Investigating the Role of Cytokines in Immunity to Marek’s Disease

by

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ABSTRACT

INVESTIGATING THE ROLE OF CYTOKINES IN IMMUNITY TO MAREK’S DISEASE

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University of Guelph, 2012

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Dr. Shayan Sharif

Marek’s disease (MD) is a lymphoproliferative disease of chickens caused by an oncogenic herpesvirus, Marek’s disease virus (MDV). Despite the availability of MD vaccines, little is known about the underlying immunological mechanisms that mediate vaccine-induced immunity. The objective of this research was to elucidate these mechanisms.

To characterize host responses in the lungs, chickens were vaccinated with herpesvirus of turkeys (HVT) and infected with MDV-RB1B. Vaccinated MDV-infected chickens had a higher accumulation of viral genome in the lungs, associated with T cell infiltration in lung tissue and an up-regulation of interferon (IFN) -γ and interleukin (IL) -10. This finding led us to conclude that IFN-γ has a role in immunity; hence, we further investigated the role of this cytokine. The hypothesis tested was that the protective efficacy of HVT against MDV-RB1B would be enhanced when combined with recombinant chicken IFN-γ (rChIFN-γ). Chicken IFN-γ coding sequence was cloned into an expression plasmid, and the bioactivity of rChIFN-γ was confirmed. Administration of this plasmid led to a significant reduction in tumour occurrence in HVT vaccinated MDV-infected chickens, suggesting enhanced vaccine-induced immunity.
To shed more light on the relevance of IFN-γ to immunity against MD, studies were designed to down-regulate the expression of IFN-γ in chicken tissues. Three small interfering (si)RNAs specific for chicken IFN-γ were selected which significantly inhibited expression of IFN-γ by up to 80% in cultured cells. These three siRNAs and a non-target control were cloned and expressed as short hairpin RNA (shRNA) using an avian adeno-associated virus (rAAAV) vector system. An MDV challenge trial was conducted once shRNA expression by the rAAAV was confirmed in vitro. It was reasoned that down-regulation of IFN-γ would lead to abrogation of immunity conferred by HVT. There was an increase in the number of chickens with tumours that received HVT and rAAAV + IFN-γ shRNA compared to the control group, though not statistically significant. However, no difference in MDV genome load in feathers was detected between vaccinated MDV-infected birds with or without rAAAV.

In summary, we have demonstrated here that cytokines are induced in the course of vaccination against Marek’s disease and that IFN-γ plays a role in vaccine-induced immunity against MD.
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Words cannot express my thanks to my family, especially my parents. To my mother, for her unconditional love and my father, who introduced me to science. I dedicate this work to you both. All my achievements are a result of your guidance and support.

To Lorraine, my wife, this simply would not have been possible without your love, support and belief in my abilities. I owe my deepest gratitude to you for everything.

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INTERFERON-GAMMA INFLUENCES IMMUNITY ELICITED BY VACCINES AGAINST VERY VIRULENT MAREK’S DISEASE VIRUS

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LIST OF ABBREVIATIONS

μg micro gram
μL micro litre
μM micro mole
AAV adeno-associated virus
AAAV avian adeno-associated virus
bp base pair
cDNA complementary DNA
CEF chicken embryo fibroblasts
CTL cytotoxic T lymphocytes
d.p.i. days post infection
DF-1 chicken fibroblast cell line
DMEM Dulbecco’s modified eagle medium
DNA deoxyribonucleic acid
dNTP deoxynucleotide triphosphate
DsiRNA dicer substrate RNA
E. coli Escherichia coli
ED18 embryo day 18
EDTA ethylenediaminetetraacetic acid
FFE feather follicle epithelium
gB glycoprotein B
HEK human embryo kidney
hr hour
HVT herpesvirus of turkeys
i.a. intra-abdominal
i.m. intramuscular
ICP4 infected cell polypeptide 4
IFN interferon
iNOS inducible nitric oxide synthase
IL- interleukin-
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>IRF</td>
<td>interferon response/regulatory factor</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>LATs</td>
<td>latency associated transcripts</td>
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<tr>
<td>MD</td>
<td>Marek’s disease</td>
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<td>MDV</td>
<td>Marek’s disease virus</td>
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<tr>
<td>meq</td>
<td>MDV <em>EcoRI</em> Q</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
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<tr>
<td>ng</td>
<td>nanogram</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>PAMP</td>
<td>pathogen associated molecular patterns</td>
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<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PFU</td>
<td>plaque forming unit</td>
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<td>pp38</td>
<td>phosphoprotein 38</td>
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<tr>
<td>rAAAV</td>
<td>recombinant AAAV</td>
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<td>rChIFN-γ</td>
<td>recombinant chicken IFN-γ</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<td>s</td>
<td>second</td>
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<td>s.c.</td>
<td>subcutaneous</td>
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<tr>
<td>S.D.</td>
<td>standard deviation</td>
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<td>S.E.M</td>
<td>standard error of mean</td>
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<td>shRNA</td>
<td>small hairpin RNA</td>
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<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>SPF</td>
<td>specific pathogen free</td>
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<td>Th</td>
<td>T helper</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
<td>U6</td>
<td>RNA polymerase III promoter</td>
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<tr>
<td>vIL-8</td>
<td>viral interleukin-8</td>
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<td>vv</td>
<td>very virulent</td>
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<tr>
<td>vv+</td>
<td>very virulent plus</td>
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DECLARATION OF WORK PERFORMED

I declare that the studies in this thesis are original and that the research reported therein has been conducted by myself or under my supervision with the exception of the item(s) listed below:

Construction of pEVA3V-LacZ-ITR, pEVA3V-RC-CMV, pHelper vectors was performed by Dr. Carlos Estevez, College of Veterinary Medicine, University of Georgia.

All work was carried out under the supervision of Dr. Shayan Sharif at the Department of Pathobiology, University of Guelph.

Kamran Haq
Marek’s disease (MD) is a highly contagious lymphoproliferative disease in chickens, which occurs worldwide. MD is characterized by immunosuppression, polyneuritis and formation of T cell lymphomas in MD susceptible birds. The causative agent is an oncogenic avian herpesvirus called Marek’s disease virus (MDV) (Witter and Schat 2003). The natural route of infection is via the respiratory tract after inhalation of cell-free virus particles by chickens, followed by transportation of MDV to lymphoid organs where the virus remains cell-associated. MDV pathogenesis constitutes an early cytolytic phase in B cells followed by a latency and neoplastic phase in T cells resulting in clinical signs such as paralysis and immune suppression. Productive infection, which only occurs in the feather follicles, leads to the release of infectious cell-free particles (Witter and Schat 2003).

Immunity against MD is the result of a combined interaction between the innate and adaptive immune responses. Due to being a highly cell-associated virus, the importance of cell-mediated immune response following vaccination has been shown; however the antibody-mediated response in immunity against natural infection or vaccine-induced protection is not definitive. It is likely that protection is dependent on the activation of T helper (T_H) 1 cells and production of pro-inflammatory cytokines. The expression of cytokines in response to MDV infection and the potential role of cytokines in immunity against MD have been described previously (Xing and Schat 2000a; Kaiser et al., 2003; Jarosinski et al., 2005). Xing and Schat (2000a), investigated the effect of
MDV infection on transcription of a number of cytokines \textit{in vitro} and \textit{in vivo}. Results showed an up-regulation of interferon (IFN)-\textgamma, interleukin (IL)-1\textbeta, IFN-\textalpha and inducible nitric oxide synthase (iNOS). Cytokine gene expression patterns associated with immunization against MD have been shown by our group (Abdul-Careem \textit{et al.}, 2007). In addition to these studies, other studies have also examined host response to MDV infection and shown stimulation of innate host responses as well as an adaptive immune response (Xing and Schat 2000b; Sarson \textit{et al.}, 2008; Abdul-Careem \textit{et al.}, 2009a; Abdul-Careem \textit{et al.}, 2009b).

MD has been successfully controlled by vaccination for over 40 years, by inducing host immune responses that prevent immunosuppression and tumour development, but not preventing infection. However, sporadic outbreaks do occur either due to emergence of new strains, vaccine immunity breakdown or poor management of poultry operations. The constant evolution of MDV has necessitated the need to develop more efficacious vaccines that can control the emerging strains (Witter 2001; Gimeno 2008). Despite the availability of MD vaccines, little is understood about the underlying immunological mechanisms that mediate immunity to MD.

The function of immune mediators in immunity to MD may be further examined by combining mediators with vaccines as adjuvants and determining if this improves vaccine efficacy. Studies have demonstrated that administration of cytokines enhances immunity against a range of infectious diseases in chickens, including coccidiosis, Newcastle disease (ND), infectious bursal disease (IBD) and infectious bronchitis (IB) (Karaca \textit{et al.}, 1998; Min \textit{et al.}, 2002; Yin \textit{et al.}, 2007; Tan \textit{et al.}, 2009; Park \textit{et al.},
2009). There is also some evidence to suggest that administration of some cytokines may enhance immunity against MD (Haq et al., 2011).

The functional role of immune mediators, including cytokines, in immunity against viral infections can be further examined in loss-of-function studies, in which the activity of a target molecule is ablated. There are several approaches available to reduce target gene expression, including gene silencing through a process known as RNA interference (RNAi). Introduction of small interfering RNA (siRNA) and short hairpin RNA (shRNA) into the cell mediates the process of gene silencing (Leung and Whittaker 2005). siRNA or shRNA, targeting specific gene sequences, can be delivered into cells by physical or chemical methods or by using viral vectors to deliver an expression cassette (Sliva and Schnierle 2010).

The objectives of the experiments described here were to (1) identify immune mediators associated with vaccine-induced protection against MD, (2) evaluate the efficacy of MD vaccines when combined with a candidate cytokine namely interferon (IFN)-γ, (3) establish an in vitro system to knockdown expression of chicken IFN-γ, (4) examine the effects of in vivo IFN-γ knockdown on expression of this cytokine and its ensuing effects on immunity to MD. The ultimate objective was to gain a better understanding of the role played by chicken IFN-γ in vaccine-induced protection.
LITERATURE REVIEW


Marek’s Disease

Background

Marek’s disease (MD), named after the Hungarian veterinarian József Marek, is a neoplastic and neuropathic disease in chickens caused by an oncogenic virus named Marek’s disease virus (MDV) (Biggs 2001). When the disease was first recognized, it was described as ‘polyneuritis’, inflammation of peripheral nerves. It was not until later that the disease was recognized as a neoplastic condition (Baigent and Davison 2004). It is now known that the disease can spread in a number of ways within a flock of birds and, the severity of MD can range from mild to severe. Following infection, susceptible birds develop clinical signs such as transient paralysis, depression, weight loss and formation of T cell lymphomas in the lymphoid and nervous tissues, skin and visceral organs.

Marek’s disease virus (MDV)

Marek’s disease virus (MDV) is a large double stranded DNA virus (~180kb), which is a member of the Herpesviridae family and belongs to the subfamily Alphaherpesvirinae and genus Mardivirus (Baigent and Davison 2004; Osterrieder and Vautherot 2004). Although originally classified as a gammaherpesvirus based on its ability to induce lymphoproliferation and its characteristics of lymphotropism, MDV has
been reclassified as an alphaherpesvirus based on its genomic structure which is similar to other alphaherpesvirus, such as Varicella-zoster virus (VZV) and herpes simplex virus-1 (HSV-1) (Buckmaster et al., 1988; Lupiani et al., 2001). Originally, MDV strains were classified into three serotypes based on antigenic and genotypic differences: serotype 1 (MDV-1) is the oncogenic strain causing MD, whereas serotype 2 (MDV-2) and serotype 3 (MDV-3; herpesvirus of turkeys, HVT) are considered non-oncogenic and have been utilized in vaccine development (Calnek and Witter, 1997; Biggs 2001). MDV-1 isolates can be further grouped into four pathotypes: 1) mild, 2) virulent (v), 3) very virulent (vv) and 4) very virulent + (vv+), reflecting their levels of pathogenicity (Witter 1997). According to the most recent nomenclature, MDV-1 is classified as Gallid herpesvirus 2 (GaHV-2), MDV-2 as Gallid herpesvirus 3 (GaHV-3), and MDV-3 is classified as Meleagrid herpesvirus 1 (MeHV-1). However, the abbreviations ‘MDV’ and ‘HVT’ are still commonly used in the literature and will be used within this thesis where applicable.

The genomes of the three serotypes are collinear and share significant sequence homology; however, they have distinctly different restriction endonuclease (RE) patterns. The genome of MDV-1 is the largest (174,076 bp) followed by MDV-2 (164,270 bp) and MDV-3 (160,673 bp) (Cebrian et al., 1982). The genome consists of a unique long (UL) and a unique short (US) segment flanked by inverted repeats known as terminal and internal repeats long (TRL and IRL, respectively) and short (TRS and IRS). The genes located in the unique regions are largely homologous to VZV and HSV-1, whereas genus and virus specific genes are located in the inverted repeat regions.
MDV pathogenesis

MDV goes through distinct phases during its replication that begins with infection in the respiratory system. Infection with MDV-1 can generally be divided into four phases: 1) early cytolytic, 2) latent, 3) late cytolytic, and 4) transformation phase (Calnek 1986). Pathogenesis of MDV has been described in detail by Calnek and Witter (1997), and as mentioned involves a cytolytic and latent phase of infection which is followed by virus reactivation in susceptible birds, leading to CD4+ T cell transformation and lymphoma development.

Infection and Cytolytic phase

The natural route of infection is through inhalation of cell-free MDV particles present in virus-contaminated dust and feather dander (Beasley et al., 1970). Infection may also occur through eye and oral mucosa (Davidson and Borenshtain 2003). Once in the lungs, virus particles may infect parenchymal cells or may be picked up by various phagocytic cells such as macrophages and taken to various lymphoid tissues via the lymph or blood (Adldinger and Calnek 1973). During the early phase, between 2 -7 days post infection (d.p.i), MDV initially infects B cells (Schat et al., 1981; Shek et al., 1983) followed by activated CD4+ T cells and resulting in the first phase of cytolytic replication. The initial target cells for cytolytic infection are B lymphocytes (Shek et al., 1983). The MDV genome harbours a spliced gene known as viral interleukin-8 (vIL-8) with general homology to IL-8. MDV-encoded vIL-8 plays an important role as a potent chemoattractant and may promote the recruitment of activated T cells to the site of infection (Liu et al., 1999; Parcells et al., 2001), facilitating the switch of infection from
B cells to T cells. During the cytolytic phase, no cell-free virus is produced, however the interaction between infected cells facilitates MDV spread from cell to cell with the virus replication peaking between 4 and 7 d.p.i. (Yunis et al., 2004). The host response mounted after initial virus infection and replication plays a significant role in the induction of the latent phase from the productive cytolytic phase around 7-8 d.p.i. (Buscaglia et al., 1988).

**Latent phase**

Herpesviruses are characterized by their ability to establish latent infection inside host cells from which the virus can reactivate and cause recurrent disease. MDV enters into a latent phase around 7 d.p.i. Volpini et al., (1996) showed that type I IFNs repress MDV antigens *in vitro* which may lead the virus into latency. The primary site of MDV latent infection is activated CD4+ T cells (Shek et al., 1983; Osterrieder et al., 2006) and latent viruses can be reactivated by *in vitro* propagation of the T cells isolated from infected chickens (Calnek et al., 1981). Previous studies have reported that thymectomy, but not bursectomy, perturbs the establishment of latency, suggesting that the cell-mediated, rather than the antibody-mediated response, is more crucial for the establishment and maintenance of MDV latency (Schat et al., 1981; Baigent and Davison 2004).

During latency, MDV expresses a number of genes such as pp14, meq (MDV *EcoRI Q*) and latency-associated transcripts (LATs) which play a role in the balance between latent and lytic infections (Parcells et al., 2003; Heidari et al., 2008), and also represses a number of genes expressed during the cytolytic phase, such as pp38 and gB
MDV LATs include two small spliced RNAs named Marek’s disease virus small RNAs (MSRs) and a 10 kb RNA. The LATs are antisense with respect to the MDV major immediate early regulatory protein, intracellular protein 4 (ICP4), and are considered to interfere with translation of ICP4, suggesting their role in suppression of lytic infection. Among the other genes expressed during latency is meq, which is unique to the pathogenic serotype and is consistently expressed. Meq has been shown to block apoptosis of latently infected CD4+ T cells and transactivate latent gene expression contributing to the maintenance of latency. Parcells and colleagues (2003) proposed a model for the regulation of MDV lytic versus latent infection, suggesting that meq-meq homodimers bind to the origin of lytic replication, repressing expression of pp38 gene product, thus maintaining latency, while transactivating promoters of transcripts associated with oncogenicity.

Late Cytolytic phase and Transformation

Marek’s disease virus can become reactivated in MD susceptible chickens resulting in a second wave of cytolytic infection followed by tumour formation (transformation phase) around 2-3 weeks after primary infection (Calnek 1986). This leads to a state of permanent immunosuppression in susceptible birds. It has been suggested that latently infected cells carry the virus to various tissues by peripheral blood lymphocytes (Baigent and Davison 2004). The disseminated virus may reactivate from latency resulting in cytolytic infection causing severe necrosis of tissue, leading to atrophy of some of the affected tissues, which is followed by tumour formation (Calnek
MDV infection can result in tumour formation within multiple organs including kidneys, spleen, gonads and proventriculus. Among these tissues, the spleen seems to be the predominant site for initial proliferation of transformed cells. *In vitro* studies have shown that MDV viral genes such ICP4 and meq are involved in transformation (Lupiani *et al.*, 2001; Levy *et al.*, 2005). Furthermore, biochemical and genetic studies have also suggested that meq is the principal oncogene of MDV (Parcells *et al.*, 2001). LATs may also be involved in cellular transformation, since their transcripts are abundantly expressed in tumour cell lines (Cantello *et al.*, 1994).

Transformation of cells leads to development of lymphomatous lesions which may appear as early as 12-14 d.p.i, and result in blindness, paralysis and mortality (Calnek 2001). There is little information available about tumour-associated or tumour-specific antigens. However, one tumour surface antigen has been identified, CD30, which is expressed by MD tumours and cell lines (Burgess *et al.*, 2004). CD30, is a member of the tumour necrosis factor receptor II family, and is expressed at very low levels by uninfected leukocytes and not expressed by resting T cells (Burgess and Davison 2002). In addition to changes in surface markers of MD tumour cells, these cells may produce immunological mediators. For example, MD tumour cells may be able to down-regulate immune responses via the expression of IL-10. A recent study showed that MD tumour cells can express IL-10 and IL-10 receptor, which may be an evasion strategy to evade host response (Buza and Burgess 2007).

Although many cell and tissue types may become infected with MDV, only the skin and the associated feather follicles (FFE) can support the production of cell-free
infectious virus (Calnek et al., 1970; Heidari et al., 2007). The FFE is unique in its epizootiologic role as the source of fully enveloped virus that can spread from bird to bird. MDV infection of the FFE can be detected as early as 4 d.p.i. by PCR amplification of the MDV meq gene (Abdul-Careem et al., 2008b). Secondary to the infection of FFE, lymphoproliferative skin lesions may also occur due to infection. Arrival of the virus initiates host responses marked by infiltration of inflammatory cells, consisting of lymphocytes and heterophils into the feather pulp region (Moriguchi et al., 1987; Abdul-Careem et al., 2008b) and expression of pro-inflammatory cytokines including IL-6, IL-8 and IFN-γ (Abdul-Careem et al., 2008c). Production and shedding of infectious cell-free virus is not influenced by genotype or vaccine-induced protection.

**Immunity against MDV**

Effective immunity to MD requires involvement and a coordinated activation of innate defense mechanisms and adaptive immune responses. The outcome of infection is not only determined by the virulence of the virus but also by the result of the complex interaction between the components of both arms of the host immune system which involve cytokines, antibodies, macrophages, natural killer (NK) cells, T helper (Th) cells, cytotoxic T lymphocytes (CTLs) and soluble factors such as nitric oxide (NO) (Schat and Markowski-Grimsrud 2001). Although the various components of the two arms may differ, there is accumulating evidence that both these host responses are in a close and constant interaction and there is also evidence to suggest that there is no distinct border between them, as originally thought. Due to the lymphotropic and highly cell-associated nature of MDV, T cell-mediated immune response is recognized as an effective immune
response. Since the nature of MDV is cell-associated, antibodies play an important role only when the virus is present in a cell-free form or when MDV antigens are expressed on cell surfaces (Sharma and Witter 1975; Omar and Schat 1996). Host responses to MDV infection within various tissues, among MD resistant or susceptible birds, as well as in response to vaccination, have been recently reviewed by Parvizí et al. (2010).

**Innate host responses**

As mentioned above, host immunity to MDV is a complex network, in that both innate and adaptive host responses are activated in infected birds. The respiratory tract is the main site of entry and uptake of MDV, although the actual site(s) and cellular mechanism involved with uptake have not yet been clearly identified. Conserved molecular patterns known as pathogen-associated molecular patterns (PAMPs), which include double-stranded RNA (dsRNA), single stranded RNA (ssRNA) and unmethylated CpG DNA are recognized by toll-like receptors (TLRs) present on the surface or in intracellular compartments of various cells. Using genomic analysis, studies have characterized the presence of avian TLRs (1, 2, 3, 4, 5, 7, 9, 15 and 21) (Kogut et al., 2005; Boyd et al., 2007; Jenkins et al., 2009); of which five (2, 3, 4, 5, and 7) are orthologs of mammalian TLRs (Brownlie and Allan 2011). TLR3, 7, 8, and 9 (or chicken TLR21) recognize viral nucleic acids and induce type I IFNs. Induction of type I interferons involves interferon regulatory factors (IRFs), a family of transcription factors involved in antiviral defence, cell growth, and immune regulation. One of the first responses to viral infection is the induction of IFN production.
In general, IFNs and more specifically type I IFNs possess antiviral activities. Effective antiviral innate responses require the detection of virus by pathogen recognition receptors, and initiation of protein signalling cascades that regulate the synthesis of IFNs. Recently, an aerosol-based infection model was developed in our laboratory to infect chicks with cell-free MDV via the respiratory route (Abdul-Careem et al., 2009a). Using this infection model, Abdul-Careem and colleagues, observed up-regulation of TLR3 and TLR7 in the lungs of infected chickens (2009a). An up-regulation of the interferon regulatory factors (IRF)-1 and IRF-3 was also seen following infection of chicken embryo fibroblasts (CEF) with either MDV or HVT (Morgan et al., 2001; Karaca et al., 2004). After infection with MDV, an increase in expression of IFN-α has been reported in susceptible chickens (Quéré et al., 2005), and in another study treatment of chicken embryonic cells with recombinant IFN-α inhibited replication of MDV *in vitro* while oral administration with recombinant IFN-α reduced *in vivo* replication (Jarosinski et al., 2001). These studies confirm the importance of interferons in immunity against MD.

*In vitro* and *in vivo* studies have shown the involvement of macrophages in MDV pathogenesis as well as immunity against MD (Kodama et al., 1979; Djeraba et al., 2000; Gimeno et al., 2001; Barrow et al., 2003). It has been shown that macrophages may inhibit MDV replication or could phagocytose MDV-infected cells. In addition, these cells are an important source of pro-inflammatory cytokines. Activated macrophages can exert their antiviral activities through the production of mediators such as NO. Up-regulation of inducible nitric oxide synthase (iNOS) after MDV infection has been shown to occur in the spleen, brain and lungs of infected chickens (Xing and Schat 2000b; Djeraba et al., 2002a; Jarosinski et al., 2005; Abdul-Careem, et al., 2009b). In the context
of MDV infection, higher concentrations of NO correspond to greater inhibition of MDV replication (Kodama et al., 1979; Xing and Schat 2000b; Djeraba et al., 2002a).

Natural killer (NK) cells, which belong to the innate immune system, have a key role in the containment of herpesvirus infections through their potent antiviral activity (Sharma and Okazaki 1981; Paludan et al., 2011). NK cells can induce rapid cell death in virus-infected cells or tumour cells via the induction of apoptosis. This may be achieved using one of two pathways: ligation of cell death receptors (such as Fas and FasL) and release of granular contents (for example granzyme and perforin which are a serine protease and a pore-forming protein, respectively). In a study by Sarson et al. (2008), an increase in the expression levels of granzyme A, NK-lysin, and perforin was noted among MDV infected birds, which suggested the role of the granzyme/perforin pathway in the host response to MDV infection. Other studies have shown an increase in the activity of NK cells in MD-resistant and vaccinated chickens when compared to unvaccinated and MD-susceptible chickens (Sharma and Okazaki 1981; Heller and Schat 1987; Garcia-Camacho et al., 2003). An in vitro study also demonstrated NK-like activity against MD tumour cells, suggesting that NK cells may play a role during the transformation phase and can target MDV-transformed cells (Quéré and Dambrine 1988). These data suggest that NK cells have an important role in protective immunity against MDV and are likely to be most active in the cytolytic phase of infection.

Adaptive immune responses

In addition to innate host responses, the adaptive immune response, which comprises cell-mediated immune responses and neutralizing antibodies, also plays a
significant role in antiviral immunity against herpesviruses (Mester and Rouse 1991). Adaptive immune responses are antigen-specific, and involve secretion of antibodies against various MDV proteins, in addition to cell-mediated immune responses initiated by the interaction of CD4+ and CD8+ T cell receptors (TCR) with the major histocompatibility complex (MHC) molecules loaded with MDV antigens.

**Role of Antibodies**

Since MDV is a highly cell-associated herpesvirus, the exact role of the antibody-mediated immune response has not been clearly established; however the role of antibodies has been implicated in protective immunity against MDV (Calnek 2001; Davison and Kaiser 2004). Antibodies are produced against MDV glycoproteins, such as gB, gE, and gI, among which anti-gB neutralizing antibodies have a known protective role via blocking virus entry into the host cells (Schat and Markowski-Grimsrud 2001; Davison and Kaiser 2004). These antibodies are believed to play a role in immunity against the virus by neutralizing cell-free viruses, blocking entry of virus into cells, and antibody-dependent cell-mediated cytotoxicity (ADCC). Chicks can passively acquire maternal antibodies from vaccinated or infected hens, or actively through virus exposure. The presence of these antibodies can alter MDV pathogenesis (Calnek 2001). Maternal antibodies have also been shown to reduce replication, tumour formation, mortality, as well as severity of MD signs (Witter and Lee 1984; Davison and Kaiser 2004). These antibodies may also inhibit cell-free virus vaccines, which would presumably reduce vaccine efficacy (Calnek 1986).
Cell-mediated immune response

Studies by Morimura et al., (1998) demonstrated an important role of CD8+ T cells by depleting them with monoclonal antibodies, resulting in a high MDV titer within CD4+ T cells. Cytotoxic T lymphocyte (CTL) responses have been shown to be directed towards cells expressing immediate-early, early and late MDV antigens. Omar and Schat (1996) originally identified the cytotoxic activity of CD8+ T cells against cells expressing MDV antigens such as pp38, meq, ICP4 and gB. This study also showed that CD4+ T cells and γδ T cells were not important for the elimination of MDV antigen expressing cells. Other studies have also confirmed the effect of CD8+ CTL against various MDV glycoproteins (Omar et al., 1998; Markowski-Grimsrud and Schat 2002). Studies from our laboratory have reported findings supporting the role of CD8+ T cells against MDV infection. Sarson et al. (2006), using a microarray approach, showed an increase in expression of granzyme A and CD8α genes after infection with MDV, suggestive of an up-regulation in CTL activity following infection.

CTL responses also play a role in genetic resistance to MD. The in vitro cytotoxicity assay developed by Schat and co-workers (1992), was also used to examine the difference in cell-mediated responses based upon MHC haplotypes of the host (Omar and Schat 1996). MDV-stimulated splenocytes demonstrated syngeneic cell-mediated immune responses against MDV proteins pp38, Meq and gB; however, cell-mediated responses against MDV protein, ICP4, were only present in the resistant line (B21) but not in the susceptible line (B19). These findings support that specific cell-mediated responses may play an important role in genetic resistance to Marek’s disease.
Cytokine and Chemokine Expression Associated with MDV Infection

Cytokines are essential for the activation, differentiation and control of the host immune system. Cytokines have been shown to be important mediators involved in cell-mediated immune responses to MDV infection and are secreted as a result of antigen presentation to T cells. Identification and characterization of chicken cytokines and chemokines was possible with the advancement in our understanding of the chicken genome and genome sequence endeavours (Wallis et al., 2004; Kaiser et al., 2005). Cytokines can be classified as: type 1, those that enhance cell-mediated immune responses (eg. IFN-γ, IL-2, IL-12), type 2, which promote antibody-mediated responses (eg. IL-3, IL-4, IL-13) as well as regulatory cytokines such as transforming growth factor (TGF)-β and IL-10. In response to MDV infection, variation in cytokine and chemokine gene expression can be monitored based on the various stages of MDV pathogenesis, genetic background of birds, as well as within various tissues and cell-types.

Production of cytokines in response to MDV infection and the potential role of cytokine in immunity have been described and reviewed elsewhere (Kaiser et al., 2003; Sarson et al., 2006; Abdul-Careem et al., 2007; Heidari et al., 2010; Haq et al., 2010b). IFN-γ expression is usually increased in the spleen of MDV-infected chickens (Xing and Schat 2000a; Djeraba et al., 2002a). However, Quéré et al. (2005) described a down-regulation of IFN-γ in the blood of susceptible but not resistant lines of chicken, although Kaiser and co-workers compared the expression of IFN-γ amongst resistant and susceptible lines and found that it was equally expressed in both lines. These findings suggest a lack of association of IFN-γ expression with resistance against MD. However, significant up-regulation of IL-6 and IL-18 was observed in splenocytes of genetically
susceptible chickens (Kaiser et al., 2003), whereas up-regulation of IL-1β and IL-8 was observed in resistant birds (Jarosinski et al., 2005). The findings from these studies demonstrate that expression of the genes encoding IL-6 and IL-18 is consistently associated with MD susceptibility, while IL-1β and IL-8 is associated with resistance. Jarosinski et al. (2005) examined the expression of IFN-γ, IL-1β, IL-6, and IL-8 in brains of infected chickens and found a correlation between the expression of cytokine genes and virulence of MDV. Birds infected with a vv+ MDV strain induced significantly higher cytokine expression compared to infection with a vMDV strain. A study by our group has shown that chickens infected with a vvMDV strain and exhibiting transient paralysis had higher IL-6, IL-12, and IFN-γ in their brain tissues when compared to asymptomatic MDV-infected chickens (Abdul-Careem et al., 2006b). These studies provide evidence that the virulence of MDV as well as the genetic background of the chicken influences the expression of cytokine genes.

Cytokines may also play a role in protection conferred by vaccines. Kano et al., (2009b) examined gene expression in splenocytes from chickens vaccinated with CVI988 and challenged with vvMDV (RB1B) and demonstrated that IFN-γ mRNA is increased in response to infection in vaccinated birds. Djeraba and co-workers (2000) reported enhanced expression of IFN-γ in splenocytes of chickens vaccinated with HVT and infected with RB1B strain of MDV. Studies from our laboratory, have also shown MDV vaccination associated with a significant increase in IFN-γ expression (Abdul-Careem et al., 2007). These studies provide useful information on the role of cytokines in response
to MDV infection, however, they do not explain the potential role of these cytokines in immunity against MD.

The direct inhibitory role of IFN-γ has been shown *in vitro* (Levy *et al.*, 1999), however IFN-γ can also have an indirect antiviral effect by its action on macrophages and through the induction of nitric oxide (NO) as well as activating CD8+ cytotoxic T cells to kill virus-infected cells (Whitmire *et al.*, 2005). Nitric oxide, produced by IFN-γ activated macrophages, plays an important role in immune responses due to its antiviral and anti-tumour functions. Inducible nitric oxide synthase (iNOS) is the enzyme that produces NO from amino acid L-arginine (Bogdan 2001). iNOS expression is primarily regulated at the transcriptional level and its expression is induced by lipopolysaccharides (LPS) and transcription factors such as nuclear factor kappa b (NF-κB), IL-1β and tumour necrosis factor alpha (TNF-α) (Aktan 2004). Following MDV infection, an increase in expression of iNOS has been reported in chicken embryo fibroblast (CEF) derived from genetically resistant birds compared to CEF from susceptible birds (Xing and Schat 2000b), and similar findings from Djeraba and co-workers showed early strong expression of cytokines (such as IFN-γ) with iNOS inducing activity in genetically resistant birds and vaccine-induced protection (Djeraba *et al.*, 2002a).

**Control and Prevention**

Vaccination of commercial chickens is an important approach to reduce disease and lower losses incurred by infection. Vaccines have been developed from whole wild-type pathogens (attenuated or killed) or genetically engineered strains, and their efficacy is improved by adjuvant.
Vaccines against MD

The routine use of vaccines since the 1970’s has successfully controlled the economic losses in the poultry industry caused by MD. Although MD vaccines have been in use for nearly 40 years, the exact mechanism of vaccine-induced immunity is not well understood (Osterrieder et al., 2006). The first successful vaccine against MD was the non-oncogenic HVT and is currently still in use either in combination with other types of MD vaccines or by itself (Witter 2001). After a decade of use, the emergence of a vvMDV strain prompted the introduction of a more effective bivalent vaccine (a mixture of serotype 2 and 3: HVT+SB-1). This was followed by the emergence of vv+ MDV pathotypes requiring the introduction of an attenuated serotype 1 strain CVI 988 vaccine (also known as ‘Rispens’) (Gimeno 2008). This continued evolution of MDV towards greater virulence was shown by a study done by Witter et al. (1997) where 31 MDV isolates of serotype 1 collected between 1987-1995 were characterized. The study found that only 3 isolates were classified as vMDV, 21 isolates were classified as vvMDV and 7 isolates were classified as vv+ MDV pathotypes, indicating an increase in virulence over time within isolates from the same serotype.

The protective efficacy of MD vaccines depends on many factors including the genetic background of the chickens (Bacon et al., 2001), presence or absence of antibodies, virulence of the infecting virus strain, and the vaccine dose administered (Witter 2001). Traditionally, vaccines were administered subcutaneously immediately after hatch. However, in the last 15 years there has been a change in the route of administration towards embryonic day (ED) 18 vaccination (also referred to as in ovo vaccination). Administering the vaccine to control MD via this route is now widely accepted and practiced
(Sarma et al., 1995). In a study by Sharma (1987), inoculation at ED18 with HVT before experimental MDV infection induced protection, however the protective ability of HVT was lost when administered with MDV or after MDV infection. The in ovo route of vaccination provides an early stimulation of protective immunity, reducing the susceptibility of chicks immediately after hatch.

Despite reducing disease incidence, virus replication and tumour formation, vaccination does not confer protection against infection and it does not reduce virus shedding from infected birds resulting in spread from vaccinated birds to other uninfected birds. Moreover, immunity induced by vaccines may be prone to breakdown. The factors that may favour the development of vaccine breakdown include evolution of viral pathogenicity and emergence of more virulent strains (Gimeno 2008). MDV virulence evolution resulting in the emergence of strains with increasing virulence can be attributed to several events that have occurred over the last few decades, such as the intensification of poultry production, changes in host genetics, and vaccination pressure (Witter 1997).

Adjuvants in vaccines

Some vaccines are generally poorly immunogenic or may not be effective due to reasons such as pathogen (virus) evolution. Some of these failures may be overcome by administering vaccines with adjuvants that target manipulation of both innate and adaptive immune systems. Traditional adjuvants such as CpG oligonucleotides, aluminum salts (commonly known as alum), incomplete Freund’s adjuvant (IFA), and oil-based adjuvants have been shown to induce various cytokines such as IFNs, IL-2, IL-12 and IL-18 that play an important role in establishing an innate host response, which in turn activates
the adaptive immune response. Recently, interest in the use of defined adjuvants such as TLR ligands or recombinant cytokines has increased. Considering the key role of cytokines in modulating host response via activation and differentiation of immune cells, enhancing immune response and inducing production of other cytokines, these molecules may serve as promising vaccine adjuvants. Successful application of recombinant cytokines in various species including humans has attracted the potential use of recombinant cytokines in chickens. In a study by Park et al. (2009), protective adjuvant effects of IL-2 and IFN-γ were reported against infectious bursal disease virus (IBDV). IL-2 has been extensively studied due to its pleotropic properties as a key T cell growth factor responsible for clonal expansion following activation. IFN-γ plays a key role in the regulation aspect of the immune response including macrophage activation, T cell and NK cell differentiation/activation, and B cell development. Tarpey and colleagues (2007) evaluated vaccine efficacy by constructing a recombinant HVT which expressed chicken IL-2. Vaccination with recombinant HVT increased the level of neutralizing antibodies but protection against vvMDV challenge did not improve. However, in a study by Djeraba and colleagues (2002b), use of chicken myelomonocytic growth factor (cMGF) improved the protective efficacy of HVT. Chickens treated with cMGF had a prolonged survival time, lower viraemia and low tumour incidence following a challenge with vvMDV. In a study with *Eimeria*, co-administration of IL-2 in combination with a pathogen protein, resulted in enhanced immunity by reducing lesions and reduction in body weight loss (Shah et al., 2011).

Chicken IFN-γ known for its properties to modulate immune response and inhibit viral replication, when co-administered with an antigen to birds resulted in a prolonged
secondary antibody response that persisted at higher levels and for a longer period (Lowenthal et al., 1997). Co-administration of duck hepatitis B virus (DHBV) with recombinant IFN-γ in ducks resulted in increased protection of birds against DHBV (Long et al., 2005). IFN-γ production is critical for the establishment of T_{H1} immunity and providing antiviral protection. Therapeutic activity of IFN-γ has also been demonstrated by the protection rendered against Eimeria infection of birds as well as reduced weight loss associated with the disease (Lillehoj and Choi, 1998). Other cytokines such as chicken IL-1β have also been assessed. When using tetanus toxoid as an antigen, administration of ChIL-1β resulted in increased antibody responses compared to when the antigen was used by itself (Schijns et al., 2000).

TLRs, Nod-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptor (CLRs) are members of a family of pathogen recognition receptors (PRR) that function as primary sensors of the innate immune system to recognise pathogens. PRR adjuvants, essentially TLR agonists, have been explored due to their properties of being powerful candidates to induce strong T_{H1} and CTL responses. Binding of agonists to these PRRs promote maturation of antigen presenting cells such as dendritic cells (DC) which in turn direct the induction of adaptive immune responses, a key reason for using TLR agonists as vaccine adjuvants. TLR ligands have been used successfully as adjuvants in vaccine formulations in various species including chicken. For instance, costimulation of chicken monocytes with TLR3 and TLR21 ligands synergistically increased the expression of IFN-γ and IL-10 and produced a stronger T_{H1} biased immune response (He et al., 2011) and TLR3 ligands also improved immunity against influenza virus in mice (Schneider-Ohrum et al., 2011). Activation of TLR3 engages a TRIF-dependent pathway.
(MyD88 independent) and activates interferon regulatory factor (IRF) that controls expression of type I IFNs and IFN-inducible genes. On the other hand TLR7 and TLR9 utilize the MyD88-TRIF pathway, which also in turn activates IRF, regulating expression of type I IFN and cytokines such as IL-1, IL-6, IL-10, IL-12 and TNF-α. TLR4 detects lipopolysaccharides and, once activated, leads to downstream release of inflammatory modulators, including TNF-α and IL-1 (Makkouk and Abdelnoor 2009). Recent reviews have summarized the application of various agonists and pathway biology in depth. However, there has not been much research about avian TLR agonists as adjuvants to improve vaccine efficacy against MDV, although recent studies have shown an immunostimulatory role for chicken TLR ligands (St. Paul *et al.*, 2011; Villanueva *et al.*, 2011; Parvizi *et al.*, submitted).

**Modulating immune response using RNA interference**

*RNA interference (RNAi)*

RNA interference (RNAi) is the term given to the post-transcriptional gene regulatory mechanism that limits the transcript level by either suppressing translation or activating a sequence-specific RNA degradation process induced by double-stranded RNA (dsRNA) in eukaryotic cells (Hammond *et al.*, 2001). The phenomenon of RNAi was first reported by Napoli *et al.* (1990) studying chalcone synthase (CHS) in petunia plants. A later study by Fire and colleagues (1998) provided an explanation for the phenomenon by demonstrating that the introduction of long dsRNA molecules into the nematode, *Caenorhabditis elegans*, resulted in ten-fold more potent gene silencing when
compared to single strand DNA (ssDNA). Experimental RNAi-mediated silencing encompasses multiple steps beginning with the introduction of dsRNA into the cytoplasm of the cell. Facilitated by the Dicer endonuclease, an RNase III enzyme complex, the dsRNA is processed into 19-21 nucleotide long small interfering RNAs (siRNA). The resulting siRNAs are incorporated into a large multicomponent RNA-protein complex which includes Argonaute 2 (AGO2) to form the RNA-induced silencing complex (RISC), which unwinds the siRNA duplex and releases the sense strand. The RISC-bound antisense strand guides the activated RISC to the target complementary mRNA sequences, resulting in subsequent mRNA cleavage and degradation.

Synthetic RNA duplexes, 19-21 nucleotides in length, have been used as experimental ‘guide’ strands to trigger RNAi. In 2005, studies demonstrated that slightly longer synthetic RNAs, which are substrates for dicer, are more potent than the traditional 21-mer siRNAs (Kim et al., 2005). These longer dsRNAs, typically 27-mer RNA duplexes known as dicer-substrate siRNAs (DsiRNAs), are processed by dicer into 21-mer siRNAs with an increased potency, which is thought to arise from the participation of dicer in RNA-induced silencing complex formation (Hefner et al., 2008). The study by Hefner and colleagues (2008) also demonstrated an increased silencing effect with a lower concentration of siRNA, which also avoids off-target effects, thus supporting an advantage for using the 27-mer siRNA. To further increase potency, synthetic siRNAs are created asymmetrically, with a strand containing a 2bp 3’-overhang. An increasing number of reports have validated the performance of the Dicer-substrate reagents in vitro and in vivo (Kim et al., 2006; Dore-Savard et al., 2008).
Use of RNAi to study function of cytokines

Utilization of siRNAs to trigger RNAi has proven to be very useful to gain a better insight into the role(s) of various genes and their encoded proteins under diverse conditions. Cytokines are soluble protein signaling molecules that exert their function by binding to high-affinity receptors located on target cells. *In vivo* studies examining cytokine expression tell us what cytokine to investigate, however, *in vivo* experimentation involving the addition or elimination of a particular cytokine is necessary to understand the impact the cytokine has on the immune response. Studies have used an RNAi approach to selectively target various molecules such as NF-κB (Pinkenburg et al., 2004; Guo et al., 2005) and cytokines including IL-23 (Li et al., 2011), IL-5 (Huang et al., 2009) IL-10, IFN-γ (Sidahmed and Wilkie 2007) and IL-12 (Hill et al., 2003) and therefore provided a useful tool to study the role these cytokines both *in vitro* and *in vivo*, as well as for therapeutic applications in some clinical studies.

Application of RNAi technology in chickens

The RNAi technology has been widely used in the chick embryo to address questions related to embryonic development. In addition, this approach has been employed to target and inhibit viral genes in chicken cell cultures or *in vivo* (Hu et al., 2002; Pekarik et al., 2003; Ge et al., 2003; Dai et al., 2005; Harpavat and Cepko 2006). There are at least three reports on the use of the RNAi technology to modulate chicken immune system genes *in vitro* by targeting iNOS, TLR3, and nuclear factor kappa B (NF-κB)-1 (Cheeseman et al., 2008; Karpala et al., 2008; Chiang et al., 2009).
Although there is no published report on the use of RNAi for silencing immune system genes in the context of Marek’s disease, there have been studies attempting to inhibit MDV replication. Chen et al. (2008) constructed three short hairpin RNA-miRNA (shRNA-mirs) targeting two MDV genes, gB and ICP4, and delivered them through replication competent retroviral vectors. The antiviral effect from the RNAi constructs targeting either gene significantly reduced viral replication. With various combinations of the gene targets in their constructs, a synergistic or non-additive effect on viral inhibition was observed in vitro. Using a lentiviral vector, Chen et al. (2009) generated birds expressing transfected with constructs expressing shRNA targeting gB and ICP4. Challenge of these birds with MDV resulted in significant reduction of MDV replication and a decrease in MDV viraemia levels as well as a reduced disease incidence (30% less when compared to scrambled shRNA or no vector). Insertion of RNAi sequences into HVT has also been reported (Lambeth et al., 2009). A recombinant HVT vaccine expressing sequences targeting gB and UL29 was used to vaccinate birds followed by infection with a very virulent MDV strain. A moderate reduction in MDV genome copy number in the peripheral blood lymphocytes (PBL), and viraemia of the challenge virus was observed in vaccinated birds compared to controls.

Delivery strategies for siRNA

Limited RNAi delivery efficiencies have been achieved in vivo due to factors such as enzymatic degradation, poor cellular uptake, oligonucleotide concentration, and site of delivery. However, various delivery methods have been developed. RNAi can be introduced into cells from intracellularly expressed shRNA, synthetic siRNAs, or
molecules incorporated into liposomes. Recently, there has been interest in utilizing viral vectors to deliver expression cassettes encoding shRNA (Sliva and Schnierle 2010). In the following section, two approaches will be described: plasmid DNA and viral vectors.

**Plasmid DNA**

Many non-viral methods have been investigated extensively for DNA transfer into cells. These methods rely on direct, physical or chemical techniques to deliver plasmid/vector into the host cells. When plasmid DNA encoding a recombinant viral gene or host protein, or synthetic siRNAs is not delivered as a complex with other molecules, it is called ‘naked’ DNA/plasmid. After transfection of the plasmid DNA, host cells can take up the plasmid DNA and express the recombinant gene and produce the corresponding protein inside the cell. A local injection of plasmid DNA in ovo or into a tissue such as muscle, skin or airways without chemical assistance has the ability to transfect cells. Vectors can be designed to deliver siRNA, microRNA (miRNA) and shRNA for post-transcriptional silencing of virtually any gene, either for understanding its biological role or for therapeutic purposes. In direct comparison, RNAi via shRNA has been shown to be more effective than antisense oligodeoxynucleotides (ODN), which are synthetic siRNA, targeted to the same gene (Miyagishi et al., 2003). Additionally, shRNA showed a longer lasting inhibitory effect (Bertrand et al., 2002). Recombinant plasmid vectors bearing transgene expression cassettes express high levels of the transgene shortly after entering the cell but the expression from the plasmid usually declines to a low level in a period of days. The primary challenge is to establish a method
that delivers the recombinant gene to selected cells where long-term expression can be achieved.

_Viral vectors_

The low delivery efficiency of naked plasmid DNA has led to the focus on viral vector delivery systems over the last decade. There has been an increasing interest towards using viral-based RNAi vectors due to the efficiency of transfection and gene delivery, both _in vitro_ and _in vivo_. Viral vectors recently engineered as shRNA delivery vehicles are comprised of major classes of viruses and include: retroviruses, herpesviruses, adenoviruses (AdV), poxviruses and adeno-associated viruses (AAV). These are based on replication-competent viruses that have been genetically modified rendering them replication defective and less immunogenic through deletion of accessory proteins. Amongst the vectors currently under development as RNAi delivery systems, one of the best candidates is the replication defective AAVs (Grimm _et al._, 2005). Several unique features that distinguish AAV from other vectors include: (i) ability to infect both dividing and non-dividing cells (ii) broad host range (iii) no association with any disease to date (iv) lack of cell-mediated immune response against the vector and (v) ability to integrate into a specific site of the host genome or persist episomally, creating potential for long term expression (Stilwell and Samulski 2003). Another factor that has played a role in encouraging the use of AAV vectors includes the discovery of new serotypes with potential tissue-specific tropisms.

Adeno-associated virus is a ssDNA virus belonging to the family _Parvoviridae_, sub-family _Parovirinae_, and genus _Dependovirus_ (Muzychka and Berns 2001). The
AAV capsid is non-enveloped, icosahedral, with affinity for certain host cell receptors. The genome of wild-type AAV is approximately 4.7 kb long containing two open reading frames (ORFs), three promoters and a polyadenylation signal, giving rise to four non-structural proteins (Rep) and three capsid proteins (Cap) through alternative splicing. The 5’ and 3’ ends of the genome are flanked by two inverted terminal repeats (ITR), of 145 bp. These ITRs contain a palindromic sequence that can form an internal T-shaped structure to self-prime for second strand synthesis during viral replication (Linden and Berns 2000). AAVs are also easily manipulated in vitro because of the small size of their genome and relatively simple organization. One of the unique features of AAV replication is that it requires co-infection by a helper virus, such as adenovirus or herpesvirus (Muzychka and Berns, 2001). Construction of recombinant AAV (rAAV) vectors was first described by Hermonat and Muzychka (1984) by inserting a neomycin resistance gene and transducing human and murine cells. The design of AAV-based vectors is straightforward; the rep and cap genes from the wild type virus are replaced with the transgene, retaining the ITRs which function as the origin of replication. Generation of rAAV requires transient transfection of cells with the vector and a plasmid providing packing genes and helper functions.

The use of recombinant AAV vectors generated from multiple serotypes has increased dramatically, especially with the isolation and characterization of newer serotypes. To date, 11 serotypes of AAV (AAV-1 to AAV-11) have been described, but so far AAV serotype 2 (AAV2) represents the most extensively studied gene delivery vector. In the literature, more than 60 clinical trials have used AAV2 as a gene delivery system.
(Mitchell et al., 2010). In vivo, AAV serotypes other than AAV2 have also shown superior transduction efficiencies, a variety of tissue tropisms, as well as different expression efficiencies determined by their capsid structure. AAV vectors have been used successfully to direct transgene expression in a variety of tissues including the respiratory system (Wootton et al., 2006; Yu et al., 2011), nervous system (Mandel and Burger 2004), metabolic system (Wang et al., 2010), and muscular system (Xiao et al., 1999). Several studies have also described the use of AAV vectors for delivering shRNA in vitro and in vivo. A recent study used rAAV to deliver shRNA targeting androgen receptor (AR), a critical factor in prostate cancer progression. Results demonstrated successful delivery of shRNA which eliminated prostate cancer cells in nude mice (Sun et al., 2010). In another similar study, rAAV expressing shRNA against a protein highly expressed in cancer (Hec1) demonstrated anti-proliferative and pro-apoptotic effects in tumour cells (Li et al., 2007). Other studies have also utilized this system to target immune mediators such as pro-inflammatory cytokines. Chronic inflammation induced by NF-κB, plays a key role in Duchenne muscular dystrophy. Recently, Yang et al. (2012) developed an AAV-9 mediated shRNA delivery system, down regulating NF-κB, and demonstrating improvement in muscle pathological lesions in mdx mice (a strain lacking dystrophin which is used as an animal model to study Duchenne muscular dystrophy). Overall, these few examples demonstrate that these vectors can efficiently introduce foreign genes (or siRNAs) in various cell types leading to expression of the transgene in various tissues.

AAVs have a wide tissue distribution and have been isolated from many species including avian species. The entire genome of two avian-AAV strains, DA-1 and VR-
865, were cloned and sequenced by Estevez and Villegas (2004). The sequence revealed a conserved distribution of structural and non-structural protein coding region consistent with strains from other species. In a later study, they constructed and generated a recombinant avian-AAV (rAAAV) expressing \textit{LacZ} gene which could infect a wide variety of tissues and be used as an efficient gene delivery system (Estevez and Villegas 2006). This was the first report of the construction of a rAAAV and vector system and its application \textit{in vitro} and \textit{in vivo}. Later studies have utilized this system to express human kallikrein (hK1) in a tissue specific manner in the oviducts of chicken (Wang \textit{et al.}, 2008), as well as express Newcastle disease virus (NDV) hemagglutinin neuraminidase gene and infectious bursal disease virus (IBDV) VP2 protein in chickens (Perozo \textit{et al.}, 2008a; Perozo \textit{et al.}, 2008b).
EXPERIMENTAL APPROACH

Objective 1

Identify immune mediators associated with vaccine-induced protection against Marek’s disease from the induction of cell-mediated immune responses in the lungs.

*Key steps involved:*

- Immunization of day old specific pathogen free (SPF) chicks with aerosolized cell-free HVT
- Infection of SPF chickens with cell-free aerosols of RB1B strain of MDV, on day 5 of age.
- Harvesting lung tissue at 1, 2, 3, 10, 21 days post infection (d.p.i)
- DNA extraction and viral genome load quantification
- RNA extraction, complementary DNA (cDNA) synthesis, and real-time PCR
- Immunohistochemical staining of T cell subsets: CD4+ and CD8+ T cells

Objective 2

Evaluate the efficacy of MD vaccines when combined with chicken interferon-gamma (IFN-γ).

*Key steps involved:*

- Designing primers to amplify full length coding sequence (CDS) of chicken IFN-γ along with a FLAG tag
• Construction of recombinant expression vector, pcDNA:chIFN-γFLAG

• Griess assay optimization, and confirmation of expression and bioactivity of recombinant chIFN-γ in vitro

• Immunization of day old SPF chicks with HVT vaccine and administration of varying doses of pcDNA:IFNγFLAG

• Postmortem examination of chickens for presence of tumours and gross lesions on day 21 post-infection

• Harvest spleens for nucleic acid extractions, cDNA synthesis

• Determine viral genome load using DNA extracts

• Determine cytokine expression by RT-qPCR

Objective 3

Develop and establish an RNA interference model for use to knockdown chicken IFN-γ in vitro and in vivo.

Key steps involved:

• Transfect DF-1 chicken fibroblast cell-line with pcDNA:IFN-γ and optimize an in vitro assay to knockdown expression of IFN-γ using three synthetic 27-mer duplex siRNAs targeting sequences within chicken IFN-γ CDS, and two negative control sequences (one universal negative sequence and another anti-GFP)

• Confirm IFN-γ gene expression knockdown by RT-PCR
- Confirm protein knockdown using chicken IFN-γ ELISA
- Construct four individual expression cassettes, each encoding chicken U6 promoter-shRNA sequence-termination sequence.
- Subclone each expression cassette into pEVA3V-ITR plasmid
- Develop an assay to generate a purified rAAAV stock by transfecting HEK293 cells and using a sucrose gradient
- Generate recombinant AAV expressing the shRNA targeting chicken IFN-γ and a non-target control (EGFP)
- Use rAAAV:U6shRNA for in vitro knockdown studies

**Objective 4**

Develop an AAV-based vector expressing shRNA targeting chicken IFN-γ, and utilize this system to knockdown IFN-γ in vivo during MDV challenge studies, to gain a better understanding of the role of this cytokine in vaccine-induced immunity.

- Assess dose and distribution of rAAAV:U6shRNA in vivo
- Administer day old SPF chicks intramuscularly (i.m.) with two different doses of rAAAV
- Harvest spleen, bursa, lung, liver and muscle tissue at 48hr-, 120hr-, 14 days- and 26 days- post administration
- DNA extraction and viral genome copy quantification
• Co-administration of day old chicks with HVT vaccine subcutaneously and/or rAAAV:U6shRNA intramuscularly.

• Infection of SPF chicks with RB1B strain of MDV at 5 days of age

• Harvest feathers at 4, 7, 10, 14 and 21 d.p.i.; Harvesting spleens at 21 d.p.i.

• DNA extraction and MDV genome load quantification
CHAPTER 2

VACCINE-INDUCED HOST RESPONSES AGAINST VERY VIRULENT MAREK’S DISEASE VIRUS INFECTION IN THE LUNGS OF CHICKENS

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Abstract

The aim of this study was to investigate the kinetics of virus replication and cellular responses in the lungs following infection with Marek’s disease virus (MDV) and/or vaccination with herpesvirus of turkey (HVT) via the respiratory route. Chickens vaccinated with HVT and challenged with MDV had a higher accumulation of MDV and HVT genomes in the lungs compared to the chickens that received HVT or MDV alone. This increase in virus load was associated with augmented expression of interferon (IFN)-γ and interleukin (IL)-10, and increased T cell infiltration. These findings shed more light on the immunological events that occur in the lungs after vaccination or infection with MDV.

Keywords: Marek’s disease virus, Herpesvirus of turkeys, lung, cell-mediated immune response, CD4+ T cell, CD8+ T cell, cytokine
Introduction

Marek’s disease (MD) in chickens is a lymphoproliferative disease (Calnek 2001; Payne 2004) caused by oncogenic (serotype 1) strains of Marek’s disease virus (MDV) belonging to the Family *Herpesviridae*. MDV goes through distinct phases during its replication that begins by the establishment of infection in the respiratory system. The initial respiratory infection is followed by an early cytolysis phase, a latent phase and finally host cell transformation leading to grossly visible lymphomas in various tissues (Witter and Schat 2003; Baigent and Davison 2004; Payne 2004).

Cell-mediated immune responses rather than antibody-mediated immune responses are critical in preventing MDV-induced tumour formation (Sharma and Witter 1975; Sharma et al., 1975). Infection of chickens with MDV leads to infiltration of CD4+ and CD8+ T cell subsets in brain (Gimeno et al., 2001), bursa of Fabricius (Abdul-Careem, et al., 2008a) and feather pulp (Abdul-Careem, et al., 2008b). Immunization of chickens with MD vaccines induces a significant expansion of CD4+ T cells in spleen (Gimeno et al., 2004), as well as infiltration of CD8+ T cell subsets in other tissues (Abdul-Careem, et al., 2008b; Gimeno et al., 2004). MDV antigens or MD vaccines induce CD8+ T cells to elicit cytotoxicity against cells expressing MDV antigens (Omar and Schat 1997; Omar et al., 1998) and CD8+ T cells play a role in reducing MDV load in MDV-infected T cells (Morimura et al., 1998).

Cytokines, produced by cells involved in innate and adaptive immunity, play a critical role in induction and modulation of cell-mediated immune response (Wigley and Kaiser 2003). A number of cytokines have been shown to be associated with MDV
infection in various tissues (Xing and Schat 2000a; Djeraba, et al., 2002; Abdul-Careem, et al., 2006b; Abdul-Careem et al., 2007; Heidari et al., 2008; Abdul-Careem et al., 2008a; Abdul-Careem et al., 2008b). Of these cytokines, the expression of interferon (IFN)-γ and interleukin (IL)-10 genes is consistently up-regulated in response to MDV infection and these cytokines are relevant in elicitation and regulation of adaptive immune response in the host.

In spite of the importance of respiratory mucosa in initial MDV establishment, very little is known about MDV pathogenesis and host responses to the virus in the respiratory system (Baigent and Davison 2004). Recently, our group has established an MDV infection model based on the inhalation of cell-free MDV containing aerosols (Abdul-Careem et al., 2009a). Based on this infection model, we have shown that MDV replication in the lungs leads to elicitation of innate host defense mechanisms dominated by the up-regulation of Toll-like receptor (TLR)3, TLR7, IL-1β, IL-8 and inducible nitric oxide synthase (iNOS) genes as well as infiltration of macrophages into the lung tissue (Abdul-Careem et al., 2009b). The objective of the present study was to study the induction of cell-mediated immune responses characterized by the infiltration of CD4+ and CD8+ T cell subsets and expression of cytokine genes in lungs following inhalation of herpesvirus of turkeys (HVT) and/or MDV. We chose HVT for this study, because this vaccine is commercially available as a cell-free virus, hence, it can be used in aerosolized form for infecting chickens via the respiratory route.
Materials and methods

Virus

The very virulent (vv) MDV strain, RB1B (passage 9) was used for infection of chickens through inhalation. The original virus stock was provided by Dr. K.A. Schat (Cornell University, NY, USA). Cell-free HVT vaccine (MD-Vac-CFL) was purchased from Fort Dodge Animal Health, Division of Wyeth, IA 50501 USA.

Experimental animals

Specific pathogen-free (SPF) White Leghorn fertile eggs were obtained from the Animal Disease Research Institute, Canadian Food Inspection Agency (Ottawa, Ontario, Canada) and hatched at the Arkell Poultry Research Center, University of Guelph. Chicks were housed in an isolation facility at the Ontario Veterinary College, University of Guelph. The use of experimental animals in this research had been approved by the University of Guelph Animal Care Committee.

Preparation of cell-free MDV suspension

Extraction of cell-free MDV was carried out using procedures described by Calnek et al., (1970) with some modifications as described elsewhere (Abdul-Careem, et al., 2009a).

Infection of chickens by inhalation of cell-free MDV

A system for generating aerosols from skin suspension originating from MDV-infected chickens was established according to the description by Sheppard et al., (2004) with modifications as described elsewhere (Abdul-Careem et al., 2009b).
Experimental design

At the day of hatch, chickens (n=25) were exposed to aerosols of cell-free HVT vaccine. At 5-days of age, these chickens were infected through inhalation of cell-free RB1B strain of MDV along with another group of age-matched chickens (n=25) acting as unvaccinated, infected controls. At 5 days of age another group of chickens (n=20) were exposed to cell-free HVT vaccine and a fourth group (n=20) were unvaccinated, uninfected controls which were not exposed to either HVT vaccine or MDV. MDV-infected chickens were exposed to aerosols of cell-free RB1B strain of MDV suspension (1280 PFU/ml) for 20 minutes. Similarly, the HVT-vaccinated chickens were exposed to cell-free HVT (28,000 PFU/ml) for 20 minutes. At 1, 2, 3, 10 and 21 days post infection (d.p.i) with vvMDV (RB1B), 5 HVT-vaccinated and MDV-infected, 5 MDV-infected, 4 HVT-vaccinated and 4 unvaccinated and uninfected chickens were necropsied and lung tissues were preserved in RNAlater (Qiagen Inc., Mississauga, Ontario, Canada) at -20°C. Lung samples collected from three chickens in each of the groups were snap frozen in embedding medium (Tissue-Tek®, Sakura Finetek Inc., Torrance, CA, USA) for immunohistochemical analysis. For sampling, we consistently used the right lung throughout our studies. For nucleic acid extraction, ~100mg of the middle 1/3 of the right lung was collected, whereas for immunohistochemistry, the distal 1/3 of the right lung was collected.
**DNA and RNA extraction**

DNA and RNA extraction from tissues collected at 1, 2, 3, 10 and 21 d.p.i was carried out using Trizol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) as has been described previously (Abdul-Careem et al., 2006a).

**Reverse Transcription**

The template for reverse transcription consisted of 2 μg of RNA. The reaction was carried out using Oligo(dT)_{12-18} primers (SuperScript™ First–Strand Synthesis System, Invitrogen Life Technologies, Carlsbad, California, USA) according to the manufacturer’s instructions.

**Primers**

The absolute MDV and HVT genome loads in various samples were quantified using primers specific for MDV-meq and HVT SORF-1 genes respectively, using DNA as the template. The relative quantification of MDV-meq and MDV-vIL-8 transcripts was done targeting MDV-meq and MDV-vIL-8 genes and normalized against expression of chicken β-actin gene. For relative quantification of viral transcripts complementary DNA (cDNA) was used as a template. The primers (Table 1) were designed based on nucleotide sequences available from the GenBank database using the Vector NTI™ software (Version 5.5, InfoMax, Inc., Frederick, Maryland, USA). The primers were synthesized by Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

**Real-time RT-PCR and PCR techniques**

All the DNA and cDNA preparations were tested in real-time PCR and RT-PCR assays, respectively, along with a dilution series of the standard that served as the calibra-
tor and a no template control. All the real-time RT-PCR and PCR runs using lung DNA or cDNA were conducted in a LightCycler® 480 multiwell Plate 384 (Roche Diagnostics GmbH, Mannheim, State of Baden-Wurttemberg, Germany) in a reaction volume of 20 µl in combination with LightCycler® 480 SYBR Green 1 Master (Roche Diagnostics GmbH) containing fast start Taq DNA polymerase for ‘hot start’ and DNA intercalating SYBR Green 1 dye. In addition, the reaction consisted of 0.25-0.05 µM of each of the gene-specific primers and 5 µl of 1:10 dilution of cDNA as template or 100 ng of DNA extracted from lungs and PCR grade water.

The optimum thermal cycling parameters for all the examined genes except HVT SORF-1 gene have been described previously (Abdul-Careem et al., 2008b; Abdul-Careem et al., 2007; Abdul-Careem et al., 2006a; Abdul-Careem et al., 2006b). Briefly, the cycles included a pre-incubation at 95°C for 10 min; 45 cycles of amplification at 95°C for 10 s, Ta°C for 5 s and 72°C for 10 s; melting curve analysis at 95°C for 10s, 65°C for 30 s and 97°C 0 s; cooling at 40°C for 10 s. Fluorescent acquisition was done at 72°C. The optimum thermal cycling parameters for HVT SORF-1 gene included pre-incubation at 95°C for 10 min; 45 cycles of amplification at 95°C for 10 s, 64°C for 5 s, and 72°C for 10 s; melting curve analysis at 95°C for 5 s, 65°C for 1 min and 97°C 0 s; cooling at 40°C for 10 s. Fluorescent acquisition was done at 72°C.

Histological observations

The lung samples preserved in embedding medium for frozen tissue specimens were sectioned (thickness 8 µm) using a cryotome (LEICA CM 3050 S, Vashaw Scientific Inc., Norcross, Atlanta, GA, USA), adhered to microslides (Superfrost plus, VWR
Labshop, Betavia, IL, USA) and preserved at -20°C until used. The immunohistochemistry technique, as described by Abdul-Careem and colleagues (2008b), was used to assess the distribution of CD4+ and CD8+ T cell infiltration in sections of lung tissue. Three animals from each time point and each group (MDV-infected and -uninfected control) were examined in order to characterize the pattern of infiltration of CD4+ and CD8+ T cells in the lungs. The degree of infiltration of T cell subsets in lungs over time was assessed quantitatively. Briefly, 5 highly infiltrated fields of 40x magnification were chosen from each section and immunoperoxidase-stained cells were enumerated by two independent observers. We reasoned that these highly infiltrated segments of the tissue would represent immune reactivity and, therefore, would be of interest for further characterization.

**Data analysis**

Quantification of MDV genome load and transcripts of MDV-meq and MDV-vIL-8 genes by real-time PCR and RT-PCR was done as described previously (Abdul-Careem et al., 2006a; Abdul-Careem et al., 2006b). Briefly, absolute number of MDV genome per 100 ng of DNA of lung was calculated based on an external standard curve. Expression of MDV-meq and MDV-vIL-8 genes was calculated relative to the expression of β-actin gene and expressed as ratios. All the data were analyzed by Student’s t-test to identify differences between observations and groups using the statistical package, MINITAB® release 14 (Minitab Inc., State College, Pennsylvania, USA). Comparisons were considered significant at $P \leq 0.05$. 

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Results

**MDV genome load in the lungs of HVT vaccinated and unvaccinated MDV-infected chickens**

Lung DNA originating from MDV-infected chickens was analyzed by real-time PCR and the data are illustrated in Figure 1 (a). At day 1 post-infection, MDV genome was detectable in all birds in HVT-vaccinated and MDV-infected birds, whereas none of the unvaccinated MDV-infected chickens showed amplification of meq. By day 2 post-infection, again all vaccinated and MDV-infected birds were positive for the presence of MDV genome in their lungs, whereas only two of the birds in the unvaccinated MDV-infected group were positive. At all other time points, all samples were positive for the presence of MDV genome. The difference in MDV genome load at 2 d.p.i between unvaccinated and vaccinated MDV-infected chickens was significant \((P=0.009)\). In the lungs of unvaccinated MDV-infected chickens, MDV genome was quantifiable from 3 d.p.i and showed an increasing trend towards the end of the study period (21 d.p.i). Higher MDV genome load was observed in the vaccinated MDV-infected group compared to the unvaccinated MDV-infected group at 10 d.p.i. \((P=0.069)\). By 21 d.p.i, the lungs of unvaccinated MDV-infected chickens had significantly higher MDV genome loads compared to the lungs of vaccinated MDV-infected chickens \((P=0.0103)\).

**MDV replication in the lungs of HVT vaccinated and unvaccinated MDV-infected chickens**

MDV replication was assessed by quantification of transcripts of MDV-meq gene that represent the latency and transformation phases (Kung et al., 2001; Heidari et al., 2008) and MDV-vIL-8 gene, which is associated with the cytolytic phase of the replication cycle (Cui et al., 2004; Heidari et al., 2008). Relative mRNA concentrations of MDV-meq and MDV-vIL-8 genes are illustrated in Figure 1 (b) and (c), respectively.
MDV-meq mRNA was quantifiable from 3 d.p.i, and increased by 21 d.p.i when maximum expression was recorded. The mRNA expression of MDV-meq was not significantly different between HVT-vaccinated and unvaccinated MDV-infected chickens at earlier time points ($P>0.05$). At 21 d.p.i a higher MDV-meq expression was observed in the lungs of unvaccinated MDV-infected chickens compared to vaccinated MDV-infected chickens although this difference was not statistically significant ($P=0.063$). MDV-meq gene expression observed at 21 d.p.i in unvaccinated MDV-infected chickens was significantly higher than that observed at other time points within the same treatment group ($P=0.0001$). The expression of MDV-vIL-8 gene was quantifiable from 1 d.p.i onwards in unvaccinated MDV-infected chickens, whereas that in HVT-vaccinated MDV-infected chickens was first observed at 2 d.p.i. The expression of MDV-vIL-8 gene gradually increased towards the end of the study in the lungs of both HVT-vaccinated and unvaccinated chickens exposed to MDV. Significantly higher expression of MDV-vIL-8 gene was observed at 10 d.p.i in the lungs of HVT-vaccinated MDV-infected chickens when compared to that in lungs of unvaccinated MDV-infected chickens ($P=0.007$).

*HVT Genome load in lungs of HVT-immunized MDV-infected and –uninfected chickens*

HVT genome load in the lungs was quantifiable at all time points from chickens vaccinated at the day of hatch and infected on day 5 of age (Figure 2). There was no amplification of HVT-SORF1 from the lungs of the chickens that received the HVT vaccine at 5 days of age, whereas the chickens that were vaccinated on the day of hatch and infected with MDV at 5 days of age had various amounts of HVT genome load beginning
from 1 d.p.i. Further investigations confirmed that the chickens exposed to HVT vaccination on day 5 of age were positive for the presence of HVT in their lungs as determined by quantification of HVT-gB transcripts in the tissue (data not shown).

Expression of cytokine genes in HVT-immunized, MDV-infected and control chickens

The expression of IFN-\( \gamma \) gene was observed in HVT-vaccinated and unvaccinated chickens, as well as in MDV-infected and -uninfected chickens (Figure 3a). The expression of IFN-\( \gamma \) gene at 10 d.p.i in the lungs of chickens that were vaccinated with HVT at the day of hatch and MDV-infected at day 5 of age was significantly higher when compared to that in the lungs of HVT-vaccinated and control chickens (\( P=0.004 \)). The expression of IFN-\( \gamma \) gene at 21 d.p.i in the lungs of chickens that were exposed only to MDV was significantly higher when compared to that in lungs of all other groups (\( P=0.002 \)). Expression of IL-10 was quantifiable from 3 d.p.i and there was an upward trend in the expression of this gene at 10 d.p.i, particularly in the lungs of chickens that were HVT-vaccinated and MDV-infected, as well as those that were unvaccinated but MDV-infected (Figure 3b). The expression of IL-10 gene in the lungs of chickens that were unvaccinated and exposed to MDV at 5 days of age was relatively high when compared to vaccinated only and unvaccinated uninfected groups, although the difference was not statistically significant (\( P=0.051 \)).

Histological observations

Infiltration of CD4+ and CD8+ cells in response to HVT vaccination and MDV infection was observed following immunohistological staining of the lung sections. The lungs of chickens that were vaccinated with HVT and then infected with MDV had more
infiltration with both CD4+ and CD8+ T cells when compared to the uninfected controls and chickens which received either the HVT vaccine or MDV (Figure 4). Stained cells in the sections were enumerated and the results are expressed as the mean number of cells observed in five fields of x20 microscopic view of the lung tissue sections (Figure 5). HVT-vaccinated MDV-infected chickens had significantly higher number of CD4+ cells in their lung sections compared to the lung section of the control chickens that were unvaccinated and uninfected from 3 d.p.i onwards ($P=0.0049$; Figure 5a). At 2 d.p.i, the lungs of chickens infected with MDV had higher numbers of CD4+ T cells when compared to the unvaccinated uninfected controls but was not significant ($P=0.0507$). At 10 d.p.i time, there was a significant difference between the degree of CD4+ T cell infiltration observed in the lungs of the chickens that were HVT vaccinated MDV-infected compared to HVT vaccinated and control groups ($P=0.0334$; Figure 5a). At the same time point, there was also a higher number of cells seen in the vaccinated MDV-infected group when compared to MDV-infected ($P=0.062$) As shown in Figure 5b, the number of CD8+ T cells in the lungs of HVT-vaccinated and MDV-infected birds was significantly higher when compared to the control group from 3 d.p.i onwards ($P=0.0336$). At 10 d.p.i, the HVT-vaccinated MDV-infected and MDV-infected group had significantly higher number of CD8+ T cells in the lungs when compared to HVT vaccinated and control groups ($P=0.0036$). Overall, there was a trend seen in the samples from infected birds showing an increase in the number of both CD4+ and CD8+ cells which peaked at 10 d.p.i and then declined by 21 d.p.i.
Discussion

Understanding the host response to infection in the lungs is important since MDV enters through the respiratory mucosa in the natural infection pattern and replicates in a diverse range of tissues in the chicken (Beasley et al., 1970; Addinger and Calnek 1973). In the present study, we found that both the vaccine (HVT) and challenge viruses (MDV) were capable of replicating in the lungs of chickens following exposure to aerosols of these viruses. The extent of replication of both viruses was higher in the chickens that were first immunized with HVT on the day of hatch, and then exposed to MDV via inhalation compared to the chickens that were exposed to each of these viruses alone. When both viruses were present in the lungs, replication of these viruses began as early as day 1 post-infection. We also discovered that HVT and MDV were capable of inducing host response at the site of virus entry, marked by the up-regulation of cytokine genes and expansion of CD4+ and CD8+ T cell subsets in the lungs. Finally, the magnitude of induced host responses was higher when both the vaccine and challenge viruses were replicating together in the lungs.

The mechanism of interaction between HVT and MDV is not known. In the current study, the extent of replication of both viruses was higher in the chickens that were co-infected with HVT and MDV. This observed phenomenon in the lungs of chickens is important since vaccination against MD does not prevent MDV infection in vaccinated chickens. Similar phenomena have been observed in other host-virus interactions where the interaction between two viruses may be promoting the replication of either one or both of the viruses. For example, Allan et al. (2000) have shown
that co-infection of porcine circovirus (PCV)-2 and porcine reproductive and respiratory syndrome (PRRSV) virus leads to higher replication of PCV-2. Further, activation of human immunodeficiency virus (HIV) from latency may be the result of the productive infection with a herpes simplex virus (Golden et al., 1992). In co-infected chickens, MDV-induced increased replication of avian leukemia virus has also been shown (Pulaski et al., 1992). Islam and Walden-Brown (2007) showed that when chickens vaccinated with Marek’s disease vaccines, such as HVT or SB-1, are infected with MDV, the shedding of vaccine viruses from feathers is increased by 38-75-fold when compared to that in chickens vaccinated with these vaccines alone. The results of our study show that a similar phenomenon, in form of an increase in HVT genome load, can occur in the lung tissue, when HVT-vaccinated chickens are infected with MDV. This observation can be explained if HVT and MDV share the same target cells in the host. Sharma (1987) has shown that the target cell for HVT replication in the embryo lungs is non-lymphoid and non-macrophage. Other studies have also shown lung epithelial cells may be targets for MDV replication (Purchase 1970; Addinger and Calnek 1973; St Hill et al., 2004). Since it is possible that HVT and MDV can replicate in a target cell simultaneously, it is also possible that replication of one virus is influenced by the other virus. Alternatively, HVT may attract macrophages and provide an increased number of targets for MDV replication.

In the present study, we did not detect the presence of HVT-SORF-1 gene in tissues of the birds that had received HVT on day 5 of age. This was probably partly due to age-dependency of permissiveness to HVT infection in the lungs, because
HVT was readily detectable in the birds that had been vaccinated on day 1 of age. The other scenario is that HVT was probably present at very low copy numbers in tissues of those birds that were vaccinated on day 5 post-hatch and, perhaps, the realtime assay for detection of SORF-1 was not sensitive enough, because we could detect low amounts of HVT-gB transcripts in the lungs of these chickens from day 2 post-HVT vaccination onwards. Altogether, the above observation of low infectivity of HVT in the lungs raises the important question as to whether the respiratory route is indeed an appropriate route for HVT vaccination in field conditions. We have conducted preliminary experiments showing that delivery of HVT via in ovo or respiratory routes confers comparable levels of protection in MDV-challenged birds (data not shown). However, there are obviously some logistical challenges associated with the use of any vaccines delivered via the respiratory route in the field compared to the in ovo route. At the same time, a potential advantage of Marek’s disease vaccines delivered via the respiratory route is that they can perhaps reduce the load of virulent MDV load in the initial phase of virus entry and replication in the host. Although we have provided evidence in the present study that the commercial HVT vaccine is not capable of reducing MDV load in the lungs, it is conceivable that other vaccines currently used, such as CVI988, might have that ability. Also, rational vaccines may be designed in the future to specifically reduce virus load in the lungs.

In addition to the innate host responses mounted against MDV infection in the lungs (Abdul-Careem et al., 2009b), antigen-specific adaptive host responses may play a role against MDV while the virus is still in the lungs. Being an intracellular pathogen, MDV
stimulates predominantly T cell-mediated immune responses (Morimura et al., 1998; Morimura et al., 1999; Markowski-Grimsrud and Schat 2002). In this regard, cytokines are important mediators involved in elicitation of cell-mediated immune responses to MDV infection. In the lungs of chickens that were exposed to HVT and MDV, as well as in the lungs of chickens that were only exposed to MDV infection, there was a significant up-regulation of IFN-γ gene at 10 and 21 d.p.i when compared to the lungs of vaccinated and control chickens. Previously, it has been shown that IFN-γ is one of the key cytokines that is increased following MDV infection or vaccination in other anatomical sites (Xing and Schat 2000a; Djeraba et al., 2002a; Kaiser et al., 2003; Jarosinski et al., 2005; Abdul-Careem et al., 2006b; Abdul-Careem et al., 2008a; Abdul-Careem et al., 2008c). Although the source of IFN-γ was not examined in the present study, this cytokine could be expressed by NK cells as well as CD4+ and CD8+ T cells (Garcia-Camacho et al., 2003; Parvizi et al., 2009a). In the present study, we observed the expansion of these T cell subsets in the lungs following MDV infection or vaccination followed by MDV infection. In addition to its direct antiviral effects, IFN-γ may activate CD8+ T cells to kill virus-infected cells (Whitmire et al., 2005). In our study, the number of CD8+ T cells was significantly increased in HVT-vaccinated and MDV-infected chickens. The expression of IL-10 was also up-regulated in response to MDV infection and vaccination with HVT followed by MDV infection in lungs. Others, as well as our group, have previously observed up-regulation of this cytokine in other tissues in response to MDV infection (Abdul-Careem et al., 2007; Heidari et al., 2008). Buza and Burgess (2007) have also shown that MDV induces the expression of IL-10 and IL-10Rα in MDV
transformed cells. This up-regulation of IL-10 may suggest an immunoevasive mechanism used by MDV to suppress the host immune system. Interestingly, in the current study the expression of IL-10 and IFN-\(\gamma\) was up-regulated at the same time points (10 d.p.i) and only in the infected groups. Furthermore, the up-regulation of these cytokines coincided with a significant increase in the number of T cell lymphocytes. The results we obtained are in agreement with other studies which have shown a similar finding using porcine reproductive and respiratory syndrome virus (PRRSV) in pigs. Suradhat and Thanawongnuwech (2003) reported an up-regulation in both IL-10 and IFN-\(\gamma\) expression associated with a concurrent increase of lymphocytes in bronchoalveolar lavage cells (BALC) around a similar time point (9 d.p.i) after infection of pigs with PRRSV.

The number of CD8+ T cells increased in the lungs in response to MDV infection or vaccination with HVT followed by MDV infection which may be in response to the up-regulation of IL-10 or the presence of the infective virus. Omar and Schat (1997) originally identified the role of CD8+ cytotoxic T lymphocytes (CTL) in killing MDV-infected cells expressing MDV pp38, meq and gB proteins. Further studies confirmed the effect of CD8+ CTL against cells expressing various glycoproteins of MDV (Omar et al., 1998; Markowski-Grimsrud and Schat 2002). Quere et al. (2005) showed that MD-susceptible chickens have significantly higher CD8+ T cells in blood in response to MDV infection. It also has been shown that CD8+ T cells are associated with MDV infection in brain (Gimeno et al., 2001), feather pulp (Abdul-Careem et al., 2008b), spleen (Baigent and Davison 1999), bursa of Fabricius (Abdul-Careem et al., 2008a), blood vessels (Njenga and Dangler 1996) and lymphoproliferative lesions (Burgess et al., 2001).
Though CD4+ T cells play a less clear role directly in clearing virus-infected cells, these cells may provide help for maturation of MDV-specific CD8+ T cells to differentiate into antigen-specific CTLs. Omar and Schat (1997) have shown that CD4+ CTLs are not important for the elimination of cells expressing the viral antigens. This observation was later confirmed by Morimura and co-workers (1999; 1998). Increased infiltration of this T cell subset has been shown to be associated with MDV infection in various tissues (Baigent and Davison 1999; Burgess et al., 2001; Gimeno et al., 2001; Abdul-Careem et al., 2008b; Abdul-Careem et al., 2008c). In agreement with these observations, we observed a significant expansion of CD4+ T cells in the lungs following MDV infection or vaccination followed by MDV infection. CD4+ T cells are also targets for MDV-induced transformation. Therefore, the possibility in this experiment cannot be excluded that the cells infiltrating the lungs, especially at the very last sampling time point, 21 d.p.i, may be MDV-transformed cells.

The increased host response in the lungs of vaccinated chickens and MDV-challenged chickens compared to the response in unvaccinated and MDV-infected birds may indicate generation of a memory response following HVT vaccination. However, the site of induction of antigen-specific immune response or the processes involved in generation and maintenance of immune memory are not known. Though information on generation of anti-MDV memory T cells is scarce, Pei et al. (2003) have shown that CD8+ T cells are critical for and can play a role as memory cells against infectious bronchitis virus in chickens. The observation of augmented immune response, seen in our vaccinated MDV-infected group, could provide evidence that HVT-induced memory
cells may be eliciting a rapid host immune response when the chickens are exposed to MDV infection.

An aspect of the present study that was not fully explored was the possibility that by day 21 post-exposure to MDV, some of the infiltrating cells in the lungs might have been MDV-transformed CD4+ T cells. If so, these cells might have contributed to some of the cellular profiles and cytokine responses that we observed in the lung tissue. However, it should be noted that we did not observe any gross tumours in the lungs of infected birds. Although this does not exclude the formation of microscopic tumours in this tissue, it reduces the possibility of involvement of transformed cells in skewing the results of our study.

In conclusion, when chickens are exposed to aerosols containing HVT prior to MDV infection, an augmented host response is generated compared to that elicited by either of the viruses alone. The response observed in the lungs was characterized by the increased expression of cytokine genes such as IFN-γ and IL-10 and increased tissue infiltration of CD4+ and CD8+ T cells. Further studies should be directed to elucidate the mechanism of elicitation of immune response at the site of virus entry.
Table 1: Oligonucleotides used for real-time PCR and RT-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Reference, Accession Number</th>
</tr>
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<tbody>
<tr>
<td>MDV meq</td>
<td>Forward: 5’- GTCCCCCCTCGATTTTCTTCTC-3’&lt;br&gt;Reverse: 5’- CGTCTGCTTCTGCCTTTCTCC-3’</td>
<td>[6]</td>
</tr>
<tr>
<td>HVT SORF-1</td>
<td>Forward: 5’- AGGCCTACGATCACGTACAGTCC-3’&lt;br&gt;Reverse: 5’- CGAGCATGCGCAGATGTCGTT-3’</td>
<td>AF282130</td>
</tr>
<tr>
<td>vIL-8</td>
<td>Forward: 5’- TATCACTGGAGAGTCTCGTGC-3’&lt;br&gt;Reverse: 5’- TCAGTCCTCTGCAGATGTCGTACC-3</td>
<td>[3], AF489276</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward: 5’- CTGAAGAACTGGACAGAGAG-3’&lt;br&gt;Reverse: 5’- CACCAGCTTCTGTAAAGATGC-3’</td>
<td>[7], X99774</td>
</tr>
<tr>
<td>IL-10</td>
<td>Forward: 5’- AGCAGATCAAGGACACGTTC-3’&lt;br&gt;Reverse: 5’- ATCAGCAGTACTCCTCGAT-3’</td>
<td>[5], AJ621614</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: 5’- CAACACAGTCTGTCTGTGTTGGTA-3’&lt;br&gt;Reverse: 5’- ATCGTACTCCTGCTTGATCC-3’</td>
<td>[7], X00182</td>
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</table>
Figure 1. MDV genome load and transcripts in the lungs of chickens infected with vvMDV or vaccinated and infected.

The groups were as follows: HVT+MDV = chickens that were exposed to aerosols of HVT at day 1 of age and MDV at 5 days of age, MDV = Chickens that were exposed to aerosols of MDV at 5 days of age. Lung tissues were obtained at 1, 2, 3, 10 and 21 days post MDV infection (d.p.i.). There were four to five chickens in each group at each time point. Mean MDV genome load (Figure 1a), meq mRNA (Figure 1b) and vIL-8 mRNA (1c) expression relative to β-actin mRNA expression are presented and the error bars represent standard error of the mean. a = significant when comparing HVT vaccinated and unvaccinated MDV-infected chickens ($P=0.009$) at 2 d.p.i. b = Significant MDV genome load difference in unvaccinated MDV-infected compared to HVT vaccinated MDV-infected chickens ($P=0.0103$). c = significant difference comparing unvaccinated MDV–infected chickens, to all other time points within that group ($P=0.0001$). d = significant difference in HVT vaccinated MDV-infected compared to unvaccinated MDV-infected chickens ($P=0.007$).
Figure 2. HVT genome load in the lungs of chickens vaccinated with HVT or vaccinated with HVT and infected with MDV.

The groups were as follows; HVT+MDV= chickens that were exposed to aerosols of HVT at day 1 of age and vvMDV strain of MDV at 5 days of age, HVT= Chickens that were exposed to aerosols of HVT at days 5 of age. Lung tissues were obtained at 1, 2, 3, 10 and 21 d.p.i. There were four to five chickens in each group at each time point. Mean HVT genome load is presented and the error bars represent standard error of the mean.
Figure 3. Expression of IFN-γ and IL-10 genes in the lungs of chickens infected with MDV, vaccinated with HVT, vaccinated with HVT and infected with MDV or control chickens that were unvaccinated and uninfected.

The groups were as follows: HVT+MDV= chickens that were exposed to aerosols of HVT at day 1 of age and MDV at 5 days of age, MDV= chickens that were exposed to aerosols of MDV at days 5 of age, HVT= chickens that were exposed to aerosols of HVT at days 5 of age, CON= unvaccinated and uninfected chickens. Figures (3a) and (3b) represent expression of IFN-γ and IL-10 genes respectively. There were four to five chickens in each group at each time point. Target gene expression is presented relative to β-actin expression and normalized to a calibrator. Error bars represent standard error of the mean. a = significant difference between vaccinated MDV-infected when compared to vaccinated uninfected and unvaccinated uninfected chickens (P=0.004). b = significant difference comparing unvaccinated MDV-infected with all other groups (P=0.002).
Figure 4. Representative immunohistochemistry images of lungs of chickens infected with vvMDV, vaccinated with HVT, vaccinated with HVT and infected with MDV and unvaccinated and uninfected control stained for CD4+ and CD8+ T cell sub sets.

The groups are the same as those indicated in the Figure 3 legend. The sections, from 10 d.p.i, were stained for CD4+ and CD8+ T cells using mouse anti-chicken monoclonal antibodies (SouthernBiotech, Birmingham, AL 35260, USA) respectively. Bar=500 μm. Arrows show examples of positively stained cells. a= air capillaries, b= Para bronchus, c =inter-parabronchial septa
Figure 5. Distribution of CD4+ (A) and CD8+ (B) subsets of T cells from immunohistochemistry slide images in chicken lungs from various groups of vaccinated and/or MDV infected groups.

The design of the experiment has been indicated in the Figure 1 legend. There were 3 chickens in each group at each time point. Group mean number of CD4+ (A) or CD8+ cells (B) is presented per x20 microscopic view and the error bars represent standard error of the mean. a = significant when HVT-vaccinated and MDV-infected group is compared to the age-matched unvaccinated uninfected controls, b = significant when comparing HVT-vaccinated and MDV-infected group to HVT-vaccinated group.
Acknowledgements

This study was carried out with financial support from Natural Sciences and Engineering Research Council of Canada, Poultry Industry Council, Canada. We thank Dr. Bruce D. Hunter for the discussions on establishment of the MDV infection model and Dr. Davor Ojkic for providing access to LC480 thermocycler.
CHAPTER 3

INTERFERON-GAMMA INFLUENCES IMMUNITY ELICITED BY VACCINES AGAINST VERY VIRULENT MAREK’S DISEASE VIRUS

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Abstract

Vaccination of chickens with herpesvirus of turkey (HVT) confers only partial protection against challenge with a very virulent Marek’s disease virus (MDV). Here, we evaluated the ability of recombinant chicken interferon-gamma (rChIFN-gamma) to enhance protective efficacy of HVT against the very virulent MDV strain, RB1B. The bioactivity of IFN-gamma expressed by a plasmid expression vector was confirmed by its ability to stimulate a chicken macrophage cell line (HD11) to produce nitric oxide (NO) in vitro. The administration of HVT with 5 μg of pcDNA:chIFN-gamma plasmid reduced the incidence of tumour development significantly when compared to vaccinated birds (77.7% in the HVT+empty vector group and 80% in HVT group versus 33.3% in the HVT+chIFN-gamma group) and significantly increased endogenous IFN-gamma expression in the splenocytes of the protected group, suggesting that rChIFN-gamma increases the potency of HVT against MDV. Further analysis demonstrated that the protected birds that received HVT vaccine and/or plasmid had lower MDV genome load and lower amounts of transcripts for meq and vIL-8 than in the birds with lesions. Similarly, lower expression of IL-10, IL-18 and IL-6 was observed in the chickens without lesions compared to the chickens that had lesions, suggesting an inverse association between up-regulation of these cytokines and vaccine-induced immunity. In conclusion, IFN-gamma can positively influence immunity conferred by HVT vaccination against challenge with a very virulent Marek’s disease virus (vvMDV) in chickens.

Keywords: Chicken; Immunology; Vaccine; Interferon-gamma; Marek’s disease; Adjuvant
Introduction

Marek’s disease (MD), which is caused by a highly contagious cell-associated alphaherpesvirus named Marek’s disease virus (MDV), is a lymphoproliferative and immune suppressive disease of chickens (Biggs 2001; Calnek, 2001; Baigent et al., 2006). There are three serotypes of MDV, serotype 1 (also known as Gallid herpesvirus-2, GaHV-2) which includes oncogenic virus strains such as RB1B and GA, serotype 2 (also known as Gallid herpesvirus-3, GaHV-3) which includes non-oncogenic strains such as SB-1, and serotype 3 (Meleagrid herpesvirus-1, MeHV-1) which includes non-oncogenic viruses of turkey also known as herpesvirus of turkeys (HVT). Early reports revealed that HVT, which is non-oncogenic for chickens and antigenetically related to MDV, can be used as a vaccine against MD (Kawamura et al., 1969; Witter et al., 1970). MD has since been controlled by vaccinating chick embryos or chickens with different vaccine formulations using HVT alone, HVT in combination with non-oncogenic strains as bivalent or trivalent vaccines, or with attenuated serotype 1 strains (Churchill et al., 1969; Okazaki et al., 1970; Rispens et al., 1972; Witter and Schat 2003). Although these vaccines do not prevent chickens from becoming infected with MDV, they have varying degrees of efficacy against disease caused by viruses of different pathotypes. For instance, while monovalent HVT vaccines are efficacious against virulent (v) MDV strains, these vaccines are not highly protective against very virulent (vv) viruses (Witter, 1997). Despite the widespread use of vaccines, MD outbreaks are still occurring due to a variety of factors including the emergence of strains with increasing virulence (Baigent et al., 2006). Evolution of MDV virulence may be related to factors such as constant administration of vaccine which tends to exert evolutionary pressure on the virus leading
it to evolve into more virulent pathotypes, a feature known as vaccine-driven evolution (Schat and Baranowski 2007). Co-administration of avian immunomodulatory molecules, including cytokines, have been proven to enhance protective host immune responses to a variety of pathogens such as *Eimeria acervulina* (Min et al., 2001), duck hepatitis B virus (DHBV) (Long et al., 2005), infectious bursal disease virus (IBDV) (Hsieh et al., 2006), *Escherichia coli* (Janardhana et al., 2007), and infectious bronchitis virus (IBV) (Wang et al., 2009). Analysis of host responses to MDV infection has led to identification of a few cytokines that play a role in immunity against the virus, hence may be included in vaccines for enhancing their immunogenicity and efficacy (Xing and Schat, 2000b; Kaiser et al., 2003; Kano et al., 2009; Parvizi et al., 2010; Haq et al., 2010b).

Interferon-gamma (IFN-γ) is a type II interferon and an important regulator of immune responses including antiviral defenses. Chicken IFN-γ (chIFN-γ) was first cloned by Digby and Lowenthal (1995), and since then has been shown to activate macrophages (Lowenthal et al., 1995; Lowenthal et al., 1997), neutralize viral replication (Digby and Lowenthal 1995; Song et al., 1997) and enhance major histocompatibility complex (MHC) class II antigen expression (Weining et al., 1996; Song et al., 1997) among other genes in chickens (Mallick et al., 2010). The expression of IFN-γ is associated with protection against MD (Xing and Schat 2000b; Jarosinski et al., 2005; Abdul-Careem et al., 2007; Kano et al., 2009; Haq et al., 2010a), making this cytokine a candidate for inclusion in Marek’s disease vaccines to enhance their protective efficacy. Recombinant chIFN-γ (rChIFN-γ) may be delivered via the use of liposomes (Kedar et al., 1997), recombinant viral vectors (Johnson et al., 2000; Yong-Ke et al., 2005), and plasmid DNA.
The immune enhancing effect of rChIFN-γ has been well studied in the coccidiosis challenge model, demonstrating that the treatment of birds with chIFN-γ reduces the oocyst production and improves resistance to *E. acervulina* (Lowenthal *et al.*, 1997; Lillehoj and Choi 1998; Min *et al.*, 2001). A recent study also showed that a DNA vaccine in combination with plasmid encoded chIFN-γ protected birds from coccidiosis, which was marked by prevention of weight loss and oocyst excretion (Shah *et al.*, 2010). The immune enhancing effects of IFN-γ in other avian species have also been recorded. For instance, administration of recombinant IFN-γ inhibited duck hepatitis B replication in hepatocytes and improved vaccine efficacy against the disease in duck (Schultz and Chisari 1999; Long *et al.*, 2005). In this study, we hypothesized that administration of IFN-γ, via the use of a plasmid encoding chIFN-γ, will enhance the protective efficacy of HVT vaccine against challenge with a very virulent strain of MDV in chickens.

**Materials and methods**

**Cell lines**

An immortal chicken fibroblast cell line, DF-1, was maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS), 1% chicken serum (CS) and 100μg/mL of penicillin and streptomycin at 37°C and 5% CO₂. Chicken macrophage cell line, HD11, was maintained in RPMI 1640 medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% FBS, 2mM glutamine, 100μg/mL penicillin and streptomycin at 41°C and 5% CO₂.
**Experimental animals**

Specific-pathogen free (SPF) eggs were obtained from the Animal Disease Research Institute, Canadian Food Inspection Agency (Ottawa, ON, Canada) and hatched at the Arkell Poultry Research Unit, University of Guelph. Hatched chicks were housed in the animal isolation facility at the Ontario Veterinary College, University of Guelph during the experimental period. All animal experiments were approved by the Animal Care Committee, University of Guelph.

**Virus and vaccine strains**

Very virulent MDV strain RB1B (passage 9) was provided by Dr. K.A. Schat (Cornell University) and was used for infecting chickens. Chickens were vaccinated subcutaneously on the day of hatch using ¼ of the recommended dose of cell-free HVT purchased from Fort Dodge Animal Health, Division of Wyeth, IA 50501 USA.

**Construction of plasmid containing chIFN-γFLAG**

The chIFN-γ coding sequence was PCR amplified from cDNA prepared from concavalin A (ConA) stimulated primary splenocytes using primers designed to span the predicted chIFN-γ coding sequence (CDS) obtained from Ensembl database (ENSGALT00000016105). The primer sequences were: forward 5’-GAACTGAGCCATCACCAAGAA-3’, reverse 5’-CGTCTTATGACCTCCTGTGCT-3’. Briefly, the PCR conditions were as follows: one cycle of 95°C for 10 minutes, 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes using High fidelity (HiFi) platinum Taq polymerase (Invitrogen Canada Inc., Burlington, ON, Canada). The amplified product and a 1xFLAG
3’ fusion tag were cloned into the pcDNA3.1(+) expression vector (Invitrogen Canada Inc., Burlington, ON, Canada), and the resulting recombinant plasmid was designated as pcDNA:chIFN-γFLAG. Plasmid DNA was prepared using a GenElute™ plasmid purification kit (Sigma-Aldrich, St. Louis, MO, USA). Recombinant plasmid pcDNA:chIFN-γFLAG was sequenced bi-directionally to verify correct insertion and sequence fidelity (Laboratory Services Division, University of Guelph).

**DNA and RNA extraction**

Spleen tissues collected on 21 days post-infection (d.p.i) were preserved in RNA later (Qiagen Inc. Mississauga, Ontario, Canada) prior to DNA and RNA extraction using Trizol reagent (Invitrogen Canada Inc. Burlington, Ontario, Canada) as described previously (Abdul-Careem et al., 2006b).

**Reverse transcription**

Complementary DNA (cDNA) was prepared from 2 μg of DNase-treated total RNA by reverse transcription using MMLV reverse transcriptase and Oligo(dT)12–18 primers (SuperScript™ First-Strand Synthesis System, Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions.

**Primers**

The absolute MDV genome load in the spleen was quantified using primers specific for the MDV-meq gene. The relative quantification of MDV-meq and MDV-vIL-8 transcripts was done using primers targeting MDV-meq and MDV-vIL-8 genes and normalized against expression of the chicken β-actin gene. The primers used to amplify MDV-meq, MDV-vIL-8, IFN-γ, IL-10, IL-6, IL-18 and β-actin have been published
previously (Abdul-Careem et al., 2006a; Abdul-Careem et al., 2006b; Abdul-Careem et al., 2007). All primers were synthesized by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

**Real-time PCR and RT-PCR**

All DNA and cDNA preparations were tested in real-time PCR and RT-PCR assays, respectively, along with a dilution series of the standard plasmids that served as the calibrator as well as a no template control. Real-time RT-PCR and PCR quantifications were performed in a LightCycler 480 Instrument (Roche Diagnostics, Laval, QC, Canada) in a reaction volume of 20 μl using LightCycler 480 SYBR Green 1 Master Mix (Roche Diagnostics). In addition, the reaction consisted of 0.25-0.5 μM of each primer and 5 μl of 1:10 dilution of cDNA or 100ng of DNA extracted from spleen as template and PCR grade water. The optimal PCR parameters for all genes have been described previously (Abdul-Careem et al., 2007; Abdul-Careem et al., 2006a). Briefly, the cycles included an initial heat-denaturing step at 95°C for 10 min, 45 cycles of amplification at 95°C for 10 s, annealing for 5 s, and elongation at 72°C for 10 s. Following amplification, melting curves were determined at 95°C for 10 s, 65°C for 30 s and 97°C for 0 s continuous acquisition. Fluorescent acquisition was done at 72°C and expression levels were normalized to β-actin expression.

**rChIFN-γFLAG expression and bioactivity**

The recombinant plasmid, pcDNA:chIFNγFLAG, was used to transfect DF-1 cells. DF-1 cells (1x10^6) were incubated 24 hours prior to transfection with pcDNA:chIFN-γFLAG or an empty vector (pcDNA3.1) as a negative control. Stock solution of
polyethyleneimine (PEI) (PolySciences, Eppenheim, Germany) was prepared in water at a final concentration of 1 mg/ml, and plasmid DNA was diluted to required concentrations. Prior to transfection, DF-1 cells were rinsed twice with PBS to remove any residual cell culture medium and 200μL of the DNA-PEI solution was added and incubated overnight (16-18 hours). At this point, the transfection mix was removed, cells were washed and complete DMEM was added to all wells. Cells and culture supernatant samples were collected 24, 48 and 72 hours post-transfection for RNA extraction and protein expression analysis. Culture supernatant was separated by 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane for Western blotting using an anti-FLAG® monoclonal antibody (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and detected using BCIP/NBT assay.

The bioactivity of the rChIFN-γFLAG was tested by a nitric oxide (NO) assay and by assessing the antiviral activity of the recombinant chIFN-γFLAG. Nitrite assay was used to determine NO production in HD11 cells after stimulating with rChIFN-γFLAG using the Griess Reagent System (Promega Corporation, Madison, WI, USA). HD11 cells (1x10^5 cells in 200 μL) were cultured overnight in sterile 96 well plates. Fifty μl of supernatant was aspirated and replaced by 50μL of transfected DF-1 culture supernatant or purified rChIFN-γFLAG, as a positive control, for 48 hours. The concentration of nitrite was then determined by adding 50 μl of experimental samples to 100 μl of Griess reagent (1:1 mixture of 1% sulphanilamide, 2.5% phosphoric acid and 0.1% naphthylethlenediamine), and absorbance was measured between 520nm-550nm with a spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). The concentration of
nitrite in test samples was determined by preparing a 0-100μM nitrite standard reference curve. All experiments were done in triplicate.

Experimental design

There were two challenge trials in this study to assess the ability of chIFN-γ expressed by a plasmid to enhance protection conferred by HVT vaccination. In the first trial, which was conducted as a pilot experiment, day old chicks (n = 5-6/group) were vaccinated with HVT and injected with two plasmid doses (1μg and 5μg). The trial included five groups of 5-6 birds each. One group served as an uninfected control, one group was infected with 250 plaque forming units (PFU) of RB1B intra-abdominally (i.a) at 5 days of age, another group was vaccinated on day 1 of hatch with HVT vaccine (Fort Dodge Animal Health, Division of Wyeth, IA 50201, USA) and infected i.a on day 5 of hatch with 250 PFU of RB1B, and the other two groups were vaccinated, MDV-infected and administered two different doses of plasmid (1μg or 5μg) subcutaneously in the leg.

In the second trial, each group had a larger number of birds (n=9-10/group) and based on the results from the pilot trial, an optimal plasmid dose (5μg) was selected and an additional control group was added. The doses and administration day were similar to the first trial. One group served as uninfected control and another group as the infected only (RB1B). The other three groups consisted of vaccinated MDV-infected (R+H), and vaccinated MDV-infected administered with 5μg of pcDNA:chIFN-γFLAG (R+H+5μg Plasmid) or an empty vector (R+H+5μg EV). The chickens were then monitored daily for any clinical signs over a 3-week period. At 21 days post-infection, all birds were weighed, necropsied and the presence of gross lesions was recorded. Based on the
presence or absence of gross lesions characteristic of Marek’s disease, chickens were further categorized as with or without lesions. To this end, all major organs including liver, heart, spleen, bursa, kidney, proventriculus, sciatic nerve, gonads, muscles and eyes were observed for presence of tumours and gross lesions. In addition, the weights of spleen and bursa of Fabricius were recorded, and the ratios of spleen/bursa of Fabricius to body weight were determined. Analysis of the viral load and gene expression profiling from the second trial is presented.

Data analysis

Quantification of MDV genome load and transcripts by real time PCR and RT-PCR was done as described previously (Abdul-Careem et al., 2006a; Abdul-Careem et al., 2006b). Briefly, absolute number of MDV genome per 100ng of DNA was calculated based on an external standard curve. Expression of MDV-meq, vIL-8, IFN-γ, IL-10, IL-6 and IL-18 was calculated relative to the expression of β-actin gene. Results are presented as with lesions and without lesions in each group. For tumour incidence data, Fisher’s exact test was used, whereas for all other data a two-tailed t-test was used to identify differences among groups. Comparisons were considered significant at $P \leq 0.05$.

Results

Construction of plasmid, expression of rChIFN-γFLAG in vitro and biological assay

The first aim of this study was to obtain a biologically active rChIFN-γ protein expressed using a eukaryotic expression system. In order to confirm the expression of cloned IFN-γ, chicken embryo fibroblast (DF-1) cells were transfected with pcDNA:chIFN-γFLAG or empty vector (mock). RNA was extracted at 24, 48 and 72
hours post transfection and transcripts were detected by real time qRT-PCR (Fig 6a). Results confirmed the expression of significantly higher chIFN-γ mRNA compared to empty vector and medium-treated cells at the three time points ($P \leq 0.05$). To confirm the identity of the protein expressed by pcDNA:chIFN-γFLAG, culture supernatants of transfected cells were analyzed by Western blotting (Fig. 6b). This analysis confirmed the presence of a band with a molecular weight of approximately 17 kDa, the position corresponding to the monomeric, non-glycosylated form of rChIFN-γ (Lillehoj and Choi, 1998; Lambrecht et al., 1999; Takehara et al., 2002). In addition, another faint band was present above the major band which most likely represents the glycosylated form of the cytokine (Fig 6b). The results confirmed expression of rChIFN-γFLAG at both the transcript and protein level. Biological activity of the recombinant protein was tested by treating HD11 cells with the supernatants from pcDNA:chIFN-γFLAG transfected DF-1 cells and measuring NO production using the Griess assay. Purified recombinant protein (positive control) and culture supernatants of pcDNA:chIFN-γFLAG transfected DF-1 cells induced high levels of nitrite production by HD11 cells (Fig. 6c). Supernatants from DF-1 cells transfected with an empty vector (mock transfected) or medium produced undetectable levels of nitrite. The average amounts of nitrite produced by HD11 cells after incubation of these cells with cell culture supernatants from transfected DF-1 cells ranged from 6.77-14.8 μM. The higher expression of rChIFN-γ mRNA seen in DF-1 transfected cells correlated with the presence of the rChIFN-γ protein in the culture supernatants from three time points (24, 48, and 72 hours), as confirmed by the Griess assay. Cells transfected with an empty vector and the negative control neither had an
increase in IFN-γ transcript expression nor did their supernatant stimulate the production of detectable levels of nitrite produced.

*Effect of pcDNA:chIFN-γFLAG on enhancing HVT conferred immunity against MDV-RB1B challenge*

Four days after HVT vaccination and administration of the plasmid encoding chIFN-γ, chicks were challenged with MDV-RB1B virus and monitored daily for development of clinical signs until 21 days post-infection. The incidence of grossly visible tumours and observable changes in organ size and condition were recorded in all the experimental groups during necropsy. Based on the presence or absence of tumours, chickens were classified as with lesions or without lesions, respectively. Table 2 summarizes the incidence of tumours among various groups in the pilot and main experiment. In the pilot study (Trial 1), the number of chickens treated with pcDNA:chIFN-γFLAG combined with HVT that developed tumours after MDV-RB1B challenge was lower compared to those that only received HVT. In the HVT+IFN-γ group, the incidence of MD tumours was 20% compared to 66.6% in the group that had only received HVT. In Trial 2, the unvaccinated RB1B-infected group, the tumour incidence was 100% whereas in the infected groups that received HVT only or HVT + pcDNA 3.1 (empty vector, EV), tumour incidence was 80% and 77.7% respectively. However, in the group that received HVT + pcDNA:chIFN-γFLAG prior to MDV infection, the tumour incidence was reduced to 33.3%. None of the birds in the unvaccinated uninfected control group developed tumours or clinical signs.

The mean ratio of bursa of Farbricius and spleen to body weight from both trials at 21 d.p.i is presented in Fig. 7. The spleens of the birds in the MDV-infected groups
were either enlarged or had tumours and resulted in increased spleen to body weight ratios, whereas the bursa to body weight ratios were lower in the MDV-infected groups compared to controls due to bursal atrophy. The spleen:body weight ratio was significantly higher in vaccinated birds compared to unvaccinated uninfected birds ($P \leq 0.05$). However, the ratio in the unvaccinated MDV-infected group was significantly higher when compared to vaccinated MDV-infected and unvaccinated uninfected control ($P \leq 0.05$ and $P \leq 0.001$ respectively). The bursa:body weight ratio was significantly lower in the unvaccinated MDV-infected group when compared to all vaccinated treatments and unvaccinated uninfected control group ($P \leq 0.05$ and $P \leq 0.001$ respectively). The bursa:body weight ratio was also significantly lower in vaccinated MDV-infected and empty vector treated groups when compared to unvaccinated uninfected group ($P \leq 0.05$), however, the ratio in the rChIFN-γ administered group was not significantly lower when compared to uninfected controls ($P > 0.05$).

**MDV genome load and MDV replication in spleens of chickens**

Total cellular DNA was isolated from spleen tissue and MDV genome load was quantified in spleen samples and compared between birds with and without lesions within each treatment group. Initial screening of spleen DNA from the uninfected control group determined that all controls were MDV-free. DNA originating from infected birds was further analyzed by real-time PCR and the data are shown in Fig 8a. MDV genome was quantifiable in all groups, however significantly less genome load was observed in the groups without lesions ($P \leq 0.05$) when compared to chickens belonging to the
unvaccinated MDV-infected group. The birds with lesions in the vaccinated groups had a higher MDV genome load when compared to groups without lesions ($P > 0.05$).

MDV replication was assessed by measuring the expression of MDV-meq and vIL-8 genes in the spleen tissue. Expression of MDV-meq and MDV-vIL-8 was quantified relative to β-actin expression, and is illustrated in Fig 8b and 8c respectively. Among all the birds without lesions in the vaccinated groups, meq transcripts were significantly lower compared to the unvaccinated MDV-infected group ($P \leq 0.001$). The birds with lesions in the vaccinated MDV-infected group also had significantly lower level of meq expression when compared to the unvaccinated MDV-infected group ($P \leq 0.05$). MDV-vIL-8 also had a trend similar to that of MDV-meq, being expressed at lower levels in the chickens without lesions compared to those with lesions, but unlike MDV-meq, the lower expression was not statistically significant.

**Cytokine expression in spleen of chickens**

The expression of IFN-γ, IL-10, IL-18 and IL-6 in spleens of all the treatment groups was analyzed and is illustrated in Fig 9a-d, respectively. There was significant up-regulation in the expression of IFN-γ in all the chickens with lesions compared to the control group (unvaccinated uninfected) (Fig. 9a). There was also a significant increase in IFN-γ expression in spleens of birds without lesions in the pcDNA:chIFN-γFLAG administered chickens that were vaccinated and MDV-infected compared to uninfected control birds ($P \leq 0.05$). Between the infected groups, however, there was no statistically significant difference in the expression of IFN-γ. Unvaccinated MDV-infected chickens had significantly higher expression of IL-10 in comparison to all vaccinated and control
groups \((P \leq 0.05)\) (Fig. 9b). The birds with lesions from the empty vector treated group also had significantly higher expression of IL-10 when compared to the control group \((P \leq 0.05)\). However, all the vaccinated groups, irrespective of presence or absence of tumours, had a lower expression of IL-10 compared to the unvaccinated challenged group. The expression of IL-18 mRNA was somewhat suppressed in the vaccinated groups that displayed no lesions compared to the groups with lesions with a significantly lower expression in the pcDNA:chIFN-\(\gamma\)FLAG treated birds \((P \leq 0.05)\). The birds without lesions in the pcDNA:chIFN-\(\gamma\) treated group also had significantly lower expression of IL-18 when compared to unvaccinated MDV infected group (Fig. 9c). IL-6 mRNA levels followed a somewhat similar pattern to that of IL-18, with low expression seen in the birds without lesions when compared to with lesions and an up-regulation in the infected only group (Fig. 9d), however, the differences were not statistically significant \((P > 0.05)\).

**Discussion**

It is widely known that some host cytokines, such as IL-1, IL-2 and IFN-\(\gamma\), act as an adjuvant and enhance immune responses elicited by vaccine formulations. One of the most widely used cytokines in adjuvant studies is IFN-\(\gamma\), which activates antigen presenting cells, up-regulates MHC class I and II expression on the surface of APC, promotes differentiation of naive T cells to become Th1 cells and, in general, is a main contributor to cell-mediated immune responses (Tovey and Lallemand 2010). In the present study, the effects of recombinant chicken IFN-\(\gamma\) on enhancing immunity elicited by HVT vaccine against the very virulent MDV-RB1B strain were examined. While
HVT is effective against virulent MDV strains, it is less protective against very virulent strains, such as RB1B. Therefore, it is important to develop novel vaccines that provide better protection against very virulent strains of MDV. The results of the present study indicate that co-administration of a plasmid encoding IFN-γ and HVT vaccine can provide enhanced protection as demonstrated by a reduction in the occurrence of tumour development following infection with vvMDV. In addition, we have demonstrated that the immunity conferred by the vaccine was associated with lower viral replication and significant reduction in MDV genome load.

IFN-γ is a pleiotropic cytokine that plays key roles in modulating immunity to infectious diseases. The mature chicken IFN-γ is a 16.8 kDa protein and has been well characterized and shown to be functionally similar to its mammalian counterpart (Digby and Lowenthal 1995; Lowenthal et al., 1995). In the present study, when chickens were co-administrated with pcDNA:chIFN-γFLAG and HVT, a significant reduction in tumour incidence was observed, signifying the protective role of IFN-γ in immunity against MD. Given that all chickens in the infected only group (MDV-RB1B) developed tumours by 21 d.p.i, we did not extend the challenge trial beyond day 21. Therefore, we cannot formally rule out the possibility that the birds with no lesions would have remained tumour-free, had we kept them longer. Therefore, the combination of IFN-γ and HVT may have only delayed the onset of tumour formation. However, based on the data presented here and considering the highly virulent nature of the virus used in this study, it is plausible to suggest that addition of IFN-γ to the vaccine had significantly reduced morbidity and, perhaps, severity of disease.
There are at least two possible mechanisms that may be involved in immunity conferred by HVT+ IFN-γ: First, IFN-γ stimulates immune system cells and expands antiviral adaptive responses elicited by the vaccine formulation. For example, it has been demonstrated that administration of IFN-γ in the form of plasmid DNA enhances immunity against viruses and tumours by activation cytotoxic T cells and steering the response towards a T helper (T\textsubscript{H1}) 1 like response (Iida et al., 2004; Ohlschlager et al., 2009). IFN-γ can also up-regulate expression of major histocompatibility complex (MHC) on the surface of tumour cells, hence rendering them more susceptible to recognition by CD4+ or CD8+ T cells (Kennedy and Celis 2008). Second, IFN-γ can directly or indirectly inhibit MDV replication. In support of this scenario, IFN-γ has been shown to activate macrophages to produce NO (Lillehoj and Li 2004), which in turn inhibits replication of various viruses including MDV (Lee 1979; Djeraba et al., 2000; Xing and Schat 2000a), NDV (Yeh et al., 1999), hepatitis B virus (HBV) (Suri et al., 2001) and DHBV (Schultz and Chisari 1999; Long et al., 2005). Importantly, we have shown here that the recombinant chicken IFN-γ expressed by plasmid DNA stimulates NO production from chicken macrophages. If indeed the plasmid-derived IFN-γ had exerted anti-viral activities in our study, this must have occurred at early stages of virus replication, because by day 21 there was no significant association between IFN-γ expression and genome load. In addition to antiviral activities, NO also has anti-tumour activities (Bogdan 2001; Blanchette et al., 2003), which in the present study might have reduced the incidence of tumours or delayed the onset of tumour formation in MDV-infected birds. The inhibitory effects of IFN-γ on viral replication and maintenance of latency have also been seen in mammals where mice deficient in IFN-γ or its receptor
have an elevated number of cells harbouring viruses reactivating from latency, producing infectious particles and large vessel vasculitis (Weck et al., 1997; Steed et al., 2006). Other studies have provided evidence of CD8+ T cells employing IFN-\(\gamma\) in maintenance of HSV-1 latency (Decman et al., 2005), suggesting that the two mechanisms described above can be intertwined. This has led us to conclude that the administration of rChIFN-\(\gamma\) encoding plasmid in our study enhances antiviral immunity induced by HVT. The exact underlying mechanisms by which rChIFN-\(\gamma\) exerts its function in the context of the present study require further examination and these experiments are underway.

In our study, the vaccine-conferred immunity was associated with significantly lower MDV genome copy numbers in the spleen which also correlated with lower expression of MDV-meq and vIL-8 genes. The above viral genes are associated with the different phases of MDV replication cycle. MDV-vIL-8 is a chemokine-like molecule that is associated with the cytolytic phase of the virus and has been shown to play a role in attracting T cells to the site of infection, in which the MDV-infected B cells are undergoing cytolysis (Liu et al., 1999; Parcells et al., 2001; Cui et al., 2004), whereas MDV-meq is associated with transformation and may also play a role in latency and anti-apoptotic properties of the virus (Jones et al., 1992; Liu et al., 1997). MDV reactivates from latency and causes a late cytolytic phase around 14 d.p.i (Calnek 1986), which may result in replication of the virus; hence higher expression of meq and vIL-8, in birds with lesions, at these time points is expected. Other studies have also shown similar significant up-regulation in spleen, lung and bursa of Fabricius of birds infected with MDV-RB1B (Abdul-Careem et al., 2007; Abdul-Careem et al., 2008a; Abdul-Careem et al., 2009a).
The expression of chIFN-γ was significantly elevated in spleens of chickens with lesions. However, an elevated expression of IFN-γ was also observed in the vaccinated chickens that had no lesions, which had also received pcDNA:chIFN-γFLAG. We could not determine whether this elevated level of IFN-γ was plasmid-derived or was produced as a native molecule by host cells or was a combination of both. It is possible that the administration of the chIFN-γ encoding plasmid may have contributed to an increase in endogenous expression of IFN-γ, in a positive feedback manner. Furthermore, we were able to detect the presence of pcDNA:chIFN-γFLAG plasmid DNA up to 7 days after intra-muscular administration in skin, muscle, bursa of Fabricius and spleen of treated chickens (data not shown). By day 21, we were still able to detect the presence of plasmid DNA in muscle and, to a lesser extent, spleen of treated birds (data not shown). Overall, it is plausible that an early increase in IFN-γ in spleens of the plasmid-administered birds may be an explanation for the low tumour incidence in this group, since IFN-γ may drive the virus into an early latency, similar to the scenario seen in resistant versus susceptible birds (Kaiser et al., 2003).

IL-10 plays a significant role in regulating the host immune response and is known to counteract the effects of IFN-γ (Endharti et al., 2005). In the present study, IL-10 expression was significantly induced in the unvaccinated RB1B infected group, in agreement with other studies (Abdul-Careem et al., 2007; Parvizi et al., 2009a; Parvizi et al., 2009b). The expression of IL-10 was also up-regulated to a lesser extent in some of the vaccinated birds that had lesions. This up-regulation in cytokine expression may be indicative of a strategy used by MDV to subvert host immune responses (Buza and
Burgess 2007), similar to what has been observed in Epstein Barr virus (EBV) infection (Marshall et al., 2003). In general, the results from IL-10 expression suggest that IL-10 expression is significantly reduced in the vaccinated chickens with and without lesions, suggesting that lower expression of IL-10 is not correlated with protection against MD.

Here, we also evaluated the expression of IL-18 and IL-6, proinflammatory cytokines shown to have an important role in the modulation of immune responses. Importantly, the lower expression of these cytokines is associated with genetic resistance against MD and vaccine-induced protection (Kaiser et al., 2003; Abdul-Careem et al., 2007). In agreement with these studies, we also observed lower expression of IL-6 and IL-18 in chickens vaccinated that had no lesions compared to unvaccinated MDV-infected chickens, confirming an inverse correlation between the expression of these cytokines and vaccine-conferred protection.

In conclusion, the findings in this study support the notion that co-administration of pcDNA:rChIFN-γ and HVT vaccine reduces tumour incidence after challenge with a vvMDV. In addition, the results demonstrate that virus replication and viral load are significantly reduced in the chickens that display no gross lesions. In the absence of any significant side effects, such as weight loss or toxicity, the use of IFN-γ as an adjuvant could be considered to enhance the protective efficacy of vaccines against Marek’s disease. However, prior to embarking upon any commercial vaccine formulations containing IFN-γ, a cost-benefit analysis would be required. We anticipate that expression of IFN-γ by a recombinant HVT may provide a cost-effective method for delivery of this cytokine.
Table 2: Tumour incidence at 21 days post infection. n = number of birds/group. EV = Empty vector

<table>
<thead>
<tr>
<th>Groups</th>
<th>Trial 1 Tumour Incidence %</th>
<th>n</th>
<th>Trial 2 Tumour Incidence %</th>
<th>n</th>
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<td>100</td>
<td>6/6</td>
<td>100</td>
<td>10/10</td>
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<td>RB1B+HVT</td>
<td>66.6</td>
<td>4/6</td>
<td>80</td>
<td>8/10</td>
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<td>-</td>
<td>77.7</td>
<td>7/9</td>
</tr>
<tr>
<td>R+H+ 1 μg pcDNA:chIFNγ</td>
<td>33.3</td>
<td>2/6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R+H+ 5 μg pcDNA:chIFNγ</td>
<td>20</td>
<td>1/5</td>
<td>33.3</td>
<td>3/9</td>
</tr>
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Figure 6. Expression of recombinant chicken IFN-γ and assessment of its bioactivity.

a) Real-time RT-PCR analysis of chIFN-γ expression in negative control cells, black bars, mock-transfected cells, white bars, (transfected with empty vector) or cells transfected with the recombinant plasmid, hatched bars, (pcDNA:chIFN-γFLAG) at three different time points; 24, 48 and 72 hours post-transfection. Data shown are representative of three replicates. * = significantly higher compared to the gene expression in control or empty vector transfected groups (P ≤ 0.05). b) Western blot analysis of FLAG tagged rChIFN-γ using anti-FLAG M2 mAb. Culture supernatant from control DF-1 cells (Lane 1), transfected with pcDNA 3.1 empty vector (Lane 2) and pcDNA:chIFN-γFLAG was analyzed (Lane 3). MW is the molecular weight marker c) NO synthesis in HD11 macrophage cells treated with supernatants of DF-1 cells transfected with pcDNA:chIFN-γFLAG. Triplicate HD11 samples were treated with positive control (1.25 μg purified rChIFN-γ expressed using a baculovirus), culture supernatants from DF-1 transfected cells, culture supernatant of empty vector transfected DF-1 cells, or negative control (culture medium). Cell activation was measured by nitrite accumulation in culture supernatant and concentration (μM) was determined by comparisons against a standard curve. Error bars represent standard error of the mean.
Figure 7. Ratios of spleen or bursa of Fabricius to body weight (S:BW and B:BW respectively) among the various treatment groups from both trials.

Treatments groups include infected only (RB1B), vaccinated MDV-infected (R+H), vaccinated MDV-infected with pcDNA:chIFN-γFLAG (R+H+5µg P) and vaccinated MDV-infected with empty vector (R+H+5µg EV). The error bars represent the standard error of the mean. *= significant difference in ratio when compared to uninfected controls ($P \leq 0.05$), ** = significant difference when comparing to uninfected control ($P \leq 0.001$).
Figure 8. MDV genome load and viral gene transcripts in the spleen of chickens from the second trial at 21 d.p.i.

Mean MDV genome load (a), meq mRNA (b), and vIL-8 mRNA (c) expression relative to β-actin mRNA expression are presented and the error bars represent standard error of the mean. MDV genome copies were calculated based upon 100 ng of spleen tissue. Black bars represent birds without lesions, and white bars with lesions. * = significantly lower expression when compared to unvaccinated MDV infected group (RB1B) ($P \leq 0.05$). **= significantly lower expression when compared to unvaccinated MDV-infected group (RB1B) ($P \leq 0.001$).
Figure 9. Expression of various cytokine genes in the spleen tissue of chickens infected, vaccinated and administered recombinant plasmid from the second trial at 21 d.p.i: a) IFN-γ, b) IL-10, c) IL-18, and d) IL-6.

Target and reference gene expression was quantified by real-time RT-PCR and expression is presented relative to β-actin expression and normalized to a calibrator. RB1B group did not have any birds without lesions. Error bars represent the standard error of the mean. a = significant up-regulation of IFN-γ when comparing birds with lesions to control birds ($P \leq 0.05$). b = significant up-regulation of IFN-γ mRNA expression comparing birds without lesions to negative control birds ($P \leq 0.05$). c = significant up-regulation of IL-10 mRNA when compared to unvaccinated uninfected birds ($P \leq 0.05$). d = significant up-regulation in birds with lesions compared to unvaccinated uninfected group ($P \leq 0.05$). e = significant down-regulation of IL-18 seen in the plasmid-treated group without lesions when compared to unvaccinated MDV-infected group. f = significant down-regulation when comparing birds with lesions and without lesions within the plasmid-treated group.
Acknowledgments

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CHAPTER 4

DOWN-REGULATING OF CHICKEN INTERFERON-GAMMA EXPRESSION USING THE SMALL INTERFERING RNA TECHNIQUE

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\textit{In preparation for submission with chapter 5}
Abstract

Interferon (IFN)-γ is a key molecule involved in induction of immune response in mammalian species. However, there is limited information available about the function of this cytokine in the avian immune system. To gain a better understanding of the biological relevance of IFN-γ in chicken immunity, loss-of-function studies may be conducted. Several techniques may be employed for down-regulating expression of host genes, including techniques that take advantage of the RNA interference (RNAi) phenomenon. RNAi is induced by small interfering RNA (siRNA), which can trigger sequence-specific gene silencing. However, sequence specificity and delivery of RNAi molecules remain critical issues. To determine whether RNAi could down-regulate expression of chicken interferon-gamma (chIFN-γ) in vitro, three 27-mer dicer substrate RNA (DsiRNA) were selected based on the coding sequence of this cytokine and transfected into a chicken fibroblast cell line expressing chIFN-γ. The expression of chicken IFN-γ transcripts was down-regulated by a cocktail containing all three siRNAs at 10nM concentration up to 72 hours post-transfection. To ensure that no off-target effects had been triggered by RNAi, the expression of chicken 2’, 5’-oligoadenylate synthetase (OAS) and interferon-alpha (IFN-α) was also examined. The data presented here suggest that the combination of three siRNAs was effective at down-regulating chicken IFN-γ expression.

Keywords: Chicken cells; RNA interference; small interfering RNA; Interferon-gamma; DF-1
Introduction

Cytokines are small pleiotropic proteins that modulate the host response to infection as well as mediate signaling between cells. These are soluble, low molecular weight polypeptides and glycoproteins produced by several cell subsets, including T cells, B cells, macrophages and dendritic cells in response to recognition of pathogens or their products (Kogut 2000). Interferons (IFNs) are a group of cytokines that play important roles in induction and regulation of immune response. Some IFNs have anti-viral activities and are produced in response to microbial infections (Goodbourn et al., 2000). The two major classes of IFN are type I (which includes IFN-α and β) and type II (which includes IFN-γ) (Kaiser et al., 2005). In addition to its anti-viral functions, chicken IFN-γ has been shown to be intimately involved in induction of T cell responses and its expression is correlated with vaccine-induced immunity (Lowenthal et al., 1995; Weining et al., 1996; Song et al., 1997; Haq et al., 2010). However, there is paucity of information about the function of IFN-γ in chickens and its causal relationship with immunity against pathogens in avian species. Therefore, to further examine the importance and relevance of IFN-γ in the chicken immune response, developing methodologies to knockdown this cytokine is warranted.

RNA interference (RNAi) is a naturally occurring mechanism in plants and animals that can be used as a molecular tool to target and suppress transcripts in a sequence-specific mechanism leading to transcript degradation or translational inhibition (Fire et al., 1998). RNAi has been employed as a tool to examine the function of specific genes and their protein products in various biological pathways. RNAi utilizes small interfering RNAs (siRNA) that can be transfected into cells or delivered via DNA-based
vectors, which upon expression are processed intracellularly to 19-21 nucleotide siRNAs (Grimm 2009). Small interfering RNAs mimic the cleavage products of double stranded RNA (dsRNA) and generally bind to and activate the RNase III–family nuclease Dicer which processes them into mature siRNA. The mature siRNAs then interact with a multi-protein RNA-induced silencing complex (RISC) and direct sequence-specific, homology-dependent gene silencing through cleavage and degradation of the mRNA. RNAi can potentially induce off-target effects through host defence mechanisms. Detection of foreign dsRNA initiates the transcription of type I IFNs as well as activation of receptors to detect dsRNA including dsRNA responsive kinase (PKR) and a family of oligo-adenylate synthetase enzymes (OAS) (Sledz et al., 2003).

Several studies have utilized the siRNA approach to study the role of various cytokines. For instance, Sidahmed and Wilkie (2007) demonstrated the use of antisense oligonucleotides (ODN) to inhibit the expression of interleukin (IL)-10 and IFN-γ in pig cells. Recently a study by Li et al. (2011) used a plasmid expressing an shRNA targeting murine IL-23. Silencing IL-23 decreased the levels of IL-17, IL-4 and IgE in serum and inhibited the development of asthma in mice. Chen et al. (2010) showed that knockdown of IL-2 led to prolonged liver allograft survival. In chickens, RNAi has been used for down-regulating immune system genes in vitro including inducible nitric oxide synthase gene (iNOS), Toll-like receptor (TLR)3 and nuclear factor kappa B (NF-κB) (Cheeseman et al., 2008; Karpala et al., 2008; Chiang et al., 2009). This approach has also been used to study the genes that play crucial roles in developmental processes in the chick embryo (Pekarik et al., 2003; Dai et al., 2005; Harpavat and Cepko 2006).
addition, the RNAi technology has been employed to inhibit viruses in chicken cells and
tissues (Chen et al., 2008; Lambeth et al., 2009).

In the present study, we hypothesized that the expression of chicken cytokines,
more specifically IFN-γ, can be down-regulated in cultured cells using the RNAi
technology. This will pave the way for future applications of this system to interrogate
fundamental questions about the importance of IFN-γ in the chicken immune system.

Materials and Methods

Cell culture

An immortal chicken fibroblast cell line, DF-1, was maintained in Dulbecco’s
modified Eagle’s medium (DMEM; Invitrogen, Burlington, ON, Canada) supplemented
with 10% fetal bovine serum (FBS), 1% chicken serum (CS) and 100 μg/mL of penicillin
and streptomycin at 37°C and 5% CO₂. Chicken macrophage cell line, HD11, was
maintained in RPMI 1640 medium (Invitrogen, Burlington, ON, Canada) supplemented
with 10% FBS, 2mM glutamine, 100 μg/mL penicillin and streptomycin at 41°C and 5%
CO₂.

Plasmid DNA construct and siRNA’s

The process for construction of the recombinant expression vector
pcDNA:chIFNγFLAG has been described previously (Haq et al., 2011). Briefly, chicken
IFN-γ coding sequence (CDS) was amplified by PCR using cDNA as template. Primers
were designed to amplify the CDS in addition to the 1xFLAG tag. Amplified products
were then cloned into the expression vector pcDNA 3.1. Orientation and sequence of the
insert were confirmed using restriction digestion and plasmid sequencing. Three Dicer-
substrate RNAs (DsiRNA) to target chicken IFN-γ CDS were designed and supplied by Integrated DNA Technologies, (Coralville, IA, USA), suspended to 20µM final concentration and stored at -80°C. Individual specific siRNA directed against chicken IFN-γ were (5’-3’), i) GGCGUGAAGAAGGUGAAAGAUAUCA; ii) GCAAGUAGUCUAAAUCUUGUUCAAC; iii) CGAUGAACGACUUGAGAAUCC AGCG. A universal negative control DsiRNA (NC-1) and a DsiRNA targeting eGFP (EGFP-S1 DS) were tested, and one was selected for later use. As a positive control the cloned expression vector, pcDNA:chIFN-γFLAG, was transfected, and as a negative control a set of PEI-treated cells (without expression plasmid or siRNAs) were run in parallel with all experiments. Design of DsiRNAs first involves site selection, which is performed using an algorithm that uses novel Dicer-substrate specific design rules. Sequences that pass this stage are next screened to minimize the potential for cross-hybridization and off-target effects (Smith-Waterman analysis) and sites that include known SNPs or alternatively spliced exons are eliminated. Finally, the local mRNA secondary structure is modeled to avoid areas with a high level of predicted structure (Kim et al., 2005).

Transfection

The recombinant plasmid, pcDNA:chIFN-γFLAG and siRNA’s were used for transfection. DF-1 cells were cultured in 6-well plates (1x10^6 cells per well) for 24 hours prior to transfection. Cells were transiently transfected using polyethylenimine (PEI; Polysciences Inc.) according to the manufacturer’s instructions. Cells were transfected or co-transfected with 2µg pcDNA:chIFN-γFLAG together with IFN-γ siRNA’s in serum-
free medium with or without IFN-γ siRNA or non-target siRNA controls and incubated for 6-8 hours at 37°C. To assess the effect of DsiRNAs, cells were transfected with 10nM, 1nM or 0.1nM chIFN-γ or 10nM non-target DsiRNA. Subsequently, the transfection medium was removed, cells were washed with PBS and fresh culture medium was added to the cells. Samples were collected at 24, 48 and 72 hours post-transfection. All transfections were done in triplicates.

**Nucleic acid isolation, reverse transcription and quantitative real-time PCR**

To measure expression levels of various genes, total RNA was harvested from DF-1 cells using TRIzol reagent (Invitrogen Inc.) according to the manufacturer’s protocol with the addition of 10 µg of glycogen (Invitrogen Inc.). Total RNA was then treated with DNase using the DNA-free kit (Ambion, Austin, TX). Complementary DNA (cDNA) was prepared from 1 µg of DNase-treated total RNA by reverse transcription using MMLV reverse transcriptase and oligo(dT)$_{12-18}$ primers (SuperScript™ First strand synthesis system, Invitrogen Inc.) according to the manufacturer’s instructions. Real-time RT-PCR was performed in a LightCycler 480 instrument (Roche Diagnostic, Laval, QC, Canada) in a reaction volume of 20 µL using SYBR Green 1 Master Mix (Roche Diagnostics). In addition, the reaction consisted of 0.25 µM of each primer and 5 µL of 1:10 dilution of cDNA. The PCR conditions included a denaturing step at 95°C for 10 min, and subjected to 40 cycles of 95°C for 10s, 60°C for 5s, 72°C for 10s followed by 72°C for 5 min. The PCR positive control consisted of 10 ng of purified pcDNA:chIFN-γFLAG as template. Expression levels were normalized to β-actin. The ratio of chicken
IFN-γ and β-actin mRNA expression was used to calculate relative reduction of IFN-γ expression.

**Chicken IFN-γ ELISA**

The concentration of IFN-γ in cell culture supernatants was assessed using a commercial chicken IFN-γ ELISA (Invitrogen) according to the manufacturer’s instructions. The concentration of IFN-γ was calculated based upon comparison with a standard curve generated with known amounts of recombinant chicken IFN-γ protein.

**Data and statistical analysis**

Data were collected and expressed as mean relative expression to normalized β-actin or, for the siRNA experiments, was converted to a percentage relative to the positive control group. Results are depicted as the mean ± standard error of the mean of three independent experiments. Significance of difference between the means was determined using the Student’s t-test in GraphPad Prism software (GraphPad software, San Diego, CA), with $P \leq 0.05$ taken to indicate significance between treatments at time points as indicated.

**Results**

**In vitro expression of rChIFN-γ**

In order to construct a plasmid that expressed chicken IFN-γ, the chIFN-γ CDS was cloned into the pcDNA 3.1 expression vector and the resulting plasmid was named pcDNA:chIFN-γFLAG. Sequencing revealed that the insert was identical to database reference sequences (Ensembl transcript: IFNG_CHICK, and GenBank NM_205149). To test whether this plasmid was functional and capable of expressing chIFN-γ, DF-1
cells were transfected and expression of IFN-γ transcripts was assessed using RT-PCR (Figure 10). Cells transfected with pcDNA:chIFN-γFLAG showed expression of IFN-γ mRNA up to 72 hours post-transfection, which was the last observation time point, while the control groups which included untreated cells and cells transfected with empty pcDNA 3.1 vector did not have detectable levels of IFN-γ transcripts. FLAG tagged IFN-γ protein was detected by western blotting, and bioactivity of the rchIFN-γ was assessed by treating HD11 cells with culture supernatant to analyze for nitric oxide (NO) production by the Griess reagent system (Haq et al., 2011).

Silencing of IFN-γ expression using RNAi

To evaluate the inhibitory effect of RNAi on chicken IFN-γ gene expression in vitro, we chose to co-transfect DF-1 cells with pcDNA:chIFN-γFLAG and a pool of three siRNAs that target the IFN-γ coding sequence. IFN-γ expression in cells transfected with pcDNA:chIFN-γFLAG was compared against cells co-transfected with the pooled IFN-γ siRNA oligonucleotides at three concentrations (10nM, 1nM and 0.1nM) or 10nM GFP targeting siRNA. Co-transfection with the pool of siRNA at three concentrations demonstrated significant reduction in IFN-γ expression using 10nM at all three time points. However, using 1nM and 0.1nM, a significant reduction was observed only at 24 and 48 hours post-transfection (Fig 11a). We then examined potential bystander effects of siRNA when administered at different concentrations. Some studies have suggested that under certain conditions, siRNAs can activate the host interferon system, potentially complicating the interpretation of RNAi gene knockdown experiments. Therefore, to examine if there was a non-specific host interferon response elicited, we examined the
expression of $2', 5'$-oligoadenylate synthetase (OAS). OAS is a dsRNA-dependent synthetase which activates endoribonuclease RNAse L to degrade ssRNA (Player and Torrence 1998). There was no significant difference in expression of $2', 5'$-OAS among groups, confirming that the siRNA oligonucleotides did not have off-target activities in a wide range of concentrations (Figure 11b).

To confirm the dose titration study and also to gain more insights into the ability of siRNA to down-regulate IFN-$\gamma$, an experiment was conducted with the dose that gave the best results in the previous experiment. When using 10nM siRNA concentration, the pool of three siRNAs was highly effective at 24 hours post-transfection, leading to a significant reduction in IFN-$\gamma$ transcripts up to approximately 80%, whereas the use of either non-target controls did not result in any significant reduction in the expression of IFN-$\gamma$ ($P \leq 0.05$) (Figure 12a). Chicken IFN-$\gamma$ in the culture supernatant was measured by ELISA from the 10nM treatment group for comparison of the protein and mRNA expression levels. Significantly lower amount of rChIFN-$\gamma$ was seen at 24 and 48 hour post-transfection when compared to both non-target and plasmid only samples. The reduction in the amount of IFN-$\gamma$ at 72 hours in the siRNA group approached significance when compared to the amount of IFN-$\gamma$ in non-target and plasmid only group ($P = 0.051$ and 0.052 respectively) (Figure 12b). Subsequently, we examined bystander effects of the siRNA cocktail by measuring transcripts of OAS and IFN-$\alpha$. No significant increase in OAS or IFN-$\alpha$ transcripts was detected in cells treated IFN-$\gamma$, siRNAs, or non-target controls when compared to non-transfected controls (Figure 13a and 13b).
Discussion

The present study demonstrated the successful knockdown of chIFN-γ using siRNA molecules designed to target the coding sequence of this cytokine. The host recognition of invading pathogens or response to vaccines may trigger the secretion of cytokines that play critical roles required for elicitation and regulation of the host immune response. A better understanding of the role of these cytokines in immunity requires loss-of-function studies. Application of RNAi, which can be induced by siRNA or short hairpin (shRNA) molecules is a recently introduced approach for sequence-specific knock-down of a target gene via mRNA degradation (Elbashir et al., 2001). Specificity of a siRNA depends only on the sequence of its nucleotides and is achieved by hybridization of the antisense (guide) strand and the target transcript. Traditionally, siRNAs are chemically synthesized as 19-21mers, however, these bypass the interaction with Dicer protein, a component of the RISC complex. In the present study, we used 27-mer dsRNA, which act as substrate for Dicer to generate mature 21-mer siRNA within the cell. These longer RNAs are processed by Dicer into 21-mer siRNAs in a predictable manner, with increased resistance to nuclease activity and increased potency which is thought to arise from the participation of Dicer in RISC formation (Kim et al., 2005).

RNAi technology has been widely used in the chick embryo to address questions related to developmental biology of the organism (Pekarik et al., 2003; Dai et al., 2005; Harpavat and Cepko 2006). There are at least three reports of the in vitro use of RNAi for down-regulating expression of chicken immune system genes involving iNOS, TLR3 and NF-κB (Cheeseman et al., 2008; Karpala et al., 2008; Chiang et al., 2009). There has been no report of the utility of this technology to modulate expression of chicken
cytokines, including IFN-γ. Here, we report inhibitory activity of three siRNAs against IFN-γ using a co-transfection model system. The results presented here demonstrated that IFN-γ mRNA expression can be down-regulated as early as 24 hours and up to 72 hours after treatment of cells with siRNA oligonucleotides.

In addition to down-regulation of IFN-γ transcripts, there was a reduction in the amount of IFN-γ protein present in the culture supernatant of IFN-γ expressing cells that were transfected with siRNA. The manufacturer of the DsiRNA oligonucleotides used in this study (Integrated DNA Technology, Coralville, IA, USA) as well as previously published studies (Wu et al., 2004) recommend that target protein levels be analyzed around 48 to 72 hours post-transfection. In the present study, however, down-regulation of IFN-γ protein was only significant at 24 and 48 hours after treatment, consistent with our observations for IFN-γ transcripts. The slight discrepancy between the temporal pattern of protein down-regulation in the present study compared to that of the previous studies may be related to the half-life of IFN-γ or the nature of the cell culture system we used. Here, we used cells transfected with a plasmid that constitutively expressed chicken IFN-γ. The CMV enhancer-promoter used in the expression plasmid for driving the expression of IFN-γ is used for high-level transgene expression and is considered a strong promoter compared to other promoters (Schlabach et al., 2010). Therefore, IFN-γ expression under the influence of this promoter might have overpowered the siRNAs. Strong enhancers / promoters such as CMV and CAG have been used for stable expression due to their ability to induce immediate and strong transcription of transgenes (Ward and Stern 2002; Nitta et al., 2005). Furthermore, it has been demonstrated in several studies that siRNA effects are temporary. For example an in vitro study
examining the kinetics of siRNA gene silencing has reported mRNA and protein levels returning to the normal range 1-2 days after transfection (Bertrand et al., 2002). In another study, the effects of siRNA remained for less than 1 week within dividing cell-lines (Bartlett and Davis 2006). In our experiments, the results were similar, with maximum knock-down that lasted 24-48 hours and thereafter diminishing in the lower dose groups.

We then asked whether modulation of IFN-\(\gamma\) expression by siRNA constructs was dose-dependent. The finding revealed that optimal siRNA concentration for IFN-\(\gamma\) knockdown was achieved using 10nM siRNA concentration, and the results revealed a dose-dependent knockdown efficiency. A similar in vitro study, using various human genes, observed >90% mRNA knockdown by 24 hours post-transfection when using duplexes at 10nM concentration (Wu et al., 2004).

One aspect of the siRNA technique that requires further attention is the off-target effects of these molecules. Studies have reported that treatment of cells with siRNAs may result in IFN-mediated activation of the Jak-STAT pathway. This activation is mediated by the dsRNA-dependent protein kinase PKR and 2’,5’-OAS, which are induced by siRNAs (Sledz et al., 2003). Therefore, the expression of two representative genes of the IFN pathway was examined to address whether there was any measurable off-target effects of treatment of cells with shRNA molecules. The studies presented here did not reveal up-regulation of IFN-\(\alpha\) and 2’,5’-OAS genes, raising the strong possibility that the treatment did not have a major off-target effect. However, a recent report suggests that DF-1 cells may not be able to produce type I IFN in response to 5’-triphosphate siRNA
due to an absence of retinoic acid-inducible gene I protein (RIG-I) in chickens (Barber et al., 2010). In a subsequent study, a chicken Mda5 (ChMda5) homolog was identified and was shown to be involved in the IFN response of chicken cells to dsRNA (Karpala et al., 2011).

Although many methods have been used for suppressing gene expression in vitro and in vivo such as antisense oligonucleotides (Gewirtz 1999) and monoclonal antibody-based techniques (Drewe and Powell 2002), RNAi has distinct advantages when compared to other methods. These include: (1) high mRNA degradation efficiency with just a few copies of dsRNA, (2) specificity: only sequences that match are degraded, (3) RNAi gene silencing provides a fast knockdown of gene expression, though it is short-term unless performing a stable gene silencing using shRNA vectors. A major research area is improving the efficient delivery of the siRNA into target cells. Two different approaches can be used to deliver RNAi: synthetic RNA duplexes can be introduced directly into cells or alternatively, dsRNA can be expressed inside the cell using a plasmid or viral vector. The first direct delivery of siRNA in vivo was by administering short duplexes of naked siRNA (McCaffrey et al., 2002). Some primary obstacles using this approach in vivo include uptake by non-target cells, poor transfection efficiency, nuclease degradation, endosomal trapping and excretion by the body. To overcome these limitations, several groups have developed viral-based vectors to deliver shRNA into host cells (Bell and Brickell 1997; Tomar et al., 2003; Bromberg-white et al., 2004; Chen et al., 2009). In the latter approach, RNAi effects can be sustained for a longer period of time.
The present *in vitro* study provides evidence that synthetic oligonucleotides (DsiRNAs) selected for IFN-γ were able to successfully reduce expression of this cytokine, without stimulating IFN-α or 2′-5′–OAS expression. Further studies are needed to develop a viral delivery system to knockdown chicken IFN-γ expression siRNA *in vivo*, and should include investigation of tissue distribution, amount of recombinant virus required and any side effects triggered by the recombinant virus.
Figure 10. Expression of chIFN-γ in cells transfected with a plasmid containing the coding sequence for chicken IFN-γ.

RT-PCR analysis of RNA extracted at the time points shown post-transfection and loaded onto a 2% agarose gel. Untransfected cells (lanes 1, 4, 7); Cells transfected with empty vector (lane 2, 5, 8) or pcDNA:chIFN-γFLAG (lanes 3, 6, 9). Control lanes: Negative PCR (no cDNA template) and positive PCR (plasmid template) are lanes 10 and 11 respectively. M = 100 bp molecular ladder. Experiment was done 3 times; a representative gel is shown.
Figure 11. *In vitro* gene silencing efficacy of siRNAs against IFN-γ.

DF-1 cells were co-transfected with a plasmid expressing chicken IFN-γ and siRNAs targeting IFN-γ or non-target GFP control siRNAs. Cells were cultured for varying amount of time and sampled for RNA expression. a) Relative reduction in IFN-γ mRNA after co-transfection of plasmid expressing IFN-γ and a pool of three siRNA’s at three concentrations (10nM, 1nM and 0.1nM) or eGFP (non-target) control at 24, 48 or 72 hours post transfection. b) Expression of 2′,5′-OAS was examined for all treatments within each time point. Errors bars represent standard error of the mean and * denotes significance ($P \leq 0.05$) when compared to non-target and positive (plasmid) controls.
Figure 12. *In vitro* gene silencing efficacy of chicken IFN-γ siRNA’s.

DF-1 cells were co-transfected with a plasmid expressing chicken IFN-γ and siRNAs targeting IFN-γ or non-target control siRNAs (NC-1 and GFP). a) Relative reduction in IFN-γ mRNA after co-transfection of plasmid expressing IFN-γ and a pool of three siRNA’s (10nM) or non-target controls (10nM) 24 hours post transfection. b) IFN-γ protein concentrations in the supernatant of transfected cells as determined by ELISA. Supernatants were collected 24, 48 and 72 hours post transfection. Experiments were done in triplicates. Negative group refers to supernatant from mock transfected cells.
Figure 13. Off-target effect of siRNA

Expression of 2', 5'-OAS and IFN-α relative to β-actin was examined for all treatments 24 hours post transfection. Experiments were done in triplicates, and error bars represent standard error of the mean. Negative refers to mock transfected cells, and positive refers to plasmid only group. There was no statistical significance in the expression of 2’, 5’-OAS and IFN-α between treatment groups.
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CHAPTER 5

GENERATION OF RECOMBINANT AVIAN ADENO-ASSOCIATED VIRUS EXPRESSING SHORT HAIRPIN RNA TARGETTING INTERFERON GAMMA AND EFFECT ON MAREK’S DISEASE

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In preparation for submission with chapter 4
Abstract

Interferon (IFN)-γ is a cytokine with a wide array of functions, including direct antiviral activities and the capacity to polarize adaptive T cells. IFN-γ has been shown to be associated with immunity to Marek’s disease virus (MDV) in chickens. The overall objective of this study was to investigate the causal relationship between IFN-γ and vaccine-conferred immunity against MDV in chickens. To achieve this objective, recombinant avian adeno-associated viruses (rAAAV) expressing small interfering RNAs (siRNAs) targeting chicken IFN-γ were constructed. Three siRNA molecules targeting the coding sequence of chicken IFN-γ, which were previously shown to reduce IFN-γ expression in vitro, and a non-target specific control siRNA targeting GFP (eGFP) were selected. A U6 promoter followed by the siRNA sequence in the form of a short hairpin RNA (shRNA) was cloned into the AAV vector, which was then transfected into HEK293 cells for generating and packaging rAAAV:U6shRNA. DF-1 cells were transfected with a recombinant plasmid expressing chicken IFN-γ followed by infection with rAAAV:U6shRNA. Significant suppression of IFN-γ transcript was observed in vitro in cells that were treated with rAAAV, expressing shRNA targeting IFN-γ. In a subsequent study, chickens were administered two doses (10^9 or 10^10 genomic copies) of rAAAV:U6shRNA, and tissue distribution of the virus was examined at 48 hours, 120 hours, 14 days and 26 days post administration, within spleen, bursa, liver, lung and muscle. An MDV challenge trial was then conducted, in which chickens were vaccinated with herpesvirus of turkey (HVT), administered rAAAV:U6shRNA and challenged with MDV (RB1B strain). An increase in the number of birds with tumours was observed in groups that were administered with the recombinant virus; however, the difference was
not significant when compared to control birds. There was no significant difference in MDV genome load in feathers of the birds that were co-treated with the vaccine and rAAAV:U6shRNA compared to vaccinated MDV-infected birds. Our results suggest that avian AAV-based vectors are efficient vectors for the delivery of siRNA into chicken cells. However, administration of the rAAAV expressing shRNA targeting chicken IFN-γ \textit{in vivo} did not seem to abrogate vaccine-induced protection.
Introduction

Marek’s disease (MD) is a highly contagious disease of poultry caused by an oncogenic virus known as Marek’s disease virus (MDV) (Calnek 2001). A number of cytokines have been shown to be associated with immunity against MDV (Haq et al., 2010), of which interferon (IFN)-γ has been shown to play an important role (Xing and Schat 2000b; Abdul-Careem et al., 2007; Haq et al., 2011). Differential expression of cytokines has been extensively investigated using techniques such as microarray and RT-PCR. However, these studies have not elucidated the functional roles played by these cytokines in immunity to MD.

The functional role of cytokines may be studied through gain- and loss-of-function experiments both in vitro and in vivo. A range of loss-of-function techniques are currently available, among which the RNA interference (RNAi) technique is of note. RNAi effects can be mediated through direct administration of short interfering RNA (siRNA) or through expression vectors, including plasmid and viral vectors (Hobel and Aigner 2010). For example, lentivirus and adenovirus-based vectors have been used for delivery of shRNA constructs in various species (Iba 2000; Grimm et al., 2005).

Successful delivery of shRNA constructs can be achieved using adeno-associated virus (AAV) based vectors. Adeno-associated viruses were first discovered in 1965 as a contaminant of simian adenovirus preparations (Gonçalves 2005). The small DNA-containing particles were shown to be antigenically different from adenoviruses (AdV). Replication of these particles only occurred when inoculated simultaneously with AdV, suggesting that these particles behaved like defective viruses. Since then AAV has been
categorized into a separate genus of the *Parvoviridae* family, designated *Dependovirus*, reflecting AAVs dependency on a helper virus for productive infection to occur. Since its discovery, many AAV serotypes have been isolated from human and non-human species, however all serotypes contain a linear single stranded DNA (ssDNA) genome of approximately 5 kb, two open reading frames (ORF: *rep* and *cap*) and inverted terminal repeats (ITR) (Stilwell and Samulski 2003). The ITRs are important elements for initiation of viral replication, integration into the host genome as well as for recombinant virus generation. The ITRs are the only signals necessary in cis for encapsulation of DNA by the structural proteins (McCarty *et al.*, 2004). Various serotypes can infect cells from specific or multiple tissues sources, which is determined by the capsid serotype. Of all the known serotypes, serotype 2 (AAV2) has been the most extensively studied to date. The avian adeno-associated virus (AAAV) was isolated from the Olson strain of quail bronchitis virus, an avian adenovirus (Yates *et al.*, 1973). Avian AAV contains a ssDNA and also requires co-infection with a helper virus for productive infection. Sequence analysis of AAAV has revealed a similar genome size and organization similar to that of other AAVs (Bossis and Chiorini 2003; Estevez and Villegas 2004).

In recent years, extensive research has been conducted to characterize and employ these replication defective paroviruses for the purpose of delivering antigens or shRNA (Estevez and Villegas 2006; Perozo *et al.*, 2008; Wang *et al.*, 2008). In general, the use of AAV is appealing because they are non-pathogenic, have the ability to infect dividing or non-dividing cells, have tissue tropisms, may integrate into the host genome or remain episomal, and can be easily engineered as a vector. The limitation to their use
is the relatively small AAAV genome, restricting the total insert size to ~4kb for optimal packaging.

The goals of this study were to develop an AAAV-based vector expressing shRNA targeting chicken IFN-γ and to utilize this system to knockdown IFN-γ expression so that the biological significance of IFN-γ in immunity against MD can be evaluated.

**Materials and Methods**

**Cell Culture**

Human embryonic kidney cells (HEK 293) were maintained in Dulbecco’s modified Eagle medium (DMEM, Invitrogen, Burlington ON, Canada) supplemented with 10% fetal bovine serum (FBS, Gibco) and 100µg/mL penicillin/streptomycin at 37°C in a 5% CO₂ air atmosphere. An immortal chicken fibroblast cell line, DF-1, was maintained in DMEM (Invitrogen, Burlington, ON, Canada) supplemented with 10% FBS, 1% chicken serum (CS) and 100µg/mL penicillin/streptomycin at 37°C in 5% CO₂.

**Experimental animals**

Specific-pathogen free (SPF) eggs were obtained from Animal Disease Research Institute, Canadian Food Inspection Agency (CFIA, Ottawa, ON, Canada) and hatched at the Arkell Poultry Research Unit, University of Guelph. Hatched chicks were housed in the animal isolation facility at the Ontario Veterinary College, University of Guelph during the experimental period. All animal experiments were approved by the Animal Care Committee, University of Guelph.
**Virus and Vaccine Strains**

Very virulent MDV strain RB1B (passage 9) was kindly provided by Dr. Karel A. Schat (Cornell University) and was used to infect chickens. Chickens were vaccinated subcutaneously on the day of hatch using the recommended dose of HVT (Fort Dodge Animal Health, Division of Wyeth, IA 50501, USA).

**Nucleic acid isolation, reverse transcription and quantitative real-time PCR**

To measure the expression level of IFN-γ and β-actin and to detect virus, total DNA and RNA were extracted from DF-1 cells and feather tips (for MDV genome copy determination) using TRIzol reagent (Invitrogen Inc) according to the manufacturer’s protocol. DNA was extracted from cells directly by addition of TRIzol reagent to the cells. Tissue samples were preserved in RNAlater (Qiagen, Inc) followed by homogenization in 1 mL TRIzol. Following chloroform extraction, the organic phase which contained DNA was separated, washed with 0.1mol/L sodium citrate in 10% EtOH, and dissolved in 8mM NaOH. RNA concentration was measured by spectrophotometry at 260 nm. Total RNA was then treated with DNase using a DNA-free kit (Ambion Inc, Austin, TX) according to the manufacturer’s instructions. Complementary DNA (cDNA) was prepared from 1 μg of DNase-treated total RNA by reverse transcription using MMLV reverse transcriptase and oligo (dT)₁₂-₁₈ primers (SuperScript™ First strand synthesis, Invitrogen Inc.) according to the manufacturer’s instruction. Real-time RT-PCR was performed in a LightCycler 480 instrument (Roche Diagnostics, Laval, QC, Canada) in a reaction volume of 20 μL using SYBR green 1 Master Mix (Roche Diagnostics). In addition, the reaction consisted of 0.25 μM of each
primer and 5 μL of 1:10 dilution of cDNA. Previously published primers were used for IFN-γ and β-actin (Abdul-Careem et al., 2006), and all primers were synthesized by Sigma-Aldrich Canada (Oakville, ON, Canada).

Construction of expression plasmid vector and recombinant avian adeno-associated virus vectors.

An siRNA expression cassette with linker ends of NdeI was made by amplifying the chicken U6 promoter region from the plasmid pRFPRNAiC (ARK-Genomics, UK). The primers used to amplify the cassette incorporated an NdeI restriction site at the 5’ and 3’ end for cloning into the expression vector (Fig. 14). The siRNA expression cassette consisted of a nucleotide sense sequence (identical to the IFN-γ target sequence), followed by a 9-bp loop, an antisense sequence and a stretch of T’s as a polymerase III transcriptional termination signal downstream of a U6 promoter. Briefly, PCR was performed using 10ng of pRFPRNAiC and the specific primers (Table 2). The PCR conditions were as follows: one cycle of 95°C for 5 min, 35 cycles of 95°C for 15s, 68°C for 15s, 72°C for 30s, followed by a final extension at 72°C for 5 min using Platinum Taq polymerase (Invitrogen, Burlington, ON Canada). The amplified product was first cloned into the pDrive cloning vector (QIAGEN). Insertion of the PCR product was verified by selecting colonies screened on LB + Ampicillin agar plates. Selected colonies were grown in 5 mL of LB overnight at 37°C. Plasmid was isolated using GenElute™ Plasmid Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA), and the construct (pDrive + U6shRNA) was verified by RE digestion on an agarose gel and sequencing of the cloned insert.
Vectors for a helper-free recombinant virus production system were generously provided by Drs. C. Estevez and P. Villegas (University of Georgia, GA). The AAAV backbone vector (pEVA3V-LacZ, 8248 bp) which includes the ITRs was digested with restriction enzyme \textit{NdeI} (NEB), to remove most of the original cassette comprising the CMV promoter and the LacZ open reading frame (ORF). The digestion reaction was resolved on a 1% agarose gel, and resulted in 2 bands: 4458 bp and 3790 bp. The larger band, 4458 bp, containing the ITRs was purified using a MinElute Gel purification kit (Qiagen, Inc). The linearized fragment was then dephosphorylated using Antarctic phosphatase (NEB) to prevent re-circularization.

The recombinant vector was constructed by ligating the linearized backbone with the \textit{NdeI} digested insert from pDrive + U6shRNA. Ligation was performed overnight at 16°C in a 10 µL reaction, using T4 DNA ligase (NEB) at a 1:5 plasmid to insert ratio. The ligation mixture was transformed into competent \textit{Escherichia coli} GT116 cells using heat-shock. These cells are a deletion strain of \textit{E. coli}, specifically engineered to support propagation of plasmid DNAs carrying hairpin structures. Briefly, the ligation mixture was added to the competent cells and incubated on ice for 30 min, heat-shocked at 42°C for 45 sec, followed by incubation on ice for 3 min. Then, 500 µL of LB medium were added and incubated at 37°C for 1 hour with shaking. Cells were then plated onto LB + Kanamycin agar plates, and incubated overnight at 37°C. Recombinant plasmid pAAAV:U6shRNA was prepared using a GenEluteTM plasmid purification kit (Sigma-Aldrich, St. Louis, MO, USA), and restriction enzyme digested to confirm ligation and orientation. The plasmids were also sequenced bi-directionally to verify correct insertion and sequence fidelity (Laboratory Services Division, University of Guelph).
Recombinant avian adeno-associated virus (rAAAV) virus generation

Recombinant AAAV viral particles expressing an individual IFN-γ siRNA or eGFP sequence were obtained by co-transfecting 3x10^6 HEK 293 cells with the AAAV backbone vector along with two packaging plasmids, which included the Rep/Cap genes and E2A, E4 and VA genes. Cells were seeded overnight in 10 cm tissue culture dishes and transfected when 70-80% confluent. For transfection, two solutions were prepared: A and B. Solution A, DNA-CaCl₂ mixture, comprised 10µg of pAAAV:U6shRNA, 10µg pRC and 10µg pHelper plasmid, 2.0M CaCl₂ and H₂O. Solution B comprised 500mM HEPES-NaOH (pH 7.1), 2.0M NaCl, 150mM Na₂HPO₄-NaH₂PO₄ (pH 7.0) and H₂O. Solution B was gently added drop-wise to solution A and incubated at room temperature for 30 min, to allow a fine precipitate to form. Then added 1mL DNA precipitate to each 10 cm dish gently, and incubated the cells for 8-10 hours at 37°C. Cells were washed with PBS and culture medium added, and incubated for a further 72 hours. Cell lysate was harvested from tissue culture plates and transferred into 50mL polypropylene tubes. The cell suspension was freeze-thawed three times by transferring the tubes between a dry ice/ethanol bath and a 37°C water bath. The cell lysate was centrifuged for 30 min at 10,000 x g at 4°C. Subsequently, the supernatant was removed and transferred into 50mL Beckman ultra-clear tubes. The supernatant was re-centrifuged for 6 hours at 24,000 x g at 4°C, followed by dilution of the virus pellet overnight at 4°C with 500µL HNE buffer (50mM Hepes (pH 7.4), 0.15M NaCl and 25mM EDTA) per tube. AAAV virus was concentrated through a 20% sucrose cushion (2:1 ratio) and centrifuged for 6 hours at 35,000 rpm and 4°C. The supernatant was carefully discarded and the pellet was dissolved gently in 500µL HNE buffer and left overnight at 4°C to dissolve. rAAAV
stocks were DNase treated, aliquoted and stored in -80°C. The rAAAV viral stocks were
titrated by quantitative RT-PCR, as described by Rohr et al., (2002). The primers
(‘rAAAV U6 titration F and R’, Table 3) used in the reaction were designed to amplify a
180 bp segment of the chicken U6 promoter region contained in the recombinant virus
dNA. For the generation of the standard curve; plasmid DNA containing one copy of the
U6 promoter sequence was serially diluted and used as template for PCR. Serial dilutions
ranged from 3 pg to 30 ng of control plasmid per 2 µL of sample. Viral titres were
recorded as genomic copies.

Cell Transfection and in vitro experiment design

DF-1 cells (1x10^6) were seeded overnight prior to transfection with
pcDNA:chIFN-γFLAG. Stock solution of polyethyleneimine (PEI) (PolySciences,
Eppenheim, Germany) was prepared in water at a final concentration of 1 mg/ml, and
plasmid DNA was diluted to the required concentrations (2 µg). A transfection solution
made with basal DMEM containing plasmid DNA and PEI was prepared and incubated at
room temperature for 15-20 min prior to transfection. DF-1 cells were rinsed twice with
PBS to remove any residual cell culture medium and 200 µL of the DNA-PEI solution
was added directly onto cells and incubated 6 – 8 hours. At this point, the transfection
mix was removed, cells were washed and complete DMEM was added to cells. Purified
rAAAV:U6shRNA-10.10 was then added into the transfected cell culture medium using
three dilution concentrations: 50µL, 5µL or 0.5µL of stock. Cells were collected 24 hours
and 48 hours post-infection for RNA extraction. A rAAAV expressing a sequence
targeting the green fluorescent protein (eGFP) was used as the negative control. Presence
of rAAAV in the cells was detected using primers to amplify a non-coding ‘stuffer’ region between the ITR’s and the coding cassette. All experiments were done in triplicates.

*Experimental design for in vivo experiments*

There were two *in vivo* trials in this study: one to assess the dose and distribution of rAAAV:U6shRNA and the second was an MDV challenge study to determine the effect of knocking down IFN-γ. In the first trial, which was conducted as a pilot study, day old chicks ($n = 16 / \text{group}$) were administered rAAAV:U6shRNA-10.10 with two different doses: Group 1, High dose ($1 \times 10^{10}$ genomic copies), Group 2, Low dose ($1 \times 10^9$ genomic copies) and Group 3, a negative control group treated with PBS ($n = 8$). rAAAV was administered intramuscularly in the right pectoral muscle. The chickens were monitored for any signs of adverse effects induced by the rAAAV. Chickens were euthanized at 48 hours, 120 hours, 14 days and 26 days post-administration, and the following tissues excised and stored in RNAlater (Qiagen Inc): Spleen, Liver, Bursa, and Muscle. Genomic DNA was extracted from approximately 25-50 mg of tissue as described above. Real-time PCR was performed to determine the genome copies of the rAAAV.

The second trial involved vaccination with a commercially available vaccine, herpesvirus of turkeys (HVT), followed by experimental infection with a very virulent MDV strain, RB1B. Based on the results from the pilot study, a high dose ($1 \times 10^{10}$ genomic copies) of rAAAV was administered. There were six groups in this trial ($n = 10 / \text{group}$); one group served as MDV-infected only (Group 1) and another as a negative
control (uninfected, unvaccinated, group 6). The other four groups consisted of vaccinated MDV-infected (Group 2, MDV + HVT), vaccinated MDV-infected and administered all 3 rAAAV:U6shRNA (Group 3, MDV + HVT + rAAAV:IFNγ shRNA), vaccinated MDV-infected and administered control shRNA (Group 4, MDV + HVT + rAAAV:U6eGFPshRNA) and an MDV-infected and administered all 3 rAAAV:U6shRNA (MDV + rAAAV:IFNγshRNA). Briefly, day-old chicks were vaccinated subcutaneously with HVT and administrated rAAAV intramuscularly in the upper third of the right pectoral muscle. At 5 days of age, birds were infected with 250 plaque forming units (PFU) of RB1B intra-abdominally. The chickens were then monitored daily for any clinical signs over a 3-week period. Feather samples were collected from 5 birds per group at each of the following time points: 4, 7, 10, 14, and 21 days post infection (d.p.i). Time points were selected based on an intra-abdominal MDV infection model system (Witter and Schat 2003), which corresponded to important phases of MDV pathogenesis. All birds were euthanized at 21 d.p.i., weighed and examined for the presence or absence of gross lesions. The weight of bursa of Fabricius and spleen was recorded during necropsy for each bird, and the ratios of spleen/bursa of Fabricius to body weight were determined. MDV genome load and transcripts in feathers were quantified using real-time PCR and RT-PCR as described previously (Abdul-Careem et al., 2006a). MDV genome load is described as the number of copies per 100 ng of DNA.
Data Analysis

For tumour incidence data, Fisher’s exact test was used, whereas for other experiments two tailed \( t \)-test and ANOVA were used to identify differences among groups. Comparisons were considered significant at \( P \leq 0.05 \).

Results

Construction of rAAAV vector and generation of rAAAV expressing shRNA

In the present study, we used the chicken U6 promoter to drive expression of siRNA targeting IFN-\( \gamma \) in chicken cells. To construct the recombinant backbone plasmid vector, chicken U6 promoter (pol III) was amplified using a 5’ forward primer upstream of the pol III promoter and a 3’ reverse primer that included the entire shRNA sequence (sense-loop-antisense) followed by a complementary sequence to the 3’ end of the promoter upstream of the +1 transcriptional start site (Figure 14). Recombinant AAAV expressing the shRNA (either targeting IFN-\( \gamma \) or the eGFP sequence) were obtained by co-transfection of HEK 293 cell culture with a plasmid containing the AAAV ITRs including the cloned expression cassette, a helper plasmid and a plasmid with the Rep and Cap genes (Figure 15).

Evaluation of IFN-\( \gamma \) mRNA down-regulation by rAAAV, in vitro

To evaluate the ability of rAAAV expressing shRNA10.10, which targets chIFN-\( \gamma \), to down-regulate expression of IFN-\( \gamma \), this virus was added to chicken fibroblast cells (DF-1) previously transfected with a plasmid containing the coding sequence of chicken IFN-\( \gamma \). Following rAAAV infection, cells were monitored for cell death. The presence of rAAAV was detected using primers which amplify a 198 bp non-coding ‘stuffer’ region
between the ITR’s, using viral extracted DNA (Figure 16a). The above result was further verified by amplifying a segment of the U6 promoter in the DNA obtained from infected cells (data not shown). Based on the PCR results, we noted that cells infected with 50µL of recombinant virus (non-target or target siRNA) had similar band intensities on an agarose gel, whereas cells infected with 5µL or 0.5µL of virus had bands with proportionately lower intensity. No amplification was detected within the untreated cells.

At 24 and 48 hours post-transfection, IFN-γ mRNA expression was determined by quantitative RT-PCR and a reduction of 50-80% in IFN-γ transcripts was observed. These results illustrated that, the expression of IFN-γ was significantly reduced compared to the positive control as well as eGFP (non-target) control, which suggested that the recombinant AAV virus expressing the shRNA targeting chicken IFN-γ could be used to down-regulate IFN-γ mRNA. Based on the IFN-γ mRNA expression results, a dose-dependent silencing ‘trend’ could also be observed (Figure 16b).

Dose and Tissue Distribution analysis of vector DNA

In order to examine tissue distribution of the recombinant AAV:U6shRNA10.10, birds were administered with the virus at 1-day of age by intramuscular (i.m.) injection and their tissues were collected at various time points post-treatment. Quantitative PCR revealed that the virus was present within 48 hours after injection in liver, spleen, lung, bursa of Fabricius and muscle (Figure 17). In chickens that received a lower dose of the virus (1x10^9 genomic copies), viral DNA was detected in muscle tissue up to 14 days post-injection, and in the spleen, liver and lungs up to 5 days post-injection. However, in the chickens that received a higher dose (1x10^{10}
genomic copies), viral DNA was detectable in spleen, liver, and lung up to 26 days post-injection, whereas in the muscle and bursa of Fabricius, viral DNA was only detected up to 14 days post-injection. There was a decline in the viral load over the course of the experiment in both of the dose groups. There was no unexpected mortality or occurrence of gross lesions in the treated birds, raising the possibility that administration of recombinant viruses in chickens is safe.

*Evaluation of IFN-γ mRNA suppression in vivo post vaccination and MDV-infection*

Chickens were administered with 1x10^10 genomic copies of three rAAAV:U6shRNA (10.6, 10.9, 10.10) or rAAAV:U6shRNA-eGFP and HVT vaccine on the day of hatch. Four days after rAAAV and HVT administration, each bird was injected intra-abdominally with 250 PFU of MDV-RB1B (or an equal volume of PBS for the sham infected group). At 21 d.p.i., chickens infected with MDV-RB1B only and MDV-RB1B + 3 rAAAV:U6shRNA weighed significantly less when compared to all the groups that were vaccinated as well as the control group (Figure 18a). At day 21 post-infection with MDV-RB1B, chickens were necropsied and examined for the presence of gross lesions. In addition, their lymphoid organs were weighed, as MD is associated with bursal atrophy and enlargement of the spleen. Figure 18b summarizes the incidence of tumours among various treatment groups. In the unvaccinated RB1B-infected group as well as the unvaccinated RB1B-infected + 3 rAAAV:U6shRNA group, the tumour incidence was 100% whereas in the infected groups that received HVT, HVT + rAAAV:U6eGFP or rAAAV:U6shRNA, tumour incidence was 40%, 20% and 55.6% respectively. None of the birds in the unvaccinated uninfected control group developed
tumours or clinical signs. Spleen weight: body weight ratio showed significant enlargement of spleen (a common gross pathological finding in MD) in the RB1B-infected only group and RB1B-infected + 3 rAAAV:U6shRNA group (Figure 18c). However, chickens vaccinated with HVT did not show enlargement of the spleen compared to uninfected control chickens. The lower bursa: body weight ratio indicated the occurrence of bursal atrophy in the MDV-infected only group and the MDV-infected chickens which were administered with the 3 rAAAV:U6shRNA (Figure 18d). In contrast, vaccinated birds did not have significant bursal atrophy when compared to control birds. There was no significant difference between vaccinated-only and vaccinated and treated with rAAAV groups with respect to bursa/body weight and spleen/body weight ratios.

MDV genome load was quantified in feather tips, which harbour fully infectious virus particles. Feather tip DNA from the different treatment groups was analyzed by real-time PCR and the data are illustrated in Figure 19. Presence of MDV was detected as early as 4 d.p.i. among all groups. At all time points, except at 21 d.p.i, the MDV-infected only group and the MDV-infected + 3 rAAAV:U6shRNA administered chickens had a higher MDV copy number compared to all vaccinated chickens. There was no genome load increase in the group vaccinated and treated with rAAAV when compared to vaccinated MDV-infected only. At 7, 10 and 14 d.p.i. all vaccinated birds had significantly lower viral load in the feather tips. At 4 and 21 d.p.i, there was no significant difference among all groups.
Discussion

Protective immunity against MDV induced by vaccination or natural infection requires a strong cell-mediated immune response, which is associated with up-regulation of IFN-γ expression in tissues (Abdul-Careem et al., 2009; Haq et al., 2010; Gimeno and Cortes 2011). In the present study, we exploited the endogenous RNAi mechanism to better understand the role of IFN-γ in vaccine-induced protection against MD.

This is the first report of down-regulation of chicken IFN-γ expression using a recombinant AAV in vivo. Earlier studies have utilized rAAAVs for the expression of a reporter gene in embryonic tissues in vitro, while other studies have shown the use of AAV vectors for expression of microRNAs or viral proteins in vivo (Estevez and Villegas 2006; Perozo et al., 2008; Wang et al., 2009). AAV-based vectors are safe and, moreover, expression cassettes up to ~4kb in length can be packaged into AAV capsids without compromising infectivity. In our study, an expression cassette containing the chicken U6 promoter and a shRNA was constructed and cloned into a plasmid vector containing the ITRs, followed by generation of recombinant AAV expressing shRNA. Sequences of the three shRNA were selected based on our earlier experiment, which examined efficacy of various siRNAs to knockdown expression of IFN-γ (Haq et al., in preparation). Several groups have described the use of small nuclear RNA promoters (H1 or U6) for the expression of siRNA in chickens (Kudo and Sutou 2005; Dai et al., 2005; Wise et al., 2007). In this regard, we chose a polymerase III promoter (U6) because this promoter transcribes endogenous small-nuclear RNAs (snRNAs) and is most commonly used to express shRNA (Kudo and Sutou 2005; Bannister et al., 2007). Mammalian pol III promoter, H1, can be used to transcribe shRNA in chicken cells.
(Yuan et al., 2006), however another study showed that chicken U6 promoter was significantly better for expressing microRNA in chicken cells compared to promoters of mammalian origin (Das et al., 2006).

In the present study, we examined tissue tropism of rAAAV and the virus was found to be distributed in all the tissues examined. Significant diversity has previously been reported in the tissue tropisms of AAV serotypes 1-9 (Wang et al., 2010). In vitro tissue tropism in avian AAV has also been determined among various avian and non-avian cell-lines, as well as primary chicken cells and human fibroblast cells. Findings by Bossis and Chiorini (2003) revealed that rAAAV had 10- to 300-fold higher transduction efficiency in avian cells when compared to rAAV2, -4, and -5. Regarding the presence of AAV, in our study, viral DNA load in tissues of the higher-dose group was detectable up to 26 days post administration. In contrast, viral DNA in the lower-dose group was not detectable in tissues other than muscle (the site of administration) after 14 days post-administration. This may be due to clearance of the virus by the host immune system. We noted that the bursa of Fabricius had a relatively lower amount of recombinant virus in both dose groups, which may suggest low tropism of AAV towards this tissue.

We then tested the hypothesis that down-regulation of IFN-γ, via the administration of rAAAV expressing shRNA, could abrogate immunity conferred by vaccination against MD. We also hypothesized that down-regulation of IFN-γ may exacerbate clinical and gross pathological lesions associated with MD. Loss of body weight in MDV-infected chickens is a characteristic sign of MD. Administration of rAAAV:U6shRNA targeting IFN-γ to vaccinated MDV-challenged birds did not have
any significant effect on the body weight of the birds. This suggests that the administration of shRNA after vaccination did not increase the severity of the disease or lower vaccine induced protection. However, both unvaccinated chicken groups infected with MDV and treated with rAAAV:U6shRNA, did have significantly lower body weight when compared to all vaccinated groups. Birds infected with MDV display bursal atrophy and spleen enlargement (Biggs 2001). Administration of rAAAV:U6shRNA to knockdown the expression of IFN-γ did not have a significant effect on bursal atrophy or enlargement of spleen when compared to unvaccinated MDV-infected chickens. HVT vaccination by itself or when co-administered with rAAAV:U6eGFP resulted in protection of 60% and 80% of birds respectively, whereas when birds were co-treated with HVT and rAAAV:U6shRNA prior to MDV-RB1B infection, only 44.4% were protected. However, the difference between groups was not statistically significant. From the results of the pilot study, recombinant AAAV was detectable up to 26 days post-administration, which correlates with 21 d.p.i, since MDV was administered at 5 days post vaccination.

IFN-γ is known as a potent activator of macrophages (Weining et al., 1996), and is also known to possess anti-tumour activities (Ikeda et al., 2002). In our earlier studies, we found that administration of rChIFN-γ, through an expression plasmid, enhanced immunity conferred by HVT leading to a reduction in tumour incidence in MDV-infected birds (Haq et al., 2011). Based on the results of our previous findings, the trend of increase in tumour incidence following administration of rAAAV:U6shRNA, supports a role for IFN-γ in the reduction of tumour development in birds. Other studies have also shown evidence for the role of IFN-γ in inhibiting tumour formation. For example,
Plachy et al. (1999) injected a congenic chicken line with ChIFN-γ followed by infection with Rous sarcoma virus (RSV), and observed a reduction in the development and tumour size in birds administered with ChIFN-γ.

The FFE is the only known tissue site from which fully formed infectious MDV particles can be shed into the environment (Calnek et al., 1970). Studies from our laboratory have examined host responses to very virulent MDV or Marek’s disease vaccines in feathers (Abdul-Careem et al., 2008b; Abdul-Careem et al., 2009c). Earlier studies showed an increase in IFN-γ expression in the FFE of vaccinated birds that correlated with a decrease in viral genome load present in the same tissue. This led us to hypothesize that down-regulating IFN-γ may lead to an increase in viral load in the FFE when compared to negative controls. The results presented here did not find a significant difference between the three vaccinated groups (MDV-infected + rAAAV:U6shRNA-eGFP, vaccinated MDV-infected, and the vaccinated MDV-infected + rAAAV:U6shRNA). The lower viral load seen in the vaccinated birds correlated with the time of MDV latency, which begins around 6-7 d.p.i. until the late cytolytic phase beginning around 14 d.p.i. (Calnek 1998). MDV-infected birds administered with rAAAV:U6shRNA had a higher viral load, although not statistically significant, when compared to MDV-infected only birds. Increase in expression of IFN-γ has been shown to correlate with an increase in inducible nitric oxide synthase expression resulting in the production of nitric oxide, which is known to inhibit viral replication (Cheeseman et al., 2008; Haq et al., 2011). This observation supports that knocking down IFN-γ expression may lead to an early increase in MDV genome load.
In conclusion, we generated three different rAAAV expressing individual siRNA targeting chicken IFN-γ as well as one rAAAV expressing a control siRNA (eGFP). We then proceeded to conduct studies with the recombinant virus to observe its tissue distribution using two different doses of the virus. This was followed by a MDV challenge study to examine the role of IFN-γ in immunity to MDV. These findings provide a basis for future studies aimed at utilizing recombinant viral vectors to deliver genes or to knockdown expression of a gene of interest and may be used to better understand roles of certain proteins or improve vaccines.
<table>
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<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
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</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>10.10 Reverse</td>
<td>CTACATATGAAAAAGCGTGAAGGATATCATCTCTTTCTTCT TGATACTTTTACCTTCTTCACGCCGACTAAGAGCATCGAGACTG</td>
</tr>
<tr>
<td>10.9 Reverse</td>
<td>CTACATATGGAAAGCAAGTAGTCTAAATCTTTGTCACTCTTTCTTCTTCTTGAAAAGATTTAGACTAAGAGCATCGAGACTG</td>
</tr>
<tr>
<td>10.6 Reverse</td>
<td>CTACATATGAAAAACGATGAAACGACTTGAGAATCCAGCTTTCTTTCTCTGCTGATTTCTCAAGTCTGTTCACTGTCGCCATCGAGACTG</td>
</tr>
<tr>
<td>shEGFP Reverse</td>
<td>CTACATATGAAAAAGCTGACCCTGAACTTTCAACTTTCTTCTTTCTAGATGACTTCAGGGTCAGCGACTAAGAGCATCGAGACTG</td>
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<td>AAAV U6 titrate F</td>
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</tr>
<tr>
<td>AAAV U6 titrate R</td>
<td>CCAATTGACGCGATTCGGCG</td>
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</table>
Figure 14. Schematic representation of the PCR cloning strategy used to produce shRNA expression vectors.

a) The yellow arrows indicate location of forward and reverse primers on the U6 promoter (red), the green indicates the sense and antisense strands and black the loop region. b) The backbone AAV vector, pEVA3V-LacZ, was digested with *Nde*I restriction enzyme and the expression cassette containing the U6 promoter and shRNA was inserted into the vector.
Figure 15. Schematic representation of the plasmid vectors for generation of rAAAVs.

The pAAAV:U6shRNA plasmid containing the expression cassette under the control of the chicken U6 promoter in addition to the helper plasmid pRC and pHelper were used together to transfect HEK 293 cells. The recombinant AAVV particles coding for the shRNA were collected and purified through a sucrose gradient.
Figure 16. Transfection of DF-1 cells with pcDNA:chIFN-γFLAG followed by infection with rAAAV expressing a shRNA targeting IFN-γ or a non-target sequence.

a) rAAAV DNA was detected by amplification of a 198 bp region in the non-coding sequence of the virus. M = 100 bp DNA marker; 1 = positive PCR control, using plasmid DNA as a template; 2 = cells infected with 50μL of DNase-treated rAAAV expressing the eGFP siRNA; 3 = cells infected with 50μL of DNase-treated rAAAV; 4 = cells infected with 5μL of rAAAV; 5 = cells infected with 0.5μL of DNase-treated rAAAV; 6 = untreated control cells; 7 = negative PCR control. b) IFN-γ expression relative to β-actin at two time points 24 and 48 hours post infection. * = Significant when compared to positive and non-target (eGFP) groups ($P \leq 0.05$).
Figure 17. Detection of rAAAV within various tissues.

The presence of rAAAV was determined in selected tissues at 48 hours, 120 hours, 14 days and 26 days post administration. The data are presented as mean virus genome copy numbers ± SEM.
**Figure 18.** Body weight, tumour incidence and lymphoid tissue weight in MDV-infected chickens.

a) Body weight (g) of chickens from various treatment groups at 21 d.p.i. b) Percentage of chickens with gross tumours in each treatment group. Ratios of spleen (c) or bursa of Fabricius (d) to body weight among the various treatment groups from the challenge trial. Treatment groups included MDV-infected only (RB1B), MDV-infected and administered 3 rAAAV targeting IFN-γ (RB1B + rAAAVshRNA), vaccinated MDV-infected (RB1B+HVT), vaccinated MDV-infected and administered non-target rAAAV (R+H+rAAAV:eGFP), vaccinated MDV-infected and administered three rAAAV targeting IFN-γ (R+H+rAAAV:IFN-γ shRNA) and an unvaccinated uninfected (control) group. Error bars represent mean of ratio ± SEM. *Significant difference when compared to all vaccinated groups and control ($P \leq 0.05$).
1: RB1B  2: RB1B + HVT  3: R + H + rAAAV:IFNγ shRNA  4: R + H + rAAAV:eGFPshRNA  5: R + rAAAV:IFN-γ
**Figure 19. MDV genome load in feather tips of chickens from various groups in the MDV challenge trial.**

Chickens were vaccinated with HVT and/or administered with rAAAV:U6shRNA on the day of hatch. Birds were infected with MDV on day 5 post hatch and sampled on 4, 7, 10, 14, 21 d.p.i. Mean MDV genome load are presented and the error bars represent SEM. * Significant when compared to unvaccinated groups (RB1B, RB1B+3 rAAAV:IFN-γ shRNA). Treatment groups include MDV-infected only (RB1B), vaccinated MDV-infected (RB1B+HVT), MDV-infected and administered 3 rAAAV targeting IFN-γ (R+ H + rAAAV:IFN-γ shRNA), vaccinated MDV-infected and administered non-target rAAAV (R+ H + rAAAVeGFP shRNA), MDV-infected and administered three rAAAV targeting IFN-γ (R+ rAAAV:IFN-γ shRNA).
Acknowledgements

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CHAPTER 6

GENERAL DISCUSSION

Marek’s disease is a lymphoproliferative disease of chickens, characterized by malignant T cell lymphoma formation caused by MDV. Because of the contagious nature of MDV, rapid disease onset and persistence in both host and environment, MD is a major concern in the poultry industry. Currently, MD is successfully controlled predominantly via vaccination which can be administered to chicks either pre- or post-hatch. Although the vaccine-induced immunity prevents development of clinical signs and histopathological lesions of the disease, it does not prevent infection. MDV provides a well-documented example of evolution in viral virulence over the last sixty years (Gimeno 2008). The reason behind the increase in virulence is unknown, although certain hypotheses have been put forward. One of the reasons for this increase in MDV virulence may be due to mass vaccinations administered to chicks worldwide, which can prompt an evolutionary increase in virulence (Gandon et al., 2001). The constant increase in virulence has led to the demand for new vaccines to be developed every decade or so to effectively control more severe MD.

The establishment of vaccine-induced immunity is under the control of host immune responses including various cytokine cascades. In chickens, as in mammals, cytokine production reflects the expansion and functionality of several cell subsets but importantly, distinct T helper cell subsets. T helper (T\textsubscript{H}) 1 cells produce IFN-\textgamma and IL-2 and give rise to cell-mediated immune responses, while T\textsubscript{H}2 cells produce IL-4 and IL-13 to help B cell antibody production. Since MDV is a highly cell associated virus, it mainly
stimulates T cell-mediated immune responses (Morimura et al., 1998). Therefore, advancing our understanding of cell-mediated immune responses will be important in developing new and more efficacious vaccines against MD.

MDV is shed by infected chickens and is transmitted to other chickens through the respiratory route; therefore, the objective of our initial study was to identify immune mediators associated with cell-mediated immune response in lung tissue. To better understand the mechanisms of immunity conferred by vaccines against MDV, chickens were vaccinated with HVT and then infected with MDV-RB1B via the respiratory route. Subsequently, virus replication kinetics in addition to cellular and cytokine responses were examined in the lungs of chickens. An earlier study examining host innate responses in the lungs following vvMDV-RB1B inhalation reported an up-regulation in the expression of toll-like receptors (TLRs), pro-inflammatory cytokines, and an increase in macrophage infiltration (Abdul-Careem et al., 2009b). Our study evaluated replication kinetics and host responses in the lungs at 1, 2, 3, 10 and 21 d.p.i after administration via the respiratory route. The 21 d.p.i was chosen as the termination point for the experiment based on our challenge model in which the incidence rate of MD in unvaccinated chickens infected with 250 PFU of MDV-RB1B is 100% by day 21 post-infection. The results from this study showed that both viruses, HVT and MDV-RB1B, were capable of replication in the lungs following infection via aerosols. Administration of HVT prior to infection with RB1B resulted in higher replication of both viruses, a phenomenon observed in other viral co-infection studies such HIV and HSV or PCV-2 and PRRSV (Golden et al., 1992; Allan et al., 2000). However, the mechanism of interaction between HVT and MDV remains unknown. The infection resulted in the induction of host
responses that were characterized by an up-regulation of cytokines and expansion of CD4+ and CD8+ cells at the site of infection. Both IFN-γ and IL-10 were up-regulated in the presence of both virus strains at 10 d.p.i. It is known that MDV reactivates between 10-14 days post-infection, which may explain the up-regulation observed at 10 d.p.i. In a study by Kano et al. (2009a), an up-regulation of IFN-γ was observed in splenocytes from CVI988-vaccinated MDV-infected birds at 10 d.p.i. Studies have shown up-regulation of IFN-γ during early phases of MDV infection, during late cytolytic phase, among genetically resistant birds, as well as in vaccinated chickens. From previous studies (Abdul-Careem et al., 2007) as well as our observations, IFN-γ was selected for its association with host immunity against MD.

Several studies have examined host cytokine response to MDV infection in tissues of specific-pathogen free chickens or in genetically resistant/susceptible birds as well as in vaccinated chickens infected with MDV. These studies have employed techniques such as microarray and RT-qPCR assays to assess alteration in cytokine gene expression. Cytokines, as immune modulators, play significant roles in host-pathogen interactions; however, little is known about the causal association between those mediators and protection from MD. One approach to better understand the role of these cytokines would be to exogenously administer them, either by an expression plasmid or as a purified protein. The use of expression plasmid DNA vectors, either encoding a protein gene for therapeutics, an adjuvant, or encoding short hairpin RNA has acquired a great deal of attention in the translational biology field. Studies have already reported that administration of a combination of vectors expressing cytokines with various vaccines
enhances immunity against certain pathogens. For example, administration of IL-2, IL-15 and IFN-\(\gamma\) to chickens enhanced immune response to \textit{Eimeria} antigens (Min \textit{et al.}, 2002). In the case of MD, when a fowlpox virus expressing chicken myelomonocytic growth factor was combined with HVT vaccination, it enhanced immunity against experimental challenge with the RB1B strain of MDV (Djeraba \textit{et al.}, 2002). To address whether the addition of IFN-\(\gamma\) would enhance protection against vvMDV (RB1B) strains, chicken IFN-\(\gamma\) coding sequence was cloned into an expression vector and administered in combination with HVT. Results from our study demonstrated a significant reduction in the occurrence of tumour development in birds following infection with MDV-RB1B and all chickens without tumours had significantly higher expression of IFN-\(\gamma\) when compared to chickens with tumours. One limitation of this study was that the source of IFN-\(\gamma\) could not be definitively identified; whether it was from the recombinant plasmid or native host IFN that resulted in the enhanced protection. Nevertheless, these data suggest an enhanced antiviral immune response when IFN-\(\gamma\) was co-administered with HVT. IFN-\(\gamma\) can exert its effects directly by inhibition of MDV replication or indirectly, by activation of macrophages, which in turn produce NO. This cytokine has been shown to have anti-tumour activities which may have been the cause for the reduced occurrence of tumours observed. However, IFN-\(\gamma\) also has been implicated in the inhibition of virus replication as well as maintenance of latency (Steed \textit{et al.}, 2007). The present study did not pursue the precise mechanism of IFN-\(\gamma\) enhanced protection; investigation into this requires further studies.
In chapters 4 and 5, a causal link between IFN-γ and vaccine-induced immunity to MD was evaluated by silencing the expression of the IFN-γ gene. To evaluate the association between IFN-γ and immunity against MD, we set out to knockdown the gene encoding IFN-γ *in vitro* and *in vivo*. The ultimate goal was to determine if, by reducing the expression of IFN-γ, immunity conferred by vaccination could be abrogated. The project involved identifying efficient siRNA sequences that could eventually be used in a vector-based shRNA delivery system to infect birds *in vivo* and determine the impact of knocking down IFN-γ on immunity. SiRNAs targeting chicken IFN-γ and non-specific controls were tested and selected based on their ability to knockdown IFN-γ. Our results indicated that a cocktail of three siRNA induced significant reduction in chicken IFN-γ mRNA and proteins levels. The corresponding controls, a non-target negative control and a GFP siRNA sequence, had no effect on the levels of IFN-γ. Double stranded RNA can activate strong antiviral defense mechanisms, which involve the IFN pathway or proteins such as 2′-5′ OAS, resulting in non-specific or off-target effects. To rule out the possibility of non-specific effects, we measured the expression levels of IFN-α and 2′-5′OAS, which were not up-regulated in cells treated with the siRNA cocktail compared to control cells.

Recent studies have demonstrated that avian viral vectors can be used to express shRNA for various purposes (Estevez *et al.* 2006; Perozo *et al.* 2008). In our next study we utilized a member of the adeno-associated virus family, avian adeno-associated virus (AAAV), which has been fully characterized and has been shown to successfully deliver genes in chicken embryo tissue and cells (Estevez and Villegas 2006). After successfully generating rAAAV expressing shRNA targeting IFN-γ, a preliminary study was
undertaken to establish the dose and tissue distribution of intramuscular administered virus over a period of 26 days. After selecting a dose based on this pilot study, an MDV-RB1B infection study was performed. Birds were vaccinated with HVT and/or administered the rAAAV followed by an MDV challenge. It was observed that birds administered with rAAAV had a higher incidence of tumours when compared to MDV infected only, suggesting an anti-tumour role of IFN-γ; however this finding was not statistically significant when compared to MDV-infected only. Since vaccine-induced immunity protects against viral replication, we then examined the viral load present in the FFE. The administration of the rAAAV did not seem to have an impact on the viral load when compared to vaccinated MDV-infected birds. Our findings have demonstrated that AAV can be used to deliver shRNA both in vitro and in vivo.

Despite the novel aspect of our work on examining the role of IFN-γ in vaccine-induced immunity, there were some caveats to these studies. Little is still known about the exact mechanism(s) underlying the immunological role played by cytokines in mediating immunity conferred by MD vaccines. One limitation we have had in the experimental model used in our laboratory was that we had to terminate infected chickens at 21 days post-infection. This was due to the fact that infected chickens would be showing clinical signs and neoplastic lesions in their internal organs. Long term studies (for example up to 8 weeks post-infection or even longer) might have been necessary for testing the adjuvant properties of IFN-γ to fully establish the long-term benefit of recombinant cytokines/adjuvants. Additionally, whether endogenous or viral delivered exogenous IFN-γ was playing a role could not be verified. There is a need to establish chicken cell lines expressing IFN-γ for a better knockdown study, rather than co-
transfecting an expression vector with a limited amount of siRNAs. Other approaches can utilize a more ‘physiological’ system, in which primary cells can be stimulated to express IFN-\(\gamma\) followed by siRNA/shRNA administration. We attempted this approach, however we were not successful due to the sensitivity of the primary splenocytes to the transfection agent, PEI (data not shown). In trials using rAAAV, further optimization needs to be investigated. A broader dose of rAAAV, as well as different sites of administration might have provided a better and more efficient delivery system. An additional caveat of the study in Chapter 5 is that to detect the full response by a rAAAV, a booster dose may be required.

Future research directions should focus on the development of more efficacious vaccines with adjuvants such as IFN-\(\gamma\) which can be delivered by expression plasmids or recombinant viral vectors. Especially, utilizing non-pathogenic viral vectors to deliver cytokines, such as IFN-\(\gamma\), would be desirable. One such viral vector may be AAV. Furthermore, since all current vaccines target to limit clinical disease and, to some extent, virus replication, novel strategies may be envisaged to prevent infection or inhibit virus shedding from vaccinated and infected birds. This can be investigated at two levels, blocking MDV entry into phagocytic cells or preventing formation of fully enveloped MDV particles at FFE. These studies can use RNAi as a major tool delivered via plasmid vectors or recombinant virus vectors expressing siRNA designed to target virus sequence or virus packaging processes, eventually reducing the infectivity of the virus. Studies have determined that DNA vaccines and plasmids injected directly into the host (or a particular tissue) are safe and may be advantageous over traditional vaccines using live
attenuated or killed virus. However, the limitation is the longevity of the plasmid DNA or siRNA. Therefore, future studies should consider the possibility of enhancing the longevity of these constructs. For example degradation of siRNA can be avoided by chemical modification of oligonucleotides or by forming complexes with cationic carriers (Spagnou et al., 2004; Zhang et al., 2006). Recent studies have shown the use of reconstituted viral envelopes (virosomes) as promising carrier systems for delivery of siRNA (Kaneda et al., 2011). Further research into developing synthetic nanoparticles such as polymers, lipids, or conjugates will have a key role in systemic application of siRNA in the clinic. Incorporation of ligands with specific tissue tropisms in combination with nanoparticles may prove valuable for targeting specific tissues. In vivo imaging systems to monitor gene carriers such as liposomes and virosomes, for a better understanding of gene delivery in local or systemic administration would be key for successful siRNA therapy.

Overall, the findings in the studies presented in this thesis shed light on the role IFN-γ in MD vaccine-induced immunity and has provided evidence for the utility of recombinant IFN-γ as a vaccine adjuvant for MD vaccines. Future studies should examine the underlying mechanisms of IFN-γ function in immunity against MD. Moreover, these studies should explore the possibility of expressing IFN-γ using viral vectors, such as AAAV or HVT, which would make or facilitate the use of this immunomodulator for MD as well as other poultry viral vaccines. The ultimate objective for development of novel vaccines for control of MD is to create a vaccine formulation that is able to control clinical signs of disease, and at the same time, is able to curtail virulent virus replication and shedding from feathers.
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