system is not complete in ED18 embryos, they are able to respond to TLR-Ls
stimulation via expression of cytokine genes, including antiviral and proinflammatory cytokines. Further, the induced responses seemed to be similar to that were induced in post-hatch chickens (St. Paul et al., 2011, 2012c). Moreover, it was revealed that encapsulated TLR-Ls stimulated innate responses in ED18 embryos particularly at 48 hps. Findings of this study suggest that stimulation of innate responses in ED18 embryos by encapsulated TLR-Ls has the potential to enable post-hatch chicks to respond to invading pathogens immediately after hatch.

Although the significant reduction of MDV-infected CEFs was observed with Poly(IC) pre-treatment in the study described in Chapter 2, due to technical difficulties, we were unable to encapsulate Poly(IC) to use in the study outlined in Chapter 3. Future investigation of the induction of innate responses in post-hatch chicks will provide more information about the lasting effect of encapsulated TLR-Ls following in ovo administration.

Once the induction of innate responses in ED18 embryos by encapsulated TLR-Ls was confirmed, we investigated the protective effect of administration of encapsulated TLR-Ls to ED18 embryos against MDV challenge in day-old chicks. In this study, three consecutive experiments were performed to determine the appropriate form of TLR-Ls (free or encapsulated), optimal frequency of administration of TLR-Ls and host responses to TLR-Ls (Chapter 4). The results of this study indicated that two-time administration, to ED18 embryos and to 14 dpi chickens, of encapsulated TLR-Ls significantly reduced tumor incidence at 21 dpi. This reduction was associated with elevated expression of IL-18 and IL-1β in spleens. However, none of the TLR-L treatments reduced MDV genome load in feathers at 21 dpi. This observation suggests that TLR-L-induced immune responses were not potent enough to inhibit the productive replication of MDV in feathers. Furthermore, two-time administration of encapsulated TLR-Ls in the second and the third experiments reduced almost similar percentage of presence of tumors
in chickens. This indicates the repeatability of the outcome of the treatment with encapsulated TLR-Ls.

Since the use of encapsulated TLR-Ls as standalone antiviral agents led to reduction of tumors in MDV-infected chickens, next we wanted to know whether administration of encapsulated TLR-L could improve the protective effect of HVT vaccine. In order to test this objective, a study was performed as highlighted in Chapter 5. Since the treatment with ECpG as a prophylactic agent caused maximum tumor reduction in the study outlined in Chapter 4, ECpG was selected for use in subsequent study. The findings of this study indicated that administration of ECpG improved, albeit not significantly, the efficacy of the HVT vaccine as indicated by a reduction in tumor incidence in MD-infected chickens at 21dpi. Reduction of tumor incidence was in both the number of tumors on a per bird basis and also finding that birds in treatment groups had no tumors. In addition, inoculation of ECpG as an adjuvant with the HVT vaccine to ED18 embryos significantly reduced MDV genome copy numbers in feathers at 21 dpi. Moreover, analysis of cytokine gene profiles suggested that there was an inverse association between the expression of IL-10 and protection provided by administration of ECpG and HVT or HVT alone. In this particular study, the possibility of interference by cross-reactive MDV-specific maternal antibodies was circumvented by the use of SPF chickens that had not received the HVT vaccine.

Despite the novel aspects of our studies about induction of host responses by in ovo administration of encapsulated TLR-Ls against MDV infection, there were some weaknesses in these studies. One of the deficiencies is that in vivo trials were carried out only until 21 dpi and we could not extend the duration further in order to comply with the guidelines of Animal Care Committee of the University of Guelph. Otherwise, we could have investigated how long the
tumor-free chickens could survive following encapsulated TLR-Ls treatment. The other constraint was that chickens were infected with MDV via the intra-abdominal route rather than the natural route, which is through the respiratory system. This might have had an effect on the pathogenesis of MDV in experimentally infected chickens in our study. Although the aerosol-based infection method of MDV challenge has been developed in our lab previously, the virus dose delivered to each bird in this model cannot be assured (Abdul-Careem et al., 2009). Further, cytokines and antiviral proteins were measured at the transcript level rather than the protein level, because commercial availability and validity of the reagents for immunological experiments in chickens are limited.

Future studies could be designed to elucidate the mechanism of action of LPS and CpG on reducing tumor incidence of MD in chickens. Examining the effect of these TLR-Ls in suppression of reactivation from latency might be one of the possibilities. Investigation of the role of CD8+ T cells in response to treatment with TLR-Ls in enhancing immune responses against MDV infection could provide more insight into the ability of these cells to eliminate MDV-infected cells and tumor cells. Further, to improve immunity against MD, a potential synergistic effect of TLR-Ls can be examined by combining various TLR-Ls. Co-stimulation with CpG and Poly(IC) could possibly improve host responses against MDV infection as combination of both TLR-Ls has been shown to induce NO production in chicken blood-derived monocytes (He et al., 2007). In addition, it is worthwhile to consider encapsulating a TLR-L and MDV-derived antigens together into PLGA nanoparticles and evaluate the use of these nanoparticles for conferring immunity against MD. Commonly, NK cells are important for the removal of virus-infected cells and tumor cells. Although NK cells are not fully characterized in chickens yet, the presence of NK-like activity has been reported. Therefore, further investigation
of induction of NK-like activity by TLR-Ls might provide more information about the role of NK-like cells in MDV infection. Moreover, decreased expression of IL-10 was associated with reduced tumor incidence and MDV load in feathers. Future experiments aimed at knocking out IL-10 using antibodies or gene editing technology will be able to confirm the observation of our study.

Previous studies have reported increased infiltration of CD8+ T cells into feather pulp following vaccination (Abdul-Careem et al., 2008b). However, it did not reduce MDV genome load in feathers. This might be due to CD8+ T cells being in a relatively unresponsive state, or there might be a suppressive milieu in the microenvironment that inhibits the effector function of CD8+ T cells. Treatment with TLR-Ls could potentially help to overcome these barriers to enhance immune responses. For example, the inhibitory activity of myeloid-derived suppressor cells (MDSC) in mice was decreased by local administration of CpG (Shirota et al., 2012). Furthermore, CpG indirectly improved CD8+ T cell function by augmenting dendritic cell maturation and differentiation even if CD4+ T cell help was lacking (Sparwasser et al., 2000). Similar events might have occurred with TLR-L treatments in MDV-infected chickens. However, future studies are needed to prove this hypothesis. For instance, characterization of CD8+ T cells and their function in feather follicles and identifying suppressive markers or cells in feathers will provide more information on mechanisms of immunity against this virus. In addition, the studies described in Chapter 5 have shown a significant reduction of MDV genome copy numbers in feathers after treatment of chickens with ECpG and the HVT vaccine. Dissecting the possibility of inducing immune responses in feather follicle to prevent the replication of infectious MDV using TLR-Ls could provide the rationale to use this method to intervene with the transmission cycle of MDV. In a previous study, immune responses were
observed in the feather pulp in response to intradermal administration of *Mycobacterium butyricum* (Erf and Ramachandran, 2016). Hence, studying the possibility of preventing MDV replication in feathers by intradermal or topical application of TLR-Ls is reasonable.

In conclusion, in the current studies, a range of functions of encapsulated TLR-Ls was evaluated, which included activation of innate responses in ED18 chicken embryos, providing protection by reducing tumor formation, reduction of MDV genome load in feathers and significant upregulation or downregulation of genes involved in immunity against MD. The induction of enhanced immune responses by *in ovo* and post-hatch administration of encapsulated TLR-Ls offers an alternative approach to control MDV infection in chickens. Another possibility is administration of ECpG with HVT at ED18 to improve immunity against MD in chickens.
References


Chow, J.C., Young, D.W., Golenbock, D.T., Christ, W.J., Gusovsky, F., 1999. Toll-like receptor-


Gobel, T.W., Schneider, K., Schärer, B., Mejri, I., Puehler, F., Weigend, S., Staeheli, P., Kaspers, B., 2003. IL-18 Stimulates the Proliferation and IFN- Release of CD4+ T Cells in


Nair, V., 2005. Evolution of Marek’s disease - A paradigm for incessant race between the


