Gamma delta T cells in Marek’s disease virus infection of chickens

by

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ABSTRACT

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Immunity against Marek’s disease in chickens is mediated by both innate and adaptive host responses. This study aimed to determine the effects of Marek’s disease virus infection on distribution and frequency of γδ T cells in tissues, as well as their expression of cytokine genes. In the spleen of infected chickens, the number of γδ T cells increased by 10 and 21 days post-infection. Additionally, nearly 100% of the splenic γδ T cells in MDV-infected birds were CD8+ by day 21 post-infection. Conversely, the number of γδ T cells in the cecal tonsils of infected birds decreased. Splenic γδ T cells had up-regulated expression of interferon-γ early in infection followed by simultaneous gene expression of interleukin-10 during the later phases. In conclusion, these results suggest a potential role for γδ T cells in immunity to MDV and further elucidate the underlying immunological mechanisms that mediate immunity to MD.
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LIST OF ABBREVIATIONS

µg Microgram
µL Microlitre
α Alpha
ABC Avidin-biotin-peroxidase complex
ACK Ammonium-chloride-potassium
ADCC Antibody-dependent cell-mediated cytotoxicity
AIV Avian influenza virus
APC Antigen presenting cells
APC Allophycocyanin
β Beta
BSA Bovine serum albumin
CD4+ cluster of differentiation 4 positive cells
CD8+ cluster of differentiation 8 positive cells
cDNA Complementary DNA
CEF Chicken embryo fibroblasts
CTL Cytotoxic T lymphocyte
delta Delta
DAB 3,3-diaminobenzidine-H2O2
DC Dendritic cells
DNA Deoxyribonucleic acid
dpi Days post-infection
ds Double-stranded
EARC Ellipsoid-associated reticular cell
EBV Epstein-Barr virus
ED Embryonic day
ER Endoplasmic reticulum
FACS Fluorescence activated cell sorting
FBS Fetal bovine serum
FFE Feather follicle epithelium
FITC Fluorescein isothiocyanate
γ Gamma
GaHV Gallid herpesvirus
gB Glycoprotein B
gE Glycoprotein E
gI Glycoprotein I
HBSS Hank’s balanced salts solution
HSP Heat-shock protein
HSV Herpes simplex virus
HVT Herpesvirus of turkey
ICP4 Intra-cellular protein-4
IFN Interferon
IL Interleukin
IR Internal repeat
iNOS  Inducible nitric oxide synthase
kDa  Kilodalton
LATs  Latency associated transcripts
LPC  Lymphoid progenitor cell
mAb  Monoclonal antibody
MD  Marek’s disease
MDV  Marek’s disease virus
MeHV  Meleagrid herpesvirus
Meq  Marek’s EcoRI-Q-encoded protein
MHC  Major histocompatibility complex
mRNA  Messenger RNA
ng  Nanogram
NK  Natural killer
NO  Nitric oxide
OCT  Optimal cutting temperature
PB  Pacific Blue
PBL  Peripheral blood lymphocytes
PBS  Phosphate-buffered saline
PCR  Polymerase chain reaction
PE  Phycoerythrin
PFA  Paraformaldehyde
PFU  Plaque forming unit
PMA  Phorbol myristate acetate
pH  Potential of Hydrogen
pp38  Phosphoprotein 38
PRR  Pattern-recognition receptors
RANTES  Regulated on activation, normal T cell expressed and secreted
RNA  Ribonucleic acid
RPMI  Rosewell Park Memorial Institute
SPF  Specific pathogen free
TAP  Transporter associated with antigen processing
TCR  T cell receptor
TE  Tris-EDTA
TGFβ  Transforming growth factor beta
Th  T helper cell
TLR  Toll-like receptor
TNF  Tumour necrosis factor
TR  Terminal repeat
UL  Unique region long
US  Unique region short
VDJ  Variable, diversity, joining
vIL-8  Viral Interleukin-8
vv  Very virulent
VV  Vaccinia virus
VZV  Varicella zoster virus
CHAPTER 1

Introduction

Marek’s disease (MD) is a highly infectious lymphoproliferative disease caused by Marek’s disease virus (MDV), an avian herpesvirus of the family *Alphaherpesviridae*. The virus causes immunosuppression in susceptible birds and may ultimately result in the formation of T cell lymphomas. MDV is first inhaled from a contaminated environment as a cell-free virus. After infecting B cells in the lungs, it spreads to secondary lymphoid organs as a cell-associated virus (Haq, Schat, & Sharif, 2013). Prior to entering a period of latency, the virus begins infecting CD4+ T cells. Upon reactivation the virus sheds from the feather follicle epithelium (FFE). As there are still many gaps in the literature pertaining to MDV, it is important to further our understanding of this virus and its interaction with the chicken’s immune system. The immune response to MDV involves the innate defense mechanism as a first line of defense followed by the adaptive immune response, which is tailored to better suit this particular viral pathogen.

Cells of the immune system are central in the activation of an immune response to invading pathogens. These cells, whether of innate or adaptive origin, interact, sometimes for several days, in lymphoid tissues in order to gauge the appropriate immune response. Lymphocytes, characterized as the backbone of the adaptive immune system, are comprised of T cells and B cells. Antigen presenting cells (APCs) interact with helper T cells by presenting pieces of foreign antigen to their T cell receptors (TCRs). Upon binding, helper T cells initiate the release of cytokines that will in turn cause B cells to secrete antibodies and cytotoxic T cells to kill infected cells. Although both arms of the immune system are recruited following MDV infection, the cell-mediated immune
response is critical as MDV is a cell-associated virus. CD4+ T cells serve as targets of transformation whereby they shift from latently infected lymphocytes to lymphoma tumour cells while CD8+ T cells have an important role in defense against MDV. The cytotoxic capabilities of CD8+ T cells have been previously described against certain MDV antigens (Schat et al., 1992). When CD8+ T cells are experimentally depleted, the result is a higher MDV load in CD4+ T cells (Morimura et al., 1998); therefore, CD8+ T cells can be characterized as having a unique and central role in the avian immune response.

In general, T cells can be classified as αβ or γδ T cells. The latter have features of both the adaptive and innate immune system making them a unique type of cell. γδ T cells arise first in ontogeny and are involved in surveillance of epithelia, which separate the internal and external milieu. The localization of these cells within the epithelia is a common feature among species and suggests a role in surveillance of mucosal surfaces. Once γδ T cells interact with, for example, inducible molecules associated with cell stress or TLR ligands, these cells lyse abnormal, stressed, or infected epithelial cells. Despite the high frequency of γδ T cells in chickens, little is known in regard to the nature and function of these cells. γδ T cells comprise about 10% of thymocytes, 20% of circulating T cells, and 30% of splenocytes in adult chickens (Bucy, Chen, & Cooper, 1991). In comparison, most research on γδ T cells has been focused on mice and humans; however, these species have a much lower frequency of γδ T cells relative to avian species (Haas, Pereria, & Tonegawa, 1993). Given the number and location of γδ T cells and the range of immune system molecules they produce, these cells are thought to be involved in immunity against pathogens, including viruses.
The ultimate goal of the present study was to elucidate the role of γδ T cells in immunity against MD in order to further our understanding of this virus and its interaction with the chicken’s immune system. The focus of the research presented in this thesis was on establishing whether γδ T cells are changed in number and frequency during the course of MDV infection. Furthermore, we set out to elucidate the role that γδ T cells may play in response to MDV by examining the cytokines that these cells express. It was hypothesized that elevated levels of γδ T cells would be found in tissues such as spleen, cecal tonsils, and lungs because of similar findings in mice infected with herpesviruses. Additionally, it was hypothesized that γδ T cell expression of genes associated with immune function varies depending on the stage of MDV pathogenesis and phases of immunity to this virus. For instance, pro-inflammatory cytokines may be present more often in the early stages whereas regulatory cytokines may appear during the later phases of infection.
Literature Review

1.1. Marek’s Disease

1.1.1. Background

József Marek, a Hungarian veterinarian, first reported the polyneuritis disease, later known as Marek’s disease (MD), in 1907 (Biggs, 2004). This disease was associated with the development of tumors in visceral organs and was later renamed *neurolymphomatosis gallinarum*. However, it was not until the late 1960s that a clear understanding of its cause was established. In 1961, P.M. Biggs classified the disease based on age group and tissue infected, as well as histopathology. Based on these distinctions it was no longer recognized as an avian sarcoma leukosis virus and was further renamed MD as the herpesvirus was determined to be the causative agent. It was during this decade of discovery that the poultry industry experienced substantial loss as a result of this virus. Mortality rose as high as 60% in certain flocks of laying hens (Morrow & Fehler, 2004). This quickly led to the development of Marek’s disease virus (MDV) vaccines in the 1970s, which proved quite successful. However, within the past 40 years, the virulence of this disease has increased dramatically; this has, therefore, provoked much discussion about the need for new vaccine strategies. It is estimated that the annual, worldwide loss due to MD is $2 billion (Smith et al., 2011a).

1.1.2. Classification

Marek’s disease is a highly infectious lymphoproliferative disease found in chickens. The etiologic agent is an avian herpesvirus known as MDV, which can result in malignant lymphoma formation. This virus was originally thought to be a member of the
*Gammaherpesvirinae* subfamily due to certain properties it shared with the Epstein-Barr virus (EBV) (i.e. lytically infects lymphocytes). Later research into the sequence data of this double-stranded DNA virus proved that, rather, it belonged to the *Alphaherpesvirinae* subfamily because it was more similar to varicella zoster virus (VZV), an α-herpesvirus, than to EBV (Baigent & Davison, 2004). Therefore, this virus is genomically similar to an α-herpesvirus but possesses the biological properties of a γ-herpesvirus.

There exist three species in the genus *Mardivirus* that are closely related: serotype 1 or gallid alphaherpesvirus 2 (GaHV-2), serotype 2 or GaHV-3, and serotype 3, HVT (turkey herpesvirus), or meleagrid alphaherpesvirus 1 (MeHV-1). While GaHV-3 and HVT are non-oncogenic in chickens (i.e. unable to cause transformation of infected cells), GaHV-2 causes disease marked by development of lymphomas in infected chickens. GaHV-2 is generally classified further according to its virulence and pathogenicity: mild (m), virulent (v), very virulent (vv), and very virulent + (vv+) (Witter, 1997).

1.1.3. Genomic structure

The MDV genome is approximately 178 kbp and encodes 103 proteins (Tulman et al., 2000). Members of the α-herpesvirus subfamily have a similar genome composition; however, they differ with respect to their restriction endonuclease patterns. The general pattern for the genome of an α-herpesvirus is composed of unique long and unique short (UL and US) segments flanked by inverted repeat sequences, terminal and internal repeats long (TRL and IRL) and terminal and internal repeats short (TRS and IRS), respectively (Roizman et al., 1992). As a result, the typical genomic organization is TRL-
U_{L-IR_{L}}-IR_{S}-U_{S}-TR_{S}. The genes within the unique segments are homologous to VZV and the genome is co-linear with that of VZV (Morrow & Fehler, 2004). The similarities expressed by these two viruses are seen from the inhalation to the shedding of this virus. This helps further reinforce the classification of MDV as an α-herpesvirus rather than a γ-herpesvirus.

1.1.4. Pathogenesis

Infection with MDV occurs shortly after hatching followed by mortality within a few weeks to months. The virus is cell-associated in tumours and most organs except the FFE from which enveloped virions shed (Calnek, Hans, and Kahn, 1970). The FFE and the skin tissue (Heidari et al., 2016) are sites from which the release of infectious cell-free MDV occurs due to the down-regulation of cytotoxic T lymphocytes (CTL) responses in these infected tissues. The virus may be shed from the debri of dead epithelial cells or moulted feathers (Carroza et al., 1973). It is classified as an airborne virus because it is inhaled by susceptible hosts from an environment contaminated with cell-free virus particles. The lungs are the portal of entry for the virus, and from there it spreads to lymphoid organs as a cell-associated virus (Haq, Schat, & Sharif, 2013). Infectivity is associated with air, dust, and litter (Witter, 1976). Horizontal transmission is the only means of infection as there is strong evidence against vertical transmission (Solomon et al., 1970). Today, due to the high-density environment in which commercial chickens are generally reared, the virus is able to spread and infect chickens easily.

MDV has a life cycle that begins within 18-20 hours post infection (h.p.i.). Depending on the stage of infection and the tissue in which the virus is residing, interaction with the host cells may be productive or non-productive (Morrow & Fehler,
The productive (lytic) phase involves invasion and take-over of host cells, resulting in production of virions. At this stage, the virus reaches the host cells by attaching to receptors on the cell surface. Viral DNA then enters the nucleus and is transcribed and translated by the host machinery. Nucleocapsids are assembled and bud from the inner nuclear membrane, gaining an envelope. Maturation of virions occurs in the Golgi apparatus followed by exocytosis. Non-productive interactions (latent) occur when gene expression of the viral genome is limited and there is neither production of virions nor cell death. This creates an economical way for the virus to persist within the host while avoiding death.

The ‘Cornell Model’, postulated by Calnek in 1986, is a widely accepted model, which describes pathogenesis of MDV (Calnek, 1986). It is important to note that the days post-infection associated with a particular phase of infection are approximate and will vary with the strain of the virus. Strains of higher virulence will lead to clinical and pathological signs earlier than strains of lower virulence by bypassing the latent phase of infection (Yunis, Jarosinski, and Schat, 2004). According to the ‘Cornell Model’, there are four phases that occur sequentially in susceptible chickens. The first phase is characterized as the early cytolytic infection, which is present from 1-7 days post-infection (d.p.i.). MDV spreads from the lungs via the bloodstream to secondary lymphoid tissues (i.e. spleen, bursa of Fabricius, thymus) within 24-36 hours post-inoculation. Macrophages act as phagocytic carriers of the virus during their transportation from the lungs. In some MDV strains, the virus is capable of infecting and replicating within macrophages as well (Barrow et al., 2003). It has also been determined that the virus can directly infect B cells in the lungs, which then carry the virus to
lymphoid organs (Baaten et al., 2009). Ellipsoid-associated reticular cells (EARCs) engulf the virus, once within lymphoid organs, leading to a cytolytic infection. Additionally, MDV up-regulates expression of major histocompatibility complex class II (MHC-II) on antigen presenting cells (i.e. B cells) to aid in activation of T cells required for infection and further spread of the virus (Niikura et al., 2007).

The second phase is latency and occurs between 5-7 d.p.i.. This phase is characterized by the loss of MDV antigen expression in lymphoid tissues due to expression of type I interferons (IFNs) by the host (Volpini et al., 1996). There are three stages to the latent phase of infection: establishment, maintenance, and reactivation. In contrast to the lytic phase, which involves B cells, the latent phase occurs within T cells. As a result, it resides mainly in CD4+ T cells but may also occur in some CD8+ T cell (Schat & Xing, 2000). During this period, transcription is not completely halted but rather limited to latency-associated transcripts (LATs) that strike a balance between latent and lytic infection (Morrow & Fehler, 2004). Marek’s EcoRI-Q-encoded protein (meq) is an important transcript involved in maintaining latency by preventing apoptosis of latent CD4+ T cells and transactivating latent gene expression (Parcells et al., 2003). A model has been postulated for the regulation of MDV lytic versus latent infection whereby Meq-Meq homodimers bind to the virus’ origin of replication, repress pp38 expression (a gene of the cytolytic phase), and transactivate transcripts associated with oncogenicity (1.8 kb, pp14, Meq oncoprotein) (Parcells et al., 2003). Meanwhile, infected lymphocytes travel to the spleen and peripheral blood. Infected peripheral blood lymphocytes (PBLs) begin spreading the virus to other organs within the host where they become reactivated in the next phase. Latent infection persists for an entire lifetime; however, in chickens resistant
to the disease, the infection level may decrease while in others, it remains high, ultimately leading to death.

The third phase of infection involves late cytolytic infection and permanent immunosuppression, leading to lymphoma formation (Calnek, 1986). Atrophy of the bursa and thymus, key organs responsible for antibody and cell-mediated immune responses respectively, leads to irreversible immunosuppression by 14-21 d.p.i.. Infected organs include the kidney, adrenal gland, proventriculus, esophagus, and some epithelial tissue (i.e. FFE). However, of these organs, only the FFE is able to harbour productive infection, which then facilitates the spread of infective virions via shedding to other birds. Between 10-12 d.p.i., infected PBLs migrate to the skin and infection is reactivated in the FFE. Around 13 d.p.i., the virus begins producing cell-free virus particles followed by apoptosis of the host cell. Shedding of this cell-free virus from the FFE persists for the entirety of the chicken’s life.

The transformation of infected lymphocytes to tumor cells is the fourth and final phase of MDV infection. The spleen is considered the predominant site for the initial proliferation of transformed cells. Lymphomatous lesions, which can be diffuse infiltrations or solid lymphomas affecting the viscera, skeletal muscle, and skin, begin to appear by 12 d.p.i. while gross lymphomas are detectable in infected chickens anywhere from 16-21 d.p.i.. CD30, a member of the tumor necrosis factor (TNF) receptor II family, is a surface antigen expressed by MD tumors (Burgess et al., 2004). Its expression by transformed lymphocytes is linked to expression of Meq (principal oncogene of MDV). It appears that MD tumors are able to down-regulate the host immune response by expression of interleukin (IL)-10 and the IL-10 receptor (Buza & Burgess, 2007). Signs
during these final stages of infection include transient paralysis due to increased pro-inflammatory cytokine expression in brain tissue resulting in encephalitis (Abdul-Careem et al., 2006b). Depression, crippling, and weight loss are other signs of infection. Paralysis and death are commonly the final outcome upon the development of lymphomatous lesions (Calnek, 2001).

1.1.5. Genetic resistance

MD is a clear example of the relationship between genetics and resistance to infectious diseases of chickens. MHC molecules play an important role in activation of the immune system; therefore, it is plausible that genetic resistance could be associated with the B-complex, which is the chicken MHC (Longenecker & Mosmann, 1981). In fact, it has been shown that different B haplotypes correlate to differing degrees of susceptibility to MD (Briles, Stone, and Cole, 1977). For example, chickens carrying the B21 haplotype are highly resistant to MD whereas those with the B19 haplotype are highly susceptible. It has been suggested that the varying degree of response to MDV based on B haplotypes could be attributed to the number of MHC class I molecules expressed on the cell surface (Kaufman, Volk, and Wallny, 1995). It is believed that MD causes strong selection for a simple MHC, coined as the “minimal essential MHC”, in order to regulate the expression level of MHC class I. This allows for regulation of virus binding and entry as well as CTL and natural killer (NK) cell responses. As a result, the B19 haplotype exhibits the highest level of surface MHC-I molecules, whereas the B21 haplotype expresses the lowest amount. Another hypothesis is the difference in the repertoire of peptides presented. For example, the B21 haplotype is able to bind peptides
with a large range of sequences, which could help explain its association with genetic resistance to MDV (Koch et al., 2007).

1.1.6. Host response to MDV

Infection within the FFE is apparent within 7-14 d.p.i.. This is the only site where the host is unable to elicit an appropriate response in order to control viral replication and curtail the spread of infectious viral particles. In fact, viral genome load is much higher in the FFE than it is in the spleen (Abdul-Careem et al., 2007). It has been suggested that a reason for this discrepancy is the lack of immune responses against MDV in the feathers (Baigent and Davison, 2004) as host responses are unable to control viral replication in the FFE. Therefore, the FFE is a site of utmost importance for studies on virus replication because it is from here that the virus sheds and infects other chickens via horizontal transmission. However, a recent study has observed that virus may also shed from the skin tissue of MDV-infected birds. A large transmigration of CD4$^+$ T cells, likely transformed, as well as macrophages occurred from blood vessels into the inflamed skin tissue of MDV infected birds at 26 d.p.i. (Heidari et al., 2016). Despite the infiltration of macrophages into infected skin tissue, the production of cell-free virions continued. Additionally, transmigrating cytotoxic T cells were present at very low numbers in infected tissue and undetectable in control samples. Meanwhile the population of migrating $\gamma\delta$ T cells increased in MDV infected skin tissue compared to controls.

The host response to infection with MDV involves both arms of the immune system: innate and adaptive, which work together in creating an appropriate response. This involves the participation of macrophages, NK cells, CD4$^+$ T cells, CD8$^+$ T cells, antibodies, pro-inflammatory cytokines, type I IFNs, antimicrobial peptides, and soluble
factors (i.e. nitric oxide). The cell-mediated immune response is considered to be the most effective response in targeting MDV because the virus is present in a cell-associated form for most phases of infection except during initial entry into the respiratory system and during shedding. However, the antibody-mediated immune response is elicited when the virus is cell-free or when MDV antigen is being expressed on cell surfaces.

1.1.7. Cytokine and chemokine expression

Cytokines are important in the activation and regulation of the immune response. The cytokine milieu helps determine the appropriate response against MD. They can be classified depending on their role in the environment. Type 1-like cytokines such as IFN-γ, IL-2, IL-12, and IL-18 promote a cell-mediated response whereas type 2-like cytokines such as IL-3, IL-4, IL-13 promote an antibody-mediated response. There also exist regulatory cytokines such as IL-10 and transforming growth factor (TGF)-β, which are involved in controlling and down-regulating the response. Gene expression of type 1- or 2-like cytokines varies throughout the different phases of infection but both can be readily found in the spleen (Heidari, Zhang, & Sharif, 2008; Xing & Schat, 2000a).

Expression of IFN-γ is up-regulated in the spleen following infection with the virus (Xing & Schat, 2000a); in fact, vaccination against MDV causes a significant increase in the expression of this cytokine (Abdul-Careem et al., 2007). It has been suggested that IFN-γ itself may act as an adjuvant by increasing the potency of an HVT vaccine against MDV (Haq et al., 2011). The result of the combined use of IFN-γ and HVT as a vaccine is lower viral genome load and lower viral transcript levels. The mechanism used by IFN-γ to confer lower MDV replication may be in up-regulation of MHC expression on infected cells thereby enhancing recognition by CTLs or activation
of macrophages to produce nitric oxide (NO) (Xing and Schat, 2000b). However, it has been suggested that there is no correlation between IFN-γ expression and genetic resistance or susceptibility to MD (Kaiser, Underwood, & Davison, 2003). In contrast, expression of certain other cytokines is associated with susceptibility and resistance. For example, IL-6 and IL-18 expression is up-regulated in genetically susceptible chickens (Kaiser, Underwood, & Davison, 2003) while IL-1β and IL-8 expression is found in resistant chickens (Jarosinski et al., 2005). Virulence also plays a part in influencing the expression of cytokine genes. Those infected with a vv+ strain of MDV have substantially higher expression of cytokines compared to those infected with a v strain (Jarosinski et al., 2005).

In general, IFN-γ, IL-18, and IL-6 expression is up-regulated at 4 and 21 d.p.i. in T cells (Parvizi et al., 2009), which indicates a type 1-like response to infection. However, GATA-3, a transcription factor for expression of type 2-like cytokines, is also up-regulated in the spleens of MDV infected chickens (Sarson et al., 2006). As a result, expression of IL-4 and IL-13 is enhanced. A third type of cytokine, IL-10, which is regulatory, is also elicited in response to MDV infection (Abdul-Careem et al., 2007). Although these are different classes of cytokines, they may, at times, be expressed simultaneously. For example, gebe expression of IFN-γ and IL-10 is up-regulated in lung mononuclear cells of MDV-infected chickens at 10 and 21 d.p.i. (Parvizi et al, 2015). The possible reason for simultaneous gene expression of IFN-γ and IL-10 is that the latter is acting in a non-regulatory fashion by inducing expression of certain cell subtypes (i.e. CD8⁺ T cells) that in turn will express IFN-γ (Chen and Zlotnik, 1991).
Chemokines are also important in innate defense against infection. Chemokine ligand CXCL14 and RANTES (Regulated on Activation, Normal T Expressed and Secreted) are involved in attracting monocytes and are expressed in MD tumor cells (Buza & Burgess, 2007). IL-8 serves as a chemoattractant for neutrophils and its gene expression is enhanced in the brain, spleen, and lungs of MDV infected chickens (Parvizi et al., 2010). There is also a virokine, named viral interleukin (vIL)-8, which is homologous to IL-8 and is involved in the early cytolytic phase of infection. This chemokine is involved in attracting T cells to the area of virus replication. It is therefore crucial for the transmission of MDV from B to T cells. Studies have found that a deletion of vIL-8 leads to a reduced number of T cells available for transformation and, as a result, decreased tumor incidence (Cui et al., 2004).

1.1.8. Innate defense mechanisms

The innate immune system serves as a ‘first line of defense’, which is a critical component of the immune response because within 12 h.p.i., the MDV replication cycle has already begun in the lungs. Upon viral infection, conserved molecular motifs, known as pathogen-associated molecular patterns (PAMPs), are recognized by pattern recognition receptors (PRRs), for example Toll-like receptors (TLRs). TLRs have an extra-cellular domain that interacts with PAMPs and, through the recruitment of adaptor molecules, causes activation of transcription factors. TLR3 and TLR7 have been shown to respond to MDV infection as they recognize viral nucleic acids (Jie et al., 2013). These PRRs are capable of inducing interferons, which are involved in defense and regulation against viruses. Additionally, administration of ligands to stimulate TLR4 and TLR21 has
proven successful in reducing MDV genome copy number and in delaying disease onset (Parvizi et al., 2014).

Macrophages are innate immune system cells that play a critical role in early and late stages of protection against MD. Macrophages are able to transport MDV from the lungs to the primary lymphoid organs during the early phase of infection. As well as being able to phagocytose pathogens, macrophages can release certain mediators such as NO and pro-inflammatory cytokines. The release of inducible NO synthase (iNOS) by macrophages has been shown to aid in inhibition of MDV replication (Xing & Schat, 2000b). However, a study by Jarosinski et al., (2005), demonstrated that infection with more virulent strains causes over expression of pro-inflammatory cytokines and NO, which may ultimately contribute to the neurological problems (i.e. paralysis) associated with MDV. Regulatory cytokines are therefore important in creating a fine balance between the appropriate levels of expression of certain mediators.

NK cells also act in the first line of defense against viral pathogens by directly lysing infected cells. Either via ligation of cell death receptors or through the release of granzyme and perforin, NK cells induce rapid cell death. Expression levels of granzyme have been shown to be up-regulated at 4 and 7 d.p.i. suggesting that NK cells play an important role during the early cytolytic phase of infection (Sarson et al., 2006). Studies have shown increased activity of NK cells in resistant or vaccinated strains of MD compared to unvaccinated or susceptible strains of chickens (Quéré & Dambrine, 1988). This suggests a link between resistance to MD and NK cell activity. This link to resistance is associated with expression of MHC class I molecules. The low expression of MHC class I in B21 haplotypes leads to reduced inhibition of NK cells. As a result, these
chickens exhibit increased levels of NK-like cells in the spleen following infection conversely to the highly susceptible B19 haplotype (Garcia-Camacho et al., 2003).

1.1.9. Adaptive immune response

Although MDV is a cell-associated virus, and therefore, cell-mediated responses are more vital than antibody-mediated, antibodies still serve in establishing immunity against MD. For example, antibodies are critical in the neonate because maternally derived antibodies, which remain in the host’s circulation for approximately 3 weeks, can help reduce MDV infection (Chubb & Churchill, 1969). However, they may also interfere with vaccination via neutralization (Calnek, 1986). In the adult chicken, antibodies are produced against MDV glycoproteins gB, gE, and gI and serve a role in establishing protective immunity by blocking virus entry into host cells (Ikuta et al., 1984). Non-neutralizing antibodies also play a role in protective immunity against MDV. These antibodies may coat the surface of lymphocytes and block viral antigen sites on the host cell membrane thereby preventing virus spread during the lytic phase of infection. This type of antibody may also induce antibody-dependent cell-mediated cytotoxicity (ADCC) to aid in the lysis of infected host cells (Schat & Markowski-Grimsrud, 2001). It has also been discovered that antibodies against self-antigens are created during MDV infection. MD lymphomas express Hodgkin’s disease antigen CD30; therefore, anti-CD30 antibodies are generated after infection in resistant chickens (Burgess et al., 2004).

T cell-mediated immune responses are generated against MDV and play a role in immunity against the virus or lymphoma formation caused by this virus. When CD8+ T cells are depleted from the host immune repertoire, the result is a high MDV load within CD4+ T cells and immunosuppression (Morimura et al., 1998), which shows that the
former cells have a critical role in controlling the virus. Cytotoxic activity of CD8$^+$ T cells has been demonstrated against certain MDV antigens (i.e. pp38, meq, ICP4, gB) expressed on host cells in order to control infection (Schat et al., 1992). CD8$^+$ T cells are further shown to be a critical component of the adaptive immune response to MDV via their up-regulation of perforin and granzyme A at 4 and 7 d.p.i., which suggests they have a role in directly lysing transformed MDV cells. Meanwhile, CD4$^+$ T cells serve as targets for cell transformation by the virus. However, both αβ T cells are involved in the induction of cytokines and hence in the creation of a cytokine milieu during different stages of MDV pathogenesis.

Although the immune response to MDV is robust, the virus has evolved ways of evading innate response mechanisms by down-regulating surface expression of MHC class I on CTLs during cytolytic infection (Hunt et al., 2001). Intracellular levels of MHC class I remain unaffected; therefore, it seems plausible that the transportation of these molecules to the cell surface is blocked, rather than regulation of transcription or translation. A recent study has in fact determined that the MDV gene MDV012 is partially responsible for blocking surface expression of MHC class I (Hearn et al., 2015). MDV012 is a transporter associated with antigen processing (TAP)-blocking MHC class I immune evasion protein that prevents transportation of the peptide into the endoplasmic reticulum (ER) lumen from the cytosol rather than inhibiting transcription or translation of MHC class I. As a result of MHC class I down-regulation, epitopes from MDV antigens are not being presented as frequently to T cells and hence, the cell-mediated immune response is not working as efficiently as possible.
1.1.10. Vaccination

MD vaccines were the first commercial vaccines to be created for the purpose of protecting against an oncogenic virus. A year after the creation of the first live attenuated MDV vaccine, HPRS-16/Att, it was replaced with non-oncogenic HVT (Witter et al., 1970). HVT is still in use today either by itself or in combination with other types of MD vaccines (i.e. CVI988 and SB-1), which form bivalent or trivalent vaccines. These types of vaccines are widely used as a result of the evolution of more virulent strains. Originally, the subcutaneous route was used for vaccination. However, this method has recently changed to an in ovo route whereby the vaccine is administered at embryonic day (ED) 18 into the amniotic fluid prior to hatching. Although vaccines are efficacious against disease, they are unable to prevent infection; therefore, the virus is still able to shed into the environment and transmit to infect other chickens.

Although MD vaccines cause the infiltration of CD8$^+$ T cells to the feather pulp area in an effort to clear virus-infected cells, this measure is unable to reach replicating virus in the FFE (Abdul-Careem et al., 2008b). The activation of CTLs may be caused by the early and strong expression of IFN-$\gamma$ in spleen and later in feather tips following vaccination (Djeraba et al., 2002). In turn, the up-regulation of IFN-$\gamma$ may be a result of activation of innate immune system cells, such as macrophages and associated NO production, as expression of the latter is increased following vaccination of infected birds (Abdul-Careem et al., 2007). A third possible source of IFN-$\gamma$ is NK cells. Research has shown that vaccination is able to increase cytotoxic granule proteins and IFN-$\gamma$ production by NK cells coupled by increased CD107a expression, an NK cell activity marker (Heidari and Hunt, 2013).
Although vaccines are highly efficacious for control of Marek’s disease, more virulent strains of MDV have emerged and evolved to outcompete current vaccine practices. For example, a study by Parvizi et al. (2015) found that HVT vaccination of birds infected with a very virulent strain of MDV caused simultaneous expression of IFN-γ and IL-10. Expression of IL-10 could be either a regulatory strategy employed by the host or an immunoevasive mechanism employed by the virus. It seems that as new effective vaccines are developed, the virus responds by increasing its virulence and overcoming protection conferred by the vaccine. For this reason, it is crucial that novel and sustainable vaccine strategies be generated in order to prevent a recurrence of the 1960s mass mortality.

1.2. Avian γδ T cells

T cells represent a component of the adaptive immune system that is responsible for recognizing a wide range of pathogens. There are numerous similarities shared between the T cells of mammals and birds including the heterodimeric αβ or γδ TCR expressed on their cell surface. Although much is known about the αβ TCR, there is still relatively little known about the γδ TCR. It was not until the mid-1980s that this second TCR subset was acknowledged upon the discovery of the gamma chain of TCR in humans and mice. Although research on this topic has focused on mice and humans, the population of γδ T cells in these species is quite low (5%) (Haas, Pereira, & Tonegawa, 1993). In contrast, there is revived interest around this topic in other species (i.e. cattle, sheep, and chickens) that have a greater representation of these cells in the peripheral blood (30%-50%) (Arstila & Lassila, 1993) and tissues (Bucy, Chen, & Cooper, 1991). In
addition to this site, chicken γδ T cells can be found in other organs such as the caecum, spleen, thymus, and bursa of Fabricius (Tregaskes et al., 1995) where they have a role in immunological surveillance (Sanchez-Garcia & McCormack, 1996). The chicken is a good model in which to study the function of γδ T cells because they have well defined thymic waves of progenitors and thymocyte progeny as well as relatively high frequencies of γδ T cells compared to other species.

1.2.1. γδ T cell receptor

It has been suggested that αβ T cells arise when the γδ TCR fails to be expressed. The deletion of the Cδ gene on both alleles is crucial for the development of αβ T cells (De Villartay et al., 1988). Therefore, the δ chain, rather than the γ chain, is of utmost importance in the segregation of γδ and αβ lineages. Monoclonal antibodies have been developed to define the three lineages of chicken T cells in the order in which they appear in the embryonic thymus: TCR1 (γδ T cells), TCR2 (αβ T cells with Vβ1 genes) and TCR3 (αβ T cells with Vβ2 genes) (Chen et al., 1988; Chen et al., 1989). Therefore, γδ T cells can be detected using TCR1, which is a heterodimer linked with a disulfide bond between the 50 kDa and 40 kDa glycoprotein chains that are further noncovalently linked to CD3 in order to establish a signal transduction unit (Sowder et al., 1988).

Each chain of a TCR gene is composed of two domains: variable (V) and joining (J) segments. Certain chains, such as the beta and delta chains, also have a diversity (D) segment. These gene segments rearrange during development in order to create a diverse TCR repertoire. Although the genes responsible for creating an αβ TCR are similar between avian and mammalian species avian α and β loci are relatively simpler because they each only contain two V subfamilies (Göbel et al., 1994; Tjoelker et al., 1990).
contrast, the avian TCRγ locus is comprised of three Vγ subfamilies (containing 8-10 members), three Jγ gene segments, and one Cγ gene (Sowder et al., 1988). There seems to be a positive relationship between the complexity of a TCRγ chain and the relative abundance of γδ T cells. Therefore, it has been suggested that the number of possible VDJ rearrangements may correlate to the relative abundance of T cells (Six et al., 1996).

In terms of the TCRδ chains, they are encoded by the Vα1 and Vα2 gene segments (Chen et al., 1996).

1.2.2. Ontogeny

1.2.2.1. Intrathymic development

Avian T cell development follows three waves in which all three subpopulations are generated in the following order: TCR1, TCR2, and TCR3 (Cooper et al., 1991). Lymphoid progenitor cells (LPC) seed the thymus for 36 hours in chickens followed by a four-day period whereby the thymus becomes unresponsive to further seeding (Jotereau & LeDouarin, 1982). The thymus receives these LPCs in three sequential waves with cyclic periodicity followed by proliferation (Coltey, Jotereau, & LeDouarin, 1987). Chicken γδ T cells (TCR1+) are the first to appear in the thymus and by ED12, five days after the initial influx of lymphocyte precursors, certain thymocytes begin expressing γδ TCR on their surface. This number reaches a peak by ED15 when γδ T cells represent 30% of the thymocyte population (Sanchez-Garcia & McCormack, 1996). At the same time, cytoplasmic expression of the Vβ1 gene begins but their corresponding thymocytes do not appear until ED15. Following hatching on ED21, the number of TCR1+ cells drops by 20%. Unlike αβ T cells, chicken γδ T cells do not undergo positive and negative selection in the thymus. Instead, they show no phenotypic changes and migrate through
the thymus very quickly and exit without undergoing clonal expansion (Cooper et al., 1991).

The generation of γδ T cells as the first of three sequential waves is a phenomenon also noticed during their migration to peripheral organs (i.e. spleen and intestine) (Dunon, Cooper, & Imhof, 1993). By ED15 and ED16, γδ T cells are the first to colonize the spleen and the embryonic intestine. In the intestine, γδ T cells have a much higher proliferation rate than in the spleen, and, therefore, require less replenishment following emigration from the thymus. Although peripheral blood γδ T cells are CD4/CD8 double negative, around two thirds of intestinal and splenic γδ T cells express the CD8 molecule on their surface (Bucy et al., 1988). No CD4⁺ γδ T cells have been reported in the chicken.

1.2.2.2. Extrathymic development

It has been suggested that the intestinal epithelium may be an extrathymic site for γδ T cell development. When the thymus was removed as a potential source of newly developed γδ T cells, the population of TCR1⁺ cells in the intestinal epithelium only drops by 50% whereas those found in the spleen, cecal tonsils, and peripheral blood are reduced by up to 90% (Cihak et al., 1993). In contrast, other researchers have found intestinal γδ T cells to be of thymic origin (Dunon, Cooper, & Imhof, 1993). The first two waves of γδ T cell thymic development migrate to and seed the intestinal epithelium and spleen where they remain for a long period of time (75 days), which suggests that, at least during the early stages of development, these γδ T cells are not derived extrathymically. These data suggest that half of TCR1⁺ cells are thymus-dependent while the other half is independent of the thymus for development.
1.2.3. Tissue distribution

γδ T cells are generally localized within epithelial structures and in the splenic red pulp (Cooper et al., 1991). This is in contrast to αβ T cells that are found in the white pulp of the spleen rather than the red pulp. The implication behind these differing T cell localizations within the spleen remains to be elucidated. However, chicken γδ T cells, similar to αβ T cells, can also be found in other peripheral lymphoid tissues with the exception of germinal centers. Although chickens do not have lymph nodes, they do have lymphoid aggregates and structures, for example cecal tonsils, which are composed of lymphoid stroma and germinal centers. TCR1+ cells are occasionally found in the lymphoid stroma but this tissue tends to be mostly dominated by TCR2+ cells (Bucy, Chen, & Cooper, 1991). Chicken γδ T cells are abundantly found in the epithelium of the intestine as opposed to the lamina propria where αβ T cells are predominantly located. The localization of these cells within the intraepithelial lymphocyte population is a feature common to a range of other species including mice, humans, and ruminants. This conservation across species suggests that γδ T cells may play a major role in immunosurveillance and protection of mucosal surfaces.

1.2.4. Phenotypic markers

Chicken γδ T cells can be identified via the expression of the TCR1/CD3 complex; however, T cells also utilize CD4 and CD8 molecules. Most chicken γδ T cells, such as the peripheral blood population, are CD4−CD8− whereas in peripheral tissues, such as the spleen and intestinal epithelium, up to 75% and 66% of γδ T cells, respectively, express CD8 (Bucy et al., 1988). The CD8 antigen, just like its mammalian counterpart, can be expressed on the cell surface as either a homodimer (CD8αα) or as a
heterodimer (CD8αβ) (Tregaskes et al., 1995). Most CD8⁺ γδ T cells are heterodimeric CD8⁺ cells in the adult chicken and are generally found in the thymus, spleen, and blood. However, 60% of CD8⁺ γδ T cells within the intestinal epithelial lymphocytes (IELs) express the homodimeric form.

At the resting stage, avian γδ T cells express diminished levels of accessory and co-stimulatory molecules. Perhaps, as a result, these cells require a higher threshold level for activation than others (Bachmann et al., 1996). Nonetheless, once activated, avian γδ T cells up-regulate expression of certain markers including CD28, CD25, MHC class II antigens, and CD5 (Koskela, Arstila, & Lassila, 1998). All CD8⁺ γδ T cells are also CD28⁺ regardless of activation. However, γδ T cells are only MHC class II positive when they are activated as well as being CD28⁺. This co-stimulatory molecule is 50% homologous to that of mammals (Vainio et al., 1991). Activation of CD28 on CD28⁺ γδ T cells has been shown to stimulate these cells without the need of exogenous growth factors (Koskela, Arstila, & Lassila, 1998). Unlike mice, chickens are able to regulate the expression of CD28 on their T cells. Although CD2 is expressed on peripheral blood αβ T cells, chicken γδ T cells appear to be devoid of this antigen (Knabel, Cihak, & Lösch, 1993). Chicken IEL populations also express the A19 integrin-like antigen at high intensities (Haury et al., 1993). After chick hatching, T cells migrate from the thymus to the intestine, after which A19 becomes expressed. It could therefore be characterized as a late activation antigen that is expressed on all T cells. Of the intestinal γδ T cell population, 83% express A19 whereas NK cells and B cells exhibit no expression of the antigen. For some of the reasons mentioned above, this integrin-like antigen has been
suggested to function as an adhesion molecule in the retention of T cells to the intestinal epithelium (Haury et al., 1993).

1.2.5. Biological function

The functional role of γδ T cells in chickens has remained unexplored; however, as a result of their high numbers in this species, the chicken is a prime candidate in which to study the function of these cells. It is believed that γδ T cells were the first T cell to evolve and, as such, performed a role in first-line of defense via recognition of stressed epithelial cells (Janeway, 1988). With the simultaneous appearance and takeover of αβ T cells as the emerging novel epithelial defense system, it has been suggested that most γδ T cells were forced to develop new roles (Janeway, 1988). As a result of their NK-like killing activity, association with the epithelia, and frequent reactivity to heat shock proteins, it is possible that the primary function of γδ T cells may be in immunosurveillance or maintenance of homeostasis. More specifically, these cells could be involved in surveillance of the maternal/fetal interface during pregnancy, as they are the first cells to appear in the thymus. Additionally, the preferential localization of these cells to epithelial and mucosal tissues suggests that some γδ T cells may still be involved in regulation of first-line defenses.

The role of γδ T cells in response to bacterial infections such as Salmonella enterica in chickens has been investigated. Pieper, Methner, and Berndt (2011) have found increased expression of immune-related proteins by CD8αα⁺ high γδ T cells. Specifically, CD8αα⁺ γδ T cells were able to up-regulate expression of IFN-γ. Furthermore, it has been determined that these CD8αα⁺ γδ T cells express increased
CD25, which suggests a robust activation of these cells in response to bacterial pathogens (Braukmann, Methner, & Berndt, 2015).

Further work has investigated the role of γδ T cells in response to viruses, although most of the work focuses on mice and humans. Mice infected with vaccinia virus (VV) had γδ T cells with increased expression of IFN-γ within 2 d.p.i. (Selin et al., 2001). This rapid expansion of γδ T cells to VV could be due to a constantly partial state of activation whereby they are sensitive to stimulation, similar to NK cells and memory CD8 T cells. However, γδ T cells seem to lack high-affinity, pathogen-specific characteristics (Hayday, 2000). As a result of this broad specificity, they must be quickly down-regulated upon completion of their task. These researchers also discovered that γδ T cells exhibit a cytotoxic state without priming with VV. These cells were then able to lyse infected targets. This work demonstrates an important role for γδ T cells in the innate immune response and initial clearing of the virus.

However, another study in mice infected with West Nile virus showed that γδ T cells provide a link between the innate and adaptive immune response by having an indirect role in the latter (Wang et al., 2006). Of the mice that survived primary infection following a TCRδ knockout, only 15% had impaired CD8⁺ αβ memory T cell responses compared to wild-type mice of which up to 85% survived. However, depletion of γδ T cells prior to secondary challenge did not affect susceptibility to West Nile virus, which suggests that these cells do not act as memory cells but rather aid in maintenance of CD8⁺ αβ memory T cells.

A similar study investigating intravaginal herpes simplex virus 2 (HSV-2) in mice also determined an indirect role for γδ T cells in establishing the adaptive immune
response (Nishimura et al., 2004). Following HSV-2 infection, accumulation of epithelial γδ T cells in the uterus and vagina resulted in expression of the IFN-γ gene. This cytokine is important in helping produce a Th1-like cytokine milieu for the differentiation of CD4+ T cells. All mice depleted of γδ T cells died by day 16 and had significantly lower gene expression of IFN-γ by CD4+ T cells. These results indicate that γδ T cells have an important role in immunity against viruses. In mice, these cells play a direct role in the innate immune response by exhibiting a quick expansion upon exposure to the pathogen. They also are critical in initiating an appropriate adaptive immune response by creating the optimal environment for Th1-like producing CD4+ T cells and establishment of CD8+ memory T cells.

1.2.6. Activation

It is currently thought that γδ T cells recognize cell surface or secreted molecules that are expressed by pathogens or stressed cells during infection. Findings in mice have led to the idea of viral recognition by γδ T cells in the absence of antigen processing (Sciammas et al., 1994). The antigens being recognized are carbohydrates, lipids, phosphorylated non-peptide antigens (Tanaka et al., 1994), as well as epitopes of stress proteins and heat shock proteins (Born et al., 1990). It may even be possible for γδ T cells to recognize self-antigens in order to maintain homeostasis. It has been demonstrated that this method of stimulation increases the number of both CD8- and CD8+ γδ T cells. Some researchers have reported the 65 kDa heat shock protein (HSP65) to be a dominant mycobacterial antigen for γδ T cell activation (Arstila, Toivanen, & Lassila, 1995). A γδ T cell clone (TgI4.4) has recently been described to recognize a viral transmembrane glycoprotein from herpes simplex virus 1 in mice (Sciammas et al., 1994). Recognition of
this glycoprotein does not require processing or presentation via antigen presenting cells. In response to infection, these clones produce IFN-γ and lyse infected target cells. A recent study found that a combination of PMA and IL-2 led to significantly increased expression of CD25 expressing CD8α− and CD8αhiβ+ γδ T lymphocytes in addition to all γδ T cells from the peripheral blood (Polasky et al., 2016).

1.2.7. Interaction with αβ T cells

γδ T cells respond poorly to stimulation by mitogens or TCR ligation, except in the presence of αβ T cells. This is due to the inability of γδ T cells to produce IL-2 on their own; they therefore are dependent on soluble factors produced by αβ T cells (Arstila, Toivanen, & Lassila, 1995). The CD8+ subpopulation of γδ T cells, which is relatively large and expresses MHC class II antigens, responds best to mitogens (Arstila, Toivanen, & Lassila, 1995). This could indicate a mutual interaction between γδ T cells and αβ T cells whereby the former, when activated, can stimulate the latter via MHC class II/TCR interaction resulting in up or down-regulation of αβ T cells. There may also be interaction between the two cell subsets during thymic selection. If αβ T cells are absent from the initial population of double negative thymocytes, CD8+ γδ T cells do not arise (Spetz, Jourvilsky, & Larsson-Sciard, 1991).
EXPERIMENTAL APPROACH

Objective 1

Identify temporal and spatial distribution of γδ T cells in response to MDV.

Key steps involved:

- Infection of SPF chickens with 250 PFU of RB1B strain of MDV at 5 days of age
- Harvest lungs, spleen, cecal tonsils, and feather tips at 4, 10, and 21 d.p.i.
- Determine viral genome load using DNA extracted from feather tips via conventional PCR and real-time PCR
- Immunohistochemical staining of γδ T cell subset in lung tissues
- Flow cytometry of spleen and cecal tonsils using the following monoclonal antibodies: TCRγδ-FITC, CD8α-PE, CD4-APC, CD3-PB and a Live/Dead® Fixable Near-IR Dead cell stain kit

Objective 2

Determine the expression of cytokine genes produced by γδ T cells during MDV infection.

Key steps involved:

- Infection of SPF chickens with 250 PFU of RB1B strain of MDV at 5 days of age
- Harvest spleen at 4, 10, and 21 d.p.i.
- Determine viral genome load using DNA
- FACS sorting of spleen cells based on TCRγδ-FITC staining
- RNA extraction and complementary DNA (cDNA) synthesis
- Real-time PCR for expression of cytokine genes
CHAPTER 2

INVESTIGATION OF GAMMA DELTA T CELL POPULATIONS IN TISSUES OF MAREK’S DISEASE VIRUS-INFECTED CHICKENS

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2.1. Abstract

Gamma delta T cells are an important T cell subset found in high numbers in avian blood and tissue. However, little research has focused on the role of these cells against viral pathogens, specifically Marek’s disease virus (MDV). Therefore, in the present study we sought to evaluate the spatial and temporal distribution of γδ T cells in MDV-infected chickens (n=32) compared to uninfected age-matched control birds (n=32). There was a significant increase in the γδ T cell numbers in the spleen by day 21 post-infection and almost all (95-99%) of these cells were CD8α⁺ γδ T cells. In contrast, in the cecal tonsils of MDV-infected chickens, there was a significant reduction in the γδ T cell numbers at days 4 and 21 post-infection. Furthermore, we observed that the number of γδ T cells in the lungs of MDV-infected chickens was significantly increased only at day 10 post-infection. Collectively, our findings suggest that avian γδ T cells may play a role during the latency and transformation phases of MDV infection in tissues such as spleen and lungs and that the dominant population at play during the transformation phase is CD8α⁺ γδ T cells. Contrary to the spleen and lungs, cecal tonsils of infected chickens underwent a reduction in γδ T cell numbers during the early cytolytic and transformation phases. In conclusion, the present study demonstrates that late activation of γδ T cells may play a role in regulation of the inflammatory response. These findings shed light on the distribution of γδ T cells in certain tissues following MDV infection.

Key words: Marek’s disease, γδ T cells, immune response, innate lymphocytes.
2.2. Introduction

Marek’s disease (MD) is a highly infectious lymphoproliferative disease of chickens that can lead to malignant lymphoma formation in unvaccinated and genetically susceptible chickens. The disease is the result of infection with an avian herpesvirus known as Marek’s disease virus (MDV). MDV is a cell-associated virus that gains entry into its host through the respiratory system where it is taken up by phagocytic cells, such as macrophages, and spread to lymphoid organs (Haq, Schat, & Sharif, 2013). In the early cytolytic phase of infection, mainly B cells become infected with the virus (Shek et al., 1983). However, prior to entering the latent phase, activated CD4+ T cells become infected with MDV (Schat & Xing, 2000). During the neoplastic phase of infection, productive infection occurs in CD4+ T cells leading to the release of cell-free virus particles from the feather follicle epithelium (FFE) and skin epithelium (Heidari et al., 2016).

Both innate and adaptive immune responses play a key role in the host defense against MDV. In this regard, cell-mediated immune responses appear to play an important role in immunity, given the cell-associated nature of the virus. The role of CTLs in immunity has been investigated (Schat et al., 1992; Morimura et al., 1998). However, the function of other T cell subsets, including γδ T cells, has yet to be determined. γδ T cells are of interest in chickens because of their relative high frequency (up to 50%) in the peripheral blood and tissues. Although little is known about γδ T cells in chickens, studies in mice and humans have revealed that the number and function of these cells are increased in the course of infection with herpesviruses (Nishimura et al., 2004; Sciammas et al., 1997). For example, humans infected with herpes simplex virus
(HSV) exhibit a 24-fold increase in the number of γδ T cells in the peripheral blood by 10 days post-infection (d.p.i.) (Bukowski, Morita, & Brenner, 1994). The latter study concluded that killing of infected target cells was primarily due to the activation of γδ T cells that resulted in the lysing of infected target cells, with very limited natural killer (NK) cell and αβ T cell contribution. Similarly, in mice, γδ T cells are able to control viral replication following intranasal HSV infection by regulating the spread of virus to spinal cord tissue (Sciammas et al., 1997). In the context of influenza infection, Carding et al. (1990) found that mice infected intranasally with influenza A virus had an inflammatory response in the lungs predominated by γδ T cells. Similar to chickens, cattle also have a large representation of circulating γδ T cells and herpesvirus type I infection in cattle causes a substantial increase in peripheral blood γδ T cells (Amadori et al., 1995). Amadori and colleagues (1995) also noted that γδ T cells developed a NK-like phenotype in their ability to directly recognize heat shock proteins induced during the inflammatory response rather than requiring antigen processing, which ultimately allows for a much more rapid immune response.

In the present study, it was hypothesized that γδ T cells have an important role to play in anti-MDV defense in chickens. Therefore, we sought to investigate the spatial and temporal distribution of γδ T cells during MDV infection in chickens. It was expected that high numbers of γδ T cells could be found in lungs, spleen, and cecal tonsils of MDV-infected chickens. These numbers should peak during the early cytolytic phase as well as post-latency in the spleen and cecal tonsils because it is at these times that the virus is active in host tissues. In the lungs, increased numbers of γδ T cells may be observed as early as 4 days post-infection because this is the site of entry for the virus.
MDV is a constantly evolving virus and the response to this has been to create increasingly potent vaccines. Perhaps by further understanding the underlying immunological mechanisms that mediate immunity to MD, the need for these potent combination vaccines will no longer be required if we are able to create novel and more effective vaccines instead.

2.3. Materials and Methods

2.3.1. Experimental Animals

Day-old specific-pathogen free (SPF) White Leghorn chickens were received from the Canadian Food Inspection Agency (CFIA, Ottawa, Canada). Upon arrival, the birds were divided into two separate rooms in the Animal Isolation Unit, University of Guelph. The birds were all housed in isolators; however, the infected and control birds were in separate rooms. The experimental protocols used in this research were approved by the University of Guelph Animal Care Committee.

2.3.2. Experimental Design

Sixty four chickens were randomly divided into two groups: infected and uninfected controls and kept in separate rooms. On day 5 of age, the infected group of birds (n=32) received an intra-abdominal injection of the very virulent (vv) strain of MDV, RB1B, at a dose of 250 plaque-forming units (PFU). The rest (n=32) were kept as uninfected controls. This dose was previously determined by colleagues in our lab through unpublished data showing that by 21 d.p.i. 250 PFU of RB1B MDV resulted in 100% disease incidence in unvaccinated chickens (Abdul-Careem et al., 2007). At 4, 10,
and 21 d.p.i. ten birds per group were euthanized by CO₂ inhalation according to the Animal Care Committee guidelines. At necropsy, spleen and cecal tonsils were taken from each of the ten birds per group and placed in ice-cold 1x Hank’s balanced salt solution (HBSS) for use in creating a single-cell suspension to then be stained with antibodies and analyzed by flow cytometry. Additionally, lung and feather tip samples were snap frozen in optimal cutting temperature (OCT) embedding medium (Clear Frozen Section Compound, VWR Scientific, Mississauga, Canada) for immunohistochemistry. DNA extraction was carried out for all chickens in each group and each time point, therefore, three feather tips per bird were taken and stored in RNAlater (Qiagen Inc., Mississauga, Canada) at -20°C.

2.3.3. Spleen and cecal tonsil single-cell suspension

Spleen and cecal tonsil tissue were used for preparing single-cell suspensions. Whole spleens were rinsed three times with room temperature 1x HBSS then crushed directly on a 40 µm cell strainer and with the flat end of a disposable 5 ml syringe. Cells were passed through a strainer with 10 ml of growth medium (RPMI with 10% FBS, 2.5 ml gentamicin, 5 ml penicillin/streptomycin, and 0.175 ml 2-mercaptoethanol) and collected in a 15 ml conical tube. After centrifugation at 100 x g for 5 minutes and 4°C, the pellet was distorted by gently flicking. 1x ACK (Ammonium-Chloride-Potassium) lysis buffer (BioWhittaker®, Lonza, VWR Scientific, Mississauga, Canada) was added at a volume of 1.5 ml per pellet and left at room temperature for 5 minutes. In order to deactivate the lysis buffer, 13.5 ml of growth medium was added and cells were pelleted
again. Spleen cells were resuspended in 5 ml of growth medium and counted to adjust cell density to $10^7$ cells/ml.

Cecal tonsils were crushed directly on wire mesh with the flat end of a disposable 5 ml syringe and passed through a 40 µm cell strainer. These cells were pelleted at 100 x g for 5 minutes and 4°C then resuspended in 1 ml of growth medium and counted to adjust the density to $10^7$ cells/ml. Cells were pelleted and resuspended in the appropriate amount of FACS (fluorescence activated cell sorting) buffer (1% BSA in PBS).

2.3.4. Flow cytometry

Cells were plated in 96 well round-bottomed plates with each well containing 100 µl, or $10^6$ cells, and 50 µl/well of staining buffer at an antibody dilution of 1:100 in FACS buffer. The following mouse anti-chicken monoclonal (mAb) antibodies (Southern Biotech, Cedarlane Laboratories, Burlington, Canada) were used: mAb mouse anti-chicken TCRγδ conjugated with Fluorescein (FITC), mAb mouse anti-chicken CD3 conjugated with Pacific Blue™ (PB), mAb mouse anti-chicken CD4-Alexa Fluor® 647, mAb mouse anti-chicken CD8α Phycoerythrin (PE), and Live/Dead® Fixable Near-IR dead cell stain kit (Thermofisher Scientific, Markham, Canada). Primary antibodies were added to each well for 30 minutes on ice and covered from the light. Cells were then washed twice in FACS buffer and pelleted at 100 x g for 5 minutes and at 4°C. Finally, cells were resuspended in 2% paraformaldehyde (PFA) (in PBS), transferred to 5 ml round-bottom polystyrene tubes (BD Falcon 352052) and kept covered on ice until analysis.
Alongside these samples were also negative controls (two replicates each), which contained unstained cells and cells treated with one of each stain to serve as compensation controls. These wells received the same 1:100 antibody dilution as the previous samples. All samples were analyzed using the FACSCanto II flow cytometer (Beckton Dickinson Biosciences, San Jose, CA, USA).

2.3.5. Data analysis

All samples within this experiment were analyzed individually using the FlowJo software (Tree Star, Ashland, OR, USA). Initial gating involved all live cells, which were detected by the Live/Dead® Fixable Near-IR dead cell stain kit. Next, using a SSC-A versus FSC-A plot, all granulocytes were gated out (i.e. heterophils, eosinophils, and basophils) in order to have a population more representative of lymphocytes. Then through a two-step process involving FSC-W versus FSC-H followed by SSC-W versus SSC-H, all doublets were eliminated in order to avoid false positive stains. Individual stains were then visualized against SSC-A (e.g. γδ TCR, CD8α, CD4). Final percentages were subsequently calculated and statistical significance was determined with a two-tailed student’s t test using GraphPad Prism software (La Jolla, CA, USA). Data are presented as a comparison between absolute numbers of control and infected groups. These numbers were achieved by back gating using the initial live cell count from whole spleens. Results were considered statistically significant if \( p \leq 0.05 (*) \), \( p < 0.01 (**) \), \( p < 0.001 (***) \), or \( p \leq 0.0001 (****) \).
2.3.6. Immunohistological observation

Lung and feather tips, preserved in OCT, were sectioned to a thickness of 5 µm using a cryotome (Leica CM 3050 S, Vashaw Scientific Inc., Norcross, Atlanta, GA, USA), adhered to superfrost-plus microscope slides (Thermofisher Scientific, Markham, Canada) and stored at in -20°C until used. The unlabeled mAb mouse anti-chicken TCRγδ (Southern Biotech, Cedarlane, Burlington, Canada) antibody was used at a 1:500 dilution in blocking buffer. To avoid nonspecific binding of the antibody, 5% goat serum made in PBS was added to the slides for 30 minutes in a humidifying chamber. The samples were incubated with the monoclonal antibody for 30 minutes in a humidifying chamber followed by rinsing and incubation with the secondary antibody, biotinylated goat anti-mouse IgG (H+L) (Vector Laboratories, Burlington, Canada) for 30 minutes. Slides were then treated with a solution of 0.3% hydrogen peroxide in 0.3% goat serum made in PBS for 10 minutes to quench for endogenous peroxidase activity. Immunoperoxidase staining was accomplished using the avidin-biotin-peroxidase complex (ABC) system (Vectastain® ABC kit, Vector Laboratories, Burlington, Canada) according to the manufacturer’s directions. Antigen labeling was visualized by incubation with 3,3-diaminobenzidine-H2O2 solution (DAB substrate kit, Vector Laboratories, Burlington, Canada) for approximately 7 minutes. Hematoxylin was used for counterstaining and lastly slides were mounted in Cytoseal-60 (Richard-Allan Scientific, Thermofisher Scientific, Markham, Canada).

All tissue samples in both groups (n=12) per time point were examined under a microscope. γδ T cell infiltration was assessed quantitatively by randomly choosing five fields at 400x magnification, counting the number of positively stained cells, and determining an average. Negative controls (no primary antibody plus secondary
antibody) and positive controls (staining on spleen samples) were also used to ensure appropriate staining.

2.3.7. DNA extraction

Two feather tips were chopped with a scalpel blade and mixed with 500 µl of cell lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 1 mM EDTA, pH 8.0 with 5% (w/v) Sarkosyl) and 100 µl of proteinase K (10 mg/ml in nuclease free water). These samples were incubated overnight in a water bath at 65°C. Then 25 µl 5 M NaCl and 2.3 ml 95% ethanol were added. These solutions were then centrifuged at 2000 x g for 2 minutes and the DNA pellet was washed twice with 70% ice-cold ethanol. Finally 100 µl Tris-EDTA (TE), pH 8.0 was added and residual ethanol was evaporated by placing open tubes in a waterbath at 65°C. DNA concentration was measured using a Nanodrop and adjusted to 50 ng/µl for all samples.

2.3.8. Conventional PCR

In order to detect presence of meq, conventional PCR was conducted with the use of a TGradient thermocycler (Biometra® GmBH, Montreal, Canada). In a total volume of 25 µl, 100 ng of template DNA was added to 1x PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 mM of the forward and reverse primer each, and 1.25 U Taq DNA polymerase. The conditions set for this reaction were as follows: pre-incubation and denaturation at 94°C for 3 min, then 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min, finishing with a final extension at 72°C for 5 min. PCR products from each
time point and both groups were run on a 2% agarose gel and stained with SYBR Safe (Invitrogen, Thermofisher Scientific, Markham, Canada).

2.3.9. Generation of standard curves

For construction of a meq standard curve, 10-fold serial dilutions ($10^{-1}$ to $10^{-9}$) of the plasmid DNA was made and assayed in triplicates. Previously designed primers (Forward 5’-GTCCCCCCTCGATCTTTCTC-3’; Reverse 5’-CGTCTGCTTCCTGCCTCCTTC-3’) specific for meq gene of MDV serotype 1 were used. The value recorded for the slope of the curve was -3.865, which yielded PCR efficiency (E) of 1.814. MDV genome load was quantified as previously described by Abdul-Careem et al. (2006a).

2.3.10. Real-time PCR

All DNA samples that were determined by conventional PCR to be meq positive were further analyzed by real-time PCR in order to quantify viral genome load. LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Laval, Canada) was used in a final volume of 20 µl including 100 ng of template DNA and 0.25 mM of both forward and reverse meq primers. A dilution of the pMeq standard was also included as well as a water control and negative control (no template DNA). The thermal cycling parameters were as follows: pre-incubation and denaturation at 95°C for 10 min; 45 cycles of amplification at 95°C for 10 s (segment 1), 64°C for 5 s (segment 2), and 72°C for 8 s (segment 3); melting at 95°C for 5 s (segment 1), 65°C for 1 min (segment 2) and 97°C for continuous acquisition (segment 3); cooling at 40°C for 10 s.
2.4. Results

2.4.1. MDV meq genome copy number in feather tips of MDV-infected chickens

As determined by conventional PCR, all the samples from uninfected control groups were meq negative. Additionally, samples from the infected chickens at 4 d.p.i. were meq negative while those at 10 and 21 d.p.i. were meq positive (Figure 1). As shown in Figure 1, infected chickens had high meq copy numbers with a mean of 2.98x10^6 in their feather follicles at 10 d.p.i.. Furthermore, the meq copy numbers were significantly higher (p ≤ 0.0001) at 21 d.p.i. indicating an increased virus shedding during the transformation stage of MDV infection.

2.4.2. Analysis of splenic γδ T cells in MDV-infected chickens

To determine changes in the splenic γδ T cell population in MDV-infected chickens, we isolated splenocytes at 4, 10, and 21 d.p.i. and stained with an anti-TCRγδ specific monoclonal antibody for flow cytometry analysis. As shown in Figure 2a, the splenic γδ T cell frequency in MDV-infected chickens was significantly reduced at all time points when compared to control birds. In contrast to their frequency, we found that the MDV-infected chickens had significantly higher absolute numbers of γδ T cells in their spleens at day 21 post-infection compared to uninfected control chickens at the same time point (Figure 2b). However, no significant changes in the number of splenic γδ T cells at days 4 and 10 post-infection were observed between infected and control chickens. Since the absolute counts, which were based on γδ T cell frequency normalized to the total number of cells collected from the whole spleen, represent a more raw and
realistic analysis of the cellular changes, our results are based on numbers rather than cell frequencies.

2.4.3. Evaluation of splenic γδ T cell subsets in MDV-infected chickens

From previous studies, it has been determined that there are three subsets of γδ T cells: CD8⁺, CD4⁺, or CD4⁻CD8⁻ double negative γδ T cells (Bucy, Chen, & Cooper, 1991). Following analysis of total γδ T cell counts in the spleens of infected birds, we sought to evaluate frequencies of splenic γδ T cell subsets in these chickens by flow cytometry. As shown in Figure 3, chickens infected with MDV had significantly higher frequencies of splenic CD8α⁺ γδ T cells by about 4 fold at day 21 post-infection compared to controls. The frequency of CD8α⁺ γδ T cells ranged between 95-100% of the total splenic γδ T cells in the infected birds (Figure 4). The normal range for splenic γδ T cell subsets in healthy birds at all the time points was 65-70% for CD4⁻CD8α⁻ double negative, 30-35% for CD8α⁺ γδ T cells and 0.5-1% for CD4⁺ γδ T cells (Figure 3).

2.4.4. Analysis of γδ T cells in cecal tonsils of MDV-infected chickens

Similar to splenic γδ T cell analysis, we further sought to analyze the changes in γδ T cell populations in the cecal tonsils collected at days 4, 10, and 21 post-infection from MDV-infected and uninfected control chickens. No changes in the frequency of cecal tonsil γδ T cells were observed at any of the three time points between infected and control chickens (Figure 5a). Unlike in spleens, we found that the MDV-infected chickens had significantly lower numbers of γδ T cells in their cecal tonsils at days 4 and 21 post-infection compared to uninfected chickens (Figure 5b).
2.4.5. Evaluation of γδ T cell subsets in cecal tonsils of MDV-infected chickens

We further evaluated the frequency of γδ T cell subsets in these chickens and found that chickens infected with MDV had relatively higher frequencies (60%) of cecal tonsil CD8α+ γδ T cells at day 21 post-infection compared to controls (Figure 6) though not significant. The normal range for γδ T cell subsets in the cecal tonsils of healthy birds was about 55-60% for CD4−CD8α− double negative, 35-40% for CD8α+ γδ T cells and 0.5-1% for CD4+ γδ T cells.

2.4.5. Evaluation of γδ T cells in lungs of MDV-infected chickens

To detect the presence of γδ T cells in the lungs of MDV-infected birds, we performed immunohistochemistry on this tissue. Representative images for control and infected lungs at each time points are shown in Figure 7. As illustrated in Figure 8, there was no significant difference at 4 d.p.i.; however, at 10 d.p.i. there was a significant increase in the number of γδ T cells in the lungs of MDV-infected birds ($p \leq 0.05$). Again, at 21 d.p.i., there was no significant difference in the number of γδ T cells between control and infected chickens.

2.5. Discussion

Different cell subsets belonging to the innate and adaptive components of the immune system have been associated with the avian host defense against MDV. Although avian γδ T cells have been implicated in antimicrobial defense (Gao et al., 2003), very little is known about their role against viral pathogens, particularly in the
context of MDV. The present study investigated the induction of γδ T cells in response to MDV infection in chickens. Three important observations emerged from the present study. First, induction of splenic γδ T cells in response to MDV infection was significantly higher at 21 d.p.i. and almost all of these cells were CD8α+ γδ T cells. Second, in contrast to the spleen, in the cecal tonsils of chickens infected with MDV, there was a significant reduction in γδ T cell numbers at 4 and 21 d.p.i. compared to uninfected controls but the subset of this population at 21 d.p.i. was predominantly of the CD8α+ phenotype. Third, the number of γδ T cells in the lungs of MDV-infected chickens was significantly increased at day 10 post-infection.

The present study demonstrated that γδ T cells may play a role in immunity against MDV; however, this role may be more apparent by 21 days post-infection in the spleen. This is in contrast to other studies investigating the role of γδ T cells against viruses. It is generally found that immediately following infection with DNA viruses, mice experience rapid expansion of γδ T cells in the peripheral blood and spleen. This observation stems from several studies investigating γδ T cell immune responses against herpes simplex virus, cytomegalovirus, hepatitis B, and vaccinia virus infections (Nishimura et al., 2004; Ninomiya et al., 2000; Sing et al., 1998; Selin et al., 2001). However, a study by Carding et al. (1990) revealed that mice infected with influenza A virus exhibited a similar late response to infection by γδ T cells. This response was paralleled by an increasing number of macrophages expressing heat shock proteins (hsp). It has been shown that hsp are dominant mycobacterial antigens that enable γδ T cell activation in mice (Born et al., 1990). Hsp are also present during the transformation phase of MDV infection, as their interaction with MDV oncoprotein meq has been shown
to inhibit apoptosis of infected cells (Zhao et al., 2009). In the challenge model used in the present study, transformation occurs in MDV-infected chickens prior to, or around, 21 d.p.i., the phase at which significantly higher levels of γδ T cells were seen in this study. These cells may be responding to increased levels of hsp during this phase of pathogenesis in an effort to resolve the MDV-induced immune response in the spleen. Although MHC down-regulation is a known strategy used by herpes viruses to evade detection by the host’s immune system (Levy et al., 2003), it is unlikely to have a role in blocking γδ T cell activation during the early phase of infection, as these cells are known to recognize surface antigens of viruses in an MHC-independent fashion (Sciammas et al., 1994). This further supports the idea that γδ T cells are involved in the later phases of viral infection involving regulation of the host response.

Although the absolute number of γδ T cells increased at 21 d.p.i., cell frequencies were found to be significantly reduced at all time points post-infection. This is likely the result of spleen enlargement due to infiltration or expansion of cells, which is a common feature of MDV infection in chickens. With the increased number of cells accumulating in the spleen at this stage of pathogenesis, there was a reduction in the percentage of γδ T cells relative to other cell subtypes. Therefore, as this was not an absolute reduction in frequency, the absolute number counts represent a more realistic picture of the events as they show the actual number of γδ T cells in the tissue rather than their frequency relative to other more frequent and increasing cell subtypes.

This present study also evaluated γδ T cell number and frequency in the cecal tonsils of MDV-infected chickens. Results were, however, reported as absolute numbers because cecal tonsils are known to undergo transient atrophy following MDV infection.
(Heidari, Fitzgerald, & Zhang, 2014). The result is a depletion of lymphoid cells during the early phases of infection followed by an intense immunological response, mediated by the host, leading to recovery of germinal centers and cell numbers (Heidari, Fitzgerald, & Zhang, 2015). In the present study, there were significantly lower numbers of γδ T cells in the cecal tonsils of chickens infected with MDV at early cytolytic as well as transformation phases of infection compared to uninfected chickens. The depletion of γδ T cells early in infection is consistent with transient cecal atrophy. The study by Heidari, Fitzgerald, and Zhang (2014) used a bacterial artificial chromosome (BAC)-cloned very virulent strain of MDV. It is possible that with the use of a wild type parent strain, such as RB1B, irreversible atrophy of cecal tonsils may result. This would provide a reasonable explanation for the continued decrease of γδ T cells at 21 d.p.i. in our study.

An important observation from the present study was that the predominant γδ T cell subset in spleens during the transformation phase was of the CD8α⁺ phenotype. This finding was more evident in the spleen in which almost all γδ T cells were CD8α⁺. In support of our finding, Berndt and Methner (2001) have previously reported similar findings where they demonstrated the participation of CD8α⁺ γδ T cells in the elimination of Salmonella enterica serovar Typhimurium in chickens. The mechanism for clearance of this pathogen was suggested to be a direct cell-to-cell contact killing of infected cells by γδ T cells. Previous reports have also demonstrated the ability of human γδ T cells to express perforin and granzyme as determined by the presence of CD107a (Qin et al., 2009; Dieli et al., 2001).

MDV gains entry to the host through the respiratory system and establishes an initial infection in the lungs followed by spreading to secondary lymphoid organs. In the
present study, we observed a significant increase in the number of γδ T cells in the lungs of MDV-infected birds at 10 days post-infection (latency). It was expected that there would be an immediate increase in the number of γδ T cells in the lungs following MDV infection because this is the site of virus entry and where macrophages become infected with the virus (Barrow et al., 2003). It is also known that there is an immediate increase in macrophage numbers in the lungs of MDV-infected birds (Abdul-Careem et al., 2009a). The increasing number of macrophages could entail an up-regulation of hsp, which would lead to infiltration of γδ T cells into the lungs in order to resolve the inflammatory response (Carding et al., 1990). Consistent with our results, a previous report also found that mice infected intranasally with Sendai virus had significantly higher numbers of γδ T cells in their lungs (Hou et al., 1992). The late increase in γδ T cells in the lungs of mice was also accompanied by a high number of macrophages expressing hsp. This again establishes a role for γδ T cells in regulating events that lead to the termination of T-cell mediated inflammatory responses.

In summary, our findings suggest that the chicken γδ T cells may be involved in host responses during latency and transformation phases of MDV infection in tissues such as spleen and lungs. This study also showed an increase in the number and frequency of CD8α+ γδ T cells during the transformation phase of infection with a possible role in killing virus-infected cells and virus clearance. Furthermore, the reduction in γδ T cell numbers in cecal tonsils of infected chickens is likely a result of MDV-induced atrophy of cecal lymphoid tissues. Although this suggests that γδ T cells are unable to exert a function in cecal tonsils of MDV-infected chickens due to lymphoid cell depletion, their late expansion in the spleen and lungs elucidates a possible role in
regulating and clearing the inflammatory response. As a prolonged inflammatory
response may trigger autoimmune diseases, it is crucial to further our understanding of
the effects of activated $\gamma\delta$ T cells during these stages of MDV infection.

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Figure 1: MDV genome copy number in feather follicles at 4, 10, and 21 d.p.i. 

Meq is a viral oncogene of MDV that was used to calculate virus copy number from 100 ng of DNA. The groups included in this representation were MDV-infected birds only as control birds lacked the presence of meq. Data presented are the mean ± SEM for ten biological replicates at each time point. Asterisks denote a significant difference between groups ($p \leq 0.0001$ (***)).
**Figure 2:** Gamma delta T cell expression in spleen represented as (2a) percentage and (2b) absolute numbers

MDV-infected and uninfected chickens were sampled at 4, 10, and 21 d.p.i.. Group mean percentage or absolute number of cells is represented and error bars indicate standard error of the mean (SEM). Data means include ten biological replicates at each time point. Black and grey bars represent uninfected and infected groups, respectively. Asterisks denote a significant difference between groups ($p \leq 0.05$ (*), or $p < 0.001$ (***)).
Figure 3: Frequency of the three subsets of gamma delta T cells represented in the spleen. Infected and uninfected chickens are shown at 4, 10, and 21 d.p.i.. Group mean is represented and includes ten biological replicates at each time point. Green, red, and blue bars represent the CD4⁻CD8⁻ double negative, CD8α⁺, and CD4⁺ populations, respectively. Y-axis indicates the percentage that each subset represents out of the total population of γδ T cells.
Figure 4: Contour plot representation of gamma delta T cells expressing the CD8 marker. This is an example of an uninfected (left) and infected (right) bird at 21 d.p.i.. SSC-A is represented as the Y-axis and the CD8α antibody as the X-axis. The number of events collected was 300,000. Data are the percentages of γδ T cells either expressing or not expressing the CD8α antibody.
Figure 5: Gamma delta T cell expression in cecal tonsils represented as (5a) percentage and (5b) absolute numbers.
Infected and uninfected chickens were sampled at 4, 10, and 21 d.p.i.. Group mean percentage or absolute number of cells is represented and error bars indicate SEM. Data means include ten biological replicates at each time point. Black and grey bars represent uninfected and infected groups, respectively. Asterisks denote a significant difference between groups ($p < 0.01 (**), p < 0.001 (***)$).
**Figure 6:** Frequency of the three subsets of gamma delta T cells in cecal tonsils
Infected and uninfected chickens are shown at 4, 10, and 21 d.p.i.. Group mean is represented and includes ten biological replicates at each time point. Green, red, and blue bars represent the CD4⁻CD8⁻ double negative, CD8α⁺, and CD4⁺ populations, respectively. Y-axis indicates the percentage that each subset represents out of the total population of γδ T cells.
Figure 7: Immunohistochemistry images of lungs of chickens infected with MDV and uninfected controls for gamma delta T cells

Sections of lungs of uninfected controls are shown in A, C, and E, whereas B, D, and F represent lung sections from MDV-infected chickens. Lung tissue was sectioned at 5µm and stained with mouse anti-chicken TCRγδ mAb using the ABC and DAB kits. γδ T cells were counted by taking the average number of positively stained cells per field of view in five random fields at 400x magnification. Bar = 20 µm. Brown spots show positively stained γδ T cells. a = inter-parabronchial septa, b = air capillaries.
**Figure 8:** Number of gamma delta T cells in lungs of chickens infected with MDV
Black and grey bars represent uninfected and infected groups, respectively. There were six chickens in each group at each time point. Five 400x microscopic fields were taken per bird and a mean was calculated. Group mean is presented and the error bars represent SEM. Asterisks denote a significant difference between groups ($p \leq 0.05$ (*)).
CHAPTER 3

CYTOKINE GENE EXPRESSION IN FRACTIONATED GAMMA DELTA T CELLS OF CHICKENS INFECTED WITH MAREK’S DISEASE VIRUS

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3.1. Abstract

Avian γδ T cells are found in high numbers in blood and tissues relative to humans and mice. Although they have been implicated as an important cell type in antimicrobial defense, very little is known about their role in defense against viruses, particular herpes viruses. In the present study, we sought to elucidate the cytokine-based immune response of chicken γδ T cells during the course of Marek’s disease virus (MDV) infection. The gene expression experiments showed that γδ T cells had significantly up-regulated expression of interferon (IFN)-γ and interleukin (IL)-13 genes during the early cytolytic phase (day 4) of infection compared to age-matched uninfected controls. In addition, at day 21 (transformation phase), gene expression of IFN-γ in MDV-infected chickens was significantly higher compared to untreated controls birds. Significant IL-10 transcriptional up-regulation was observed at days 10 (latency phase) and 21 post-infection. Furthermore, we also found that γδ T cells harvested at day 4 had significantly higher Toll-like receptor (TLR)3 gene expression. Collectively, our data suggest that while the γδ T cell responses to MDV infection in chickens induce largely an IFN-γ mediated response, the later course of their response is mostly of a regulatory phenotype, marked by expression of IL-10 gene. The present study also signifies the possible involvement of TLR3 in the recognition of MD virus by γδ T cells.

Key words: avian γδ T cells, Marek’s disease, cytokines, Toll-like receptor immunity.
3.2. Introduction

Marek’s disease is a highly infectious lymphoproliferative disease caused by Marek’s disease virus (MDV). MDV is a member of the genus *Mardivirus* and classified as gallid herpesvirus type 2 (GaHV-2), or MDV-1. MDV enters the host through the respiratory system as it is inhaled from an environment contaminated with the virus. It is then transported from the lungs to secondary lymphoid organs by macrophages (Barrow et al., 2003). During this first phase of infection, known as the early cytolytic phase, B cells become infected with the virus (Baaten et al., 2009). With the switch from active infection to latency, between 7 and 10 days post-infection (d.p.i.), MDV begins infecting mostly the activated CD4\(^+\) T cells (Schat & Xing, 2000). When the virus becomes reactivated, it is released from infected CD4\(^+\) T cells undergoing lysis and sheds from the feather follicle epithelium (FFE). There is a fourth phase that may occur, known as transformation. This phase is characterized by the formation of lymphomatous lesions whereby death is commonly the final outcome.

The host response to MDV infection first involves the innate immune response followed by a more specialized adaptive immune response. The spleen is an important tissue to examine in studying the immune response to MDV because the viral genome load is high in this tissue, particularly later in infection (Abdul-Careem et al., 2007). The response in the spleen is composed of many immune system cells interacting and eliciting appropriate responses. Such responses initially include recognition of the virus by Toll-like receptors (TLRs) followed by an adaptive response that is tailored to respond to a particular pathogen, such as MDV. For example, CD4\(^+\) T cells can influence the environment by creating different cytokine milieus depending on the stage of MDV
pathogenesis. Both T helper (Th1- and Th2-like cytokines have been shown to be up-regulated in the spleen of MDV-infected chickens at different phases of infection (Parvizi et al., 2009; Sarson et al., 2006; Abdul-Careem et al., 2007). A study by Parvizi et al. (2009) examined the expression profile of splenic CD4$^+$ and CD8$^+$ T cells and suggested that these cells tend to elicit more of a Th1-like response during the early cytolytic (day 4) and transformation (day 21) phases. However, another study (Heidari, Zhang, & Sharif, 2008) found that cytokines expressed in the whole splenic tissue showed a Th2-like immune response during the early cytolytic phase of infection. Due to the fact that CD4$^+$ and CD8$^+$ T cells were shown to elicit a Th1 immune response, the findings from the latter study suggest that there could be other cell subsets at play in the spleen steering the overall response towards a Th2 immune response. Other cell subsets that remain to be studied in response to MDV include γδ T cells. In fact, a recent study by Heidari et al. (2016), demonstrated a large transmigration of γδ T cells towards the skin tissues of MDV infected birds, which suggests a role in clearing the virus.

It has been demonstrated that murine γδ T cells exhibit plasticity in their response depending on the nature of the pathogen (Ferrick et al., 1995). In the study by Ferrick et al. (1995), the intracellular bacterium, *Listeria monocytogenes*, caused an increase in gene expression of IFN-γ by γδ T cells in mice whereby the extracellular parasite, *Nippostrongylus brasiliensis*, induced gene expression of IL-4 by γδ T cells. Furthermore, research on avian CD8$^+$ γδ T cells has shown mRNA expression of IFN-γ but not IL-4 in response to *Salmonella*, which classifies these immune system cells as Th1 polarized during Salmonellosis (Pieper, Methner, & Berndt, 2008). This plasticity in response demonstrates that γδ T cells are able to discriminate between parasites and
bacteria that are posing a risk to the host and respond in the appropriate fashion. Therefore, it is important to determine whether they are Th1 or Th2 cytokine-producing cells. Previous work has also shown that chicken γδ T cells are able to express certain TLRs, in particular TLR3 (Iqbal, Philbin, & Smith, 2005). With the ability to express pattern recognition receptors (PRRs) such as TLRs and produce cytokines, chicken γδ T cells should be capable of detecting and responding to a viral invasion thereby establishing a first line of defense. The cytokines produced by γδ T cells in response to infection may also aid in creating a cytokine milieu with the aim of influencing the differentiation of CD4+ T cells into a Th1 or Th2 subset.

The range of cytokines produced by γδ T cells following infection with MDV has not yet been studied. We hypothesized an initial increase in the gene expression of pro-inflammatory cytokines as γδ T cells initiate the immune response and establish a Th1 cytokine milieu. However, later in infection we expect there should be an up-regulation of regulatory cytokines as γδ T cells attempt to control the immune response and prevent an exaggerated response caused by over expression of pro-inflammatory cytokines. Therefore, in the present study, we sought to investigate the immune response of chicken γδ T cells during the course of MDV infection.

3.3. Materials and Methods

3.3.1. Experimental Animals

Day-old specific-pathogen free (SPF) White Leghorn chickens were received from the Canadian Food Inspection Agency (CFIA, Ottawa, Canada). Upon arrival, the birds were separated into isolators in one room in the Animal Isolation Unit, University
of Guelph. The experimental protocols used in this research were approved by the University of Guelph Animal Care Committee.

3.3.2. Experimental Design

73 chickens were randomly divided into two groups: infected and uninfected controls. On day five of age, the infected group of birds (n=37) received an intra-abdominal injection of the very virulent (vv) strain of MDV, RB1B, at a dose of 250 plaque-forming units (PFU). The rest (n=36) were kept as uninfected controls. This dose was previously determined by colleagues in our lab through unpublished data showing that by 21 d.p.i. 250 PFU of RB1B MDV resulted in 100% disease incidence in unvaccinated chickens (Abdul-Careem et al., 2007). The number of birds euthanized at each time point differed because of the size of the spleen at that age such that younger birds had smaller spleens with insufficient numbers of cells. A total of six samples were required from both control and infected groups at each time point. Therefore, on day 4 post-infection, 18 random birds from both groups were euthanized and spleens were pooled in order to harvest an adequate number of cells. At day 10 post-infection, 10 birds per group were euthanized and by day 21 post-infection, 6 spleens per group were sufficient. Birds were euthanized by CO₂ inhalation according to the Animal Care Committee guidelines. At necropsy, the whole spleen was taken from each bird and placed in ice-cold 1x Hank’s balanced salt solution (HBSS) for use in creating a single-cell suspension. Additionally, three feather tips per bird were taken from the left wing tip and stored in RINalater (Qiagen, Mississauga, Canada) at -20°C. Feather tips were used
for DNA extraction for the purpose of MDV genome quantification (Abdul-Careem et al., 2006a).

3.3.3. Spleen single-cell suspension

Spleens were used to create a single-cell suspension. The spleen tissues were rinsed three times with room temperature 1x HBSS then crushed directly on a 40 µm cell strainer and with the flat end of a disposable 5 ml syringe. For days 4 and 10 post-infection, two to three spleens were pooled to create one sample. Cells were passed through the strainer with 10 ml of growth medium (RPMI with 10% FBS, 2.5 ml gentamicin, 5 ml penicillin/streptomycin) and collected in a 15 ml conical tube. After centrifugation at 400 x g for 10 minutes and 4°C, the pellet was distorted by gently flicking. Cells were overlaid on Histopaque®1077 at a 1:1 ratio. After centrifugation at room temperature for 30 minutes and 400 x g, the interface was collected and added to 10 ml growth medium. Cells were washed twice at 4°C for 10 minutes at 400g and finally resuspended in 5 ml of growth medium. They were then counted and cell viability was determined to be greater than 90% using Trypan Blue. Cell density was then adjusted to 2E7 live cells/ml.

3.3.4. T cell fractionation

To obtain a pure population of γδ T cells, single-cell suspensions of splenocytes were stained with the mouse anti-chicken TCRγδ monoclonal antibody (mAb) conjugated with fluorescein isothiocyanate (FITC) (Southern Biotech, Cedarlane, Burlington, Canada) for 30 minutes on ice. Cell were then washed twice in FACS buffer and pelleted
at 400 x g for 6 minutes at 4°C. A total of 4x10^7 live cells per bird (n = 12) were sorted using the FACSARia™ Ilu flow cytometry machine (BD Biosciences, CA). The sorted cells were collected in a round-bottom polystyrene tube (BD Falcon 352052) and an aliquot of which was further analyzed to note the purity of the γδ T cell population.

3.3.5. RNA extraction

The sorted cells were pelleted and frozen at -80°C in the cell lysis buffer of Qiagen RNeasy extraction kit (Qiagen, Missisauga, Canada). This kit was used to extract RNA following the manufacturer’s instructions. For DNase digestion, the associated Qiagen RNase-free DNase set (Qiagen, Missisauga, Canada) was used. Following this, the quality and quantity of RNA was measured using NanoDrop® ND-1000 spectrophotometry (Thermo Fisher Scientific, Markham, Canada) to ensure all samples fell within the range of 1.8-2.0 for the 260/280 ratio of absorbance.

3.3.6. cDNA synthesis

Reverse transcription of 50 ng of RNA was carried out using the Superscript® II protocol (Invitrogen, Thermofisher Scientific, Markham, Canada). RNA was mixed with oligo dT primers and dNTPs, then heated in a thermocycler to 65°C for 5 minutes. DTT, Superscript II, and RNaseOUT™ were added to the samples and allowed to incubate at 42°C for 50 minutes. The reaction was then inactivated by heating to 70°C for 15 minutes followed by cooling and storing at -20°C.
3.3.7. Primers

The absolute MDV genome load was quantified using primers specific for MDV-\textit{meq}, using DNA as the template. The previously published primers were used for relative gene quantification of target cytokines IFN-\textit{γ}, IL-10, IL-6, IL-13, TLR3, and TLR21 and normalized against expression of \textit{β}-actin, using cDNA as the template. All primers were synthesized by Sigma-Aldrich Canada Ltd. (Oakville, Canada) (table #1).

3.3.8. DNA extraction

Two feather tips were chopped with a scalpel blade and mixed with 500 µl of cell lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 1 mM EDTA, pH 8.0 with 5\% (w/v) Sarkosyl) and 100 µl of proteinase K (10 mg/ml in nuclease free water). These samples were incubated overnight in a water bath at 65°C. Then 25 µl 5M NaCl and 2.3 ml 95\% ethanol were added. These solutions were then centrifuged at 100 x g for 2 minutes and the DNA pellet was washed twice with 70\% ice-cold ethanol. Finally 100 µl TE, pH 8.0 was added and residual ethanol was evaporated by placing open tubes in a water bath at 65°C. DNA concentration was measured using a Nanodrop and adjusted to 50 ng/µl for all samples.

3.3.9. Conventional PCR

In order to detect presence of the \textit{meq} gene in feather tips, conventional PCR was conducted with the use of a TGradient thermocycler (Biometra® GmBH, Gottingen, Germany). In a total volume of 25 µl, 100 ng of template DNA was added to 1x PCR buffer, 1.5 mM MgCl\textsubscript{2}, 0.2 mM dNTPs, 0.2 mM of the forward and reverse primer each,
and 1.25 U Taq DNA polymerase. The conditions set for this reaction were as follows: pre-incubation and denaturation at 94°C for 3 min, then 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min, finishing with a final extension at 72°C for 5 min. PCR products from each time point and both groups were run in a 2% agarose gel and stained with SYBR Safe.

3.3.10. Real-time PCR

All DNA samples that were determined by conventional PCR to be meq+ samples were further analyzed by real-time PCR in order to quantify viral genome load. LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Laval, Canada) was used in a final volume of 20 µl including 5 µl of 1:10 diluted cDNA and 0.25 µM of both forward and reverse primers. The thermal cycling parameters varied according to the gene and consisted of: pre-incubation and denaturation at 95°C for 10 min; 45 cycles (β-actin, IFN-γ, meq, TLR 3, and TLR 21), 50 cycles (IL-13), 55 cycles (IL-6), and 60 cycles (IL-10) of amplification at 95°C for 10 s (segment 1), 64°C for 5 s (segment 2), and 72°C for 8 s (meq) and 10 s (all other genes) (segment 3); melting at 95°C (β-actin, IFN-γ, IL-10, IL-13, IL-6, meq) and at 97°C (TLR 3 and TLR 21) for 5 s (segment 1), 65°C for 1 min (segment 2) and 97°C for continuous acquisition (segment 3); cooling at 40°C for 10 s.

3.3.11. Data analysis

The relative expression of all target genes was calculated relative to the housekeeping gene β-actin using the LightCycler® 480 Software (Roche Diagnostics,
Laval, Canada). The β-actin gene was used because it is constitutively expressed as it expressed in all cells under homeostatic conditions. Statistical significance was determined using an unpaired student’s t-test. Results were considered statistically significant if \( p \leq 0.05 \) (*), \( p < 0.01 \) (**), \( p < 0.001 \) (***) or \( p \leq 0.0001 \) (****).

### 3.4. Results

#### 3.4.1. T cell fractionation

In total there were twelve spleens sorted per time point, six were used as untreated controls and another six as MDV-infected birds. The number of sorted γδ T cells per sample, including pooled spleens, was approximately \( 1 \times 10^6 \) for control groups and \( 6 \times 10^5 \) for MDV-infected groups from a total of \( 4 \times 10^7 \) splenocytes. The purity of sorted cells was 97% or higher.

#### 3.4.2. MDV meq gene expression in feather tips of MDV-infected chickens

DNA was extracted from the feather tips of control and infected birds and expression of the meq gene was first analyzed by conventional PCR then subsequently by real-time qRT-PCR. All control samples across the three time points were meq negative as well as the infected samples from 4 d.p.i.. However, infected samples from 10 and 21 d.p.i. were meq positive and therefore used in real-time qRT-PCR to quantify copy number. The viral gene was detectable in infected samples at 10 d.p.i. (mean = \( 4.4 \times 10^4 \)) and 21 d.p.i. (mean = \( 9.1 \times 10^5 \)) as shown in Figure 9. There was a significant increase \( (p < 0.001) \) in meq transcription from day 10 to 21 post-infection.
3.4.3. IFN-γ and IL-6 cytokine gene expression in γδ T cells

IFN-γ expression was significantly up-regulated during the early cytolytic (day 4) ($p \leq 0.0001$) and transformation (day 21) ($p \leq 0.05$) phases of infection when comparing MDV-infected birds to controls at each time point (Figure 10a). However, at 10 d.p.i., although not statistically significant, γδ T cells from the infected chickens showed slightly elevated IFN-γ transcription compared to those from the uninfected control birds. Similarly, although IL-6 gene expression in γδ T cells from infected birds was higher at 4 and 21 d.p.i. than the controls, no statistical difference was found between these two groups of birds (Figure 10b).

3.4.4. IL-10 and IL-13 cytokine gene expression in γδ T cells

As illustrated in Figure 11a, there was a significant increase in the expression of IL-10 transcripts in γδ T cells from MDV-infected birds compared to controls at days 10 (latency phase) ($p \leq 0.05$) and 21 (transformation) ($p < 0.01$) post-infection. No significant increase of IL-10 transcription was observed in γδ T cells during the early cytolytic phase of the MDV infection. Gene expression for IL-13, a cytokine considered to indicate a Th2 response, was significantly increased at day 4 post-infection in the γδ T cells of MDV-infected birds compared to controls ($p \leq 0.05$) (Figure 11b). However, the IL-13 expression was seen decreasing drastically to an undetectable level by day 21 post-infection.
3.4.5. TLR3 and TLR21 cytokine gene expression in γδ T cells

It is generally noted that avian TLR3 and TLR21 are involved in the virus recognition process mediated by cells of the innate immune system. To this end, we observed a significant increase in gene expression of TLR3 at 4 d.p.i. \((p \leq 0.05)\) in purified γδ T cells isolated from the MDV-infected chickens compared to controls (Figure 12a). No statistical difference was noted in TLR21 gene expression in γδ T cells between the infected and uninfected birds (Figure 12b).

3.5. Discussion

Cells of the innate immune system play a critical role in the recognition of viruses and thereby, initiate an orchestrated immune response against viruses. Although avian γδ T cells are found in abundance in blood and tissues of chickens, very little work has been done to investigate the role of γδ T cells, particularly the response phenotype of these cells, in defense against viruses. The studies presented here revealed that avian γδ T cells may utilize the TLR3-dependent pathway for recognition and response to the virus. This was manifested by expression of TLR3 in cells obtained from infected chickens at 4 d.p.i.. Moreover, the results demonstrated that IFN-γ expression by γδ T cells is increased at 4 and 21 d.p.i.. Expression of TLR3 and IFN-γ may contribute to induction and regulation of anti-MDV responses.

TLRs play a key role in the early phases of infection that involve pathogen recognition and induction of innate immune responses. In the context of viral pathogens, TLR3 is widely implicated in the recognition of double stranded RNA (dsRNA). It has been shown that MDV encodes microRNAs for viral replication and establishment of latency (Hu et al., 2014). dsRNA is then detected by TLR3 on antigen presenting cells. A
previous report examining the response of TLR3 in lungs of MDV-infected chickens found a significant increase in the expression of this receptor during the early cytolytic phase of infection (Abdul-Careem et al., 2009a). It has also been reported that TLR3 is enhanced during the early phases of infection in the thymus of MDV-infected birds (Hu et al., 2014). These studies are in agreement with our findings whereby TLR3 expression in the purified γδ T cells was significantly elevated during the early cytolytic phase of infection (4 d.p.i.). In addition, we hypothesize that the overall increase in splenic TLR3 expression correlates to an abundance of γδ T cells present in these tissues. We found that there was a sharp decline in γδ T cell gene expression of TLR3 at both days 10 and 21 post-infection, suggesting that TLR3 had a role to play primarily during the early cytolytic phase of infection. Recently Hu et al. (2016) also found a similar decline in TLR3 gene expression in chicken embryo fibroblast (CEF) cells infected with RB1B MDV in vitro. In the present study, the decline in TLR3 expression in γδ T cells during the latency (day 10) and transformation (day 21) phases of MDV infection was associated with a dramatic increase in virus copy numbers. TLR3 also plays a role in tumor suppression whereby the tumor suppressor p53 binds and regulates the p53 site on the TLR3 promoter during MDV infection (Hu et al., 2016). It has been shown that meq, a viral protein of MDV, can inhibit expression of p53 and hence dampen TLR3 transcription in infected chickens (Deng et al., 2010). Therefore, it is hypothesized that this immunoevasive property of MDV may have caused a rapid drop in γδ T cell gene expression of TLR3. Nevertheless, our study suggests the possibility of TLR3 involvement in γδ T cell recognition of MDV.
Another important observation from this study was the significant increase in the transcription of the IFN-γ gene in splenic γδ T cells during the early cytolytic and transformation phases of MDV infection. It has previously been noted that MDV-infected birds have an increased splenic gene expression of IFN-γ during the cytolytic phases of infection (Xing & Schat, 2000a). It is possible that an increased IFN-γ transcription in γδ T cells contributes to an increase in the overall gene expression of IFN-γ as γδ T cells are found in very high numbers in spleen tissues. One possible reason for an increased IFN-γ transcription in γδ T cells during the early cytolytic phase of infection is perhaps to drive the response towards a more Th1-biased pro-inflammatory phenotype. This type of response may drive the virus into early latency thereby leading to a reduction in clinical disease as suggested by Kaiser, Underwood, & Davison (2003) who also found early high splenic gene expression of IFN-γ in MDV infected birds between 3 and 10 d.p.i.. IFN-γ expression also causes the differentiation and expansion of cytotoxic T lymphocytes (CTLs) to directly lyse transformed cells (Whitmire, Tan, & Whitton, 2005). This early interaction provides an immediate source of IFN-γ for subsequent Th1 differentiation of surrounding naïve CD4 helper T cells. Our finding can further be supported by a previous report that used an intracellular bacterium (Listeria monocytogenes) infection model in mice to show expression of IFN-γ by γδ T cells within 24 hours post-infection (Ferrick et al., 1995). It is of note that we also observed an increased transcription of IL-13 in splenic γδ T cells during only the early cytolytic phase of MDV infection. This observation could explain the γδ T cell capacity of inducing both Th1 (IFN-γ) and Th2 (IL-13) responses to different MDV antigens early on during the infection followed by a finely tuned and desirable IFN-γ dominated response during the late cytolytic phase of
MDV infection, as similarly found in splenocytes of MDV-infected chickens (Xing & Schat, 2000a).

In addition to producing effector cytokines, γδ T cells have also been shown to play a regulatory role in controlling excessive immune responses via secretion of IL-10. For example, Skeen et al. (2001) found that in the absence of γδ T cells following murine infection with *Listeria monocytogenes*, systemic expression of pro-inflammatory cytokines was significantly higher and resulted in an exaggerated innate response. In the context of MDV infection in chickens, Abdul-Careem et al. (2007) previously suggested that infection with MDV causes an up-regulation of IL-10 gene expression in the spleen of chickens at 21 d.p.i.. Another study has also observed an increased splenic IL-10 transcription during the latency phase of MDV infection in chickens (Heidari, Zhang, & Sharif, 2008). An observation from studies of Parvizi et al. (2009) was that while IL-10 gene expression was increased in purified CD4⁺ T cells during all phases of infection (4, 10, and 21 d.p.i.), purified CD8⁺ T cell gene expression of IL-10 was found only during the transformation phase (21 d.p.i.). In the present study, IL-10 transcription in γδ T cells was significantly enhanced during the latency (day 10) and transformation (day 21) phases of MDV infection with simultaneously increased IFN-γ gene expression at day 21.

Considering the fact that IL-10 has been shown to down-regulate expression of IFN-γ and thereby the proliferation of CD8⁺ T cells (Endharti et al., 2005) in order to inhibit inflammatory responses (Heidari, Zhang, & Sharif, 2008), IL-10 expression of γδ T cells in the present study suggests a possible regulatory role for these cells during the later phases of MDV infection. Alternatively, based on the observation that there was an enhanced co-expression of IFN-γ and IL-10 at 21 d.p.i., it is also possible that IL-10 may
have a non-regulatory role of attracting CD8\(^+\) T cells to the site of pathogen entry (Redpath, Ghazal, and Gascoigne, 2001). Two previous studies investigating cytokine responses during MDV infection also found gene expression of IFN-\(\gamma\) and IL-10 during the transformation phase of MDV infection (Parvizi et al., 2015; Abdul-Careem et al., 2008a). However, further studies are required to determine if IFN-\(\gamma\) and IL-10 may exert a concerted role in the immune response against MDV during the later course of MDV infection in chickens.

The present study investigated \(\gamma\delta\) T cell-mediated cytokine responses during different phases of MDV infection in chickens. These responses are marked by early expression of IFN-\(\gamma\) (4 d.p.i.) followed by simultaneous expression of IL-10 and IFN-\(\gamma\) in late stages of MDV infection (21 d.p.i.). This study also highlights the ability of \(\gamma\delta\) T cells to recognize PAMPs early in infection through expression of PRRs such as TLR3 and TLR21, key components of innate immune system cells. Additional research is needed to further elucidate the role of \(\gamma\delta\) T cells in MDV infection and the possibility of whether these cells become infected with the virus.

### 3.6. Acknowledgements

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Table 1: Sequences and annealing temperatures for real-time PCR primers

<table>
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<tr>
<th>Target gene</th>
<th>Primer sequence</th>
<th>Accession number/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>F: 5'- CAACACAGTGTCTGCTGGTGTA -3' R: 5'- ATCGTACTCTCGTCTGATCC -3'</td>
<td>X00182</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>F: 5'- ACACTGACAAAGTCAGGCGCACA -3' R: 5'- AGTCCTCAGCAGCTTGGGC -3'</td>
<td>X99774</td>
</tr>
<tr>
<td>IL-10</td>
<td>F: 5'- TTTGGCTGGCTGCTTGTC -3' R: 5'- CTCATCCATCTTTCTCAGTC -3'</td>
<td>NC_006113.4</td>
</tr>
<tr>
<td>IL-13</td>
<td>F: 5'- ACTTGTCAGTGCTAAGCTGCTGTC -3' R: 5'- TCTTTGGAAGCTGCTGTC -3'</td>
<td>AJ621250</td>
</tr>
<tr>
<td>IL-6</td>
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<td>NM_204628.1</td>
</tr>
<tr>
<td>TLR 3</td>
<td>F: 5'- TCAGGACATTTGTAACACCCGCGC -3' R: 5'- GCGTCATATACAACACTGC -3'</td>
<td>DQ780341</td>
</tr>
<tr>
<td>TLR 21</td>
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<td>AJ720600.1</td>
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<tr>
<td>meq</td>
<td>F: 5'- GTCCCCCTCAGATCTTTCTC -3' R: 5'- GTTCTGCTTCTGCTGCTTTC -3'</td>
<td>Abdul-Careem et al., 2006</td>
</tr>
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</table>
**Figure 9:** MDV genome copy number in feather follicles at 4, 10, and 21 d.p.i.

*Meq* is a viral oncogene of MDV that was used to calculate virus copy number from 100 ng of DNA. The groups included in this representation were MDV-infected birds only as control birds lacked the presence of *meq*. Data presented are the mean ± SEM for six biological replicates at each time point. Asterisks denote a significant difference between groups ($p < 0.001 (***)$).
**Figure 10**: Relative expression of pro-inflammatory cytokines (10a) IFN-gamma and (10b) IL-6 in gamma delta T cells

γδ T cells are from spleens of SPF chickens infected experimentally with MDV-RB1B. Infected and uninfected chickens were sampled at 4, 10, and 21 d.p.i.. Relative expression of IFN-γ and IL-6 from splenic γδ T cells of infected and uninfected chickens are presented here. Data represented are the mean relative expression ± SEM for six biological replicates at each time point. Black and grey bars represent uninfected and infected groups, respectively. Asterisks denote a significant difference between groups ($p \leq 0.05$ (*) or $p < 0.001$ (***)).
Figure 11: Relative expression of (11a) IL-10 and (11b) IL-13 in gamma delta T cells γδ T cell subsets are from spleens of SPF chickens infected experimentally with MDV-RB1B. Infected and uninfected chickens were sampled at 4, 10, and 21 d.p.i. Relative expression of IL-10 and IL-13 from splenic γδ T cells of infected and uninfected chickens are presented here. Data represented are the mean relative expression ± SEM for six biological replicates at each time point. Black and grey bars represent uninfected and infected groups, respectively. Asterisks denote a significant difference between groups ($p \leq 0.05$ (*)) or $p < 0.01$ (**)).
Figure 12: Relative expression of (12a) TLR3 and (12b) TLR21 in gamma delta T cells γδ T cell subsets are from spleens of SPF chickens infected experimentally with MDV-RB1B. Infected and uninfected chickens were sampled at 4, 10, and 21 d.p.i.. Relative expression of TLR3 and TLR21 from splenic γδ T cells of infected and uninfected chickens are presented here. Data represented are the mean relative expression ± SEM for six biological replicates at each time point. Black and grey bars represent uninfected and infected groups, respectively. Asterisks denote a significant difference between groups (p ≤ 0.05 (*)).
CHAPTER 4

4.1. General discussion

Marek’s disease (MD) causes substantial economic losses to the poultry industry every year. Due to its highly contagious nature and ease of infection, MD is quite difficult to control. Commercial chicken flocks are vaccinated using non-oncogenic turkey herpesvirus (HVT) either alone or in combination with other MD vaccines (Witter, 2001). Although vaccines are capable of preventing immunosuppression and tumour incidence, they are unable to confer protection against infection or shedding of the virus. As a result, the virus is still present in many poultry farms and most vaccinated chicks will be challenged with MDV within a few days of their arrival from hatcheries. The use of bivalent and trivalent vaccines has largely provided relief from outbreaks; however, the use of increasingly potent vaccine programs has driven MDV to increasing virulence (Davison & Nair, 2005).

The classic pathogen trade-off model predicted by Davison and Nair (2005) indicates that all pathogens have an expected range of virulence within which natural selection creates an optimal balance. Nevertheless, vaccination can relax this favoured selection thereby altering the optimal level of virulence, leading to increased pathogenicity (Davison & Nair, 2005). The creation of combination vaccines in the 1980s as an answer to decreased HVT vaccine efficacy has exacerbated the problem by creating a self-perpetuating cycle whereby more potent vaccines are followed by more virulent strains of MDV. As there is still much to discover regarding the underlying immunological mechanisms that mediate immunity against MD, there is a need to further
characterize host responses and define cell populations following MDV infection in order to create novel and effective solutions.

It is known that upon entry into the host’s respiratory system, macrophages transport the virus from the lungs to secondary lymphoid organs, such as the spleen, cecal tonsils, and bursa. Once the virus reaches these tissues, it begins infecting B cells during the early cytolytic phase (Baigent & Davison, 2004). B cells are then responsible for causing activation of CD4\(^+\) T cells, which are targets of infection for MDV. Upon CD4\(^+\) T cell infection, a period of latency begins (Schat & Xing, 2000). Virus may then be carried to the skin where productive infection occurs in the feather follicle epithelium (FFE) causing shedding of infectious cell-free virus (Calnek, Hans, & Kahn, 1970). MDV mainly causes the activation of T cell-mediated immune responses because of the cell-associated nature of this virus while causing cytolytic infection within the host (Morimura et al., 1998). CD4\(^+\) T cells serve mostly as targets of transformation while cytotoxic T lymphocytes (CTLs) have been implicated in MDV antigen recognition and destruction of infected cells (Schat et al., 1992). Another T cell subset that has been understudied, particularly in the context of MDV, is γδ T cells. Most mouse studies have shown an expansion of γδ T cells following viral infections (Nishimura et al., 2004; Ninomiya et al., 2000; Sing et al., 1998; Selin et al., 2001); however, little work has focused on chicken γδ T cells. These cells comprise about 10% of thymocytes, 20% of circulating T cells, and 30% of splenocytes in adult chickens (Bucy, Chen, & Cooper, 1991). Due to the high frequency of these cells in the blood and tissues of chickens, it is likely that these cells play an important role in defense against pathogens.
Therefore, this study was undertaken in order to elucidate the role of γδ T cells in the context of MDV. As there was no previous literature on this topic, it was initially imperative to determine the distribution of γδ T cells following MDV infection. Chickens were infected with MDV-RB1B and tissues such as spleen, cecal tonsils, and lungs were harvested for flow cytometric analysis of γδ T cell numbers and frequency compared to uninfected control chickens. Spleens were also harvested for FACS sorting to obtain a population of γδ T cells to be used for analysis of cytokine gene expression. Both studies evaluated γδ T cell responses in the aforementioned tissues at 4, 10, and 21 days post-infection (d.p.i.). These phases correlate to the early cytolytic, latent, and transformation phases of MDV infection. The results from this study showed an increase in the absolute number of γδ T cells at 21 d.p.i. in MDV-infected birds compared to controls in the spleen. Furthermore, it was determined that sorted populations of splenic γδ T cells significantly up-regulated expression of IFN-γ at 4 and 21 d.p.i.. Although γδ T cells were not significantly increased in numbers at 4 d.p.i., their up-regulation of IFN-γ at this time is likely an inflammatory response to early phases of infection. Similar findings were reported in mice infected with herpes simplex virus (HSV) 2 and further depleted of γδ T cells (Nishimura et al., 2004). These mice experienced a 90% mortality rate by day 11 and reduced IFN-γ expression by CD4+ T cells resulting in an impaired Th1 response. These results demonstrate the importance of γδ T cells in initiating the immune response and establishing an appropriate environment for the activation of a Th1 response.

The increased number of γδ T cells at 21 d.p.i. and associated up-regulation of both IFN-γ and IL-10 suggests a non-regulatory role for the latter cytokine. A possible non-regulatory role for IL-10 that has been suggested by other studies is in the attraction
of CD8\(^+\) T cells to the site of infection (Parvizi et al., 2015; Abdul-Careem et al., 2008a). This is further supported by our study in which we found high expression of CD8 on γδ T cells during the transformation phase of infection when IL-10 expression is significantly up-regulated. CD8\(^+\) γδ T cells are suggested to be crucial in pathogen clearance by killing of infected cells in chickens (Berndt and Methner, 2001), which is further supported by the presence of CD107a on human γδ T cells (Qin et al., 2009). Another attraction for CD8\(^+\) γδ T cells to the site of infection at 21 d.p.i., other than IL-10 expression, may be expression of heat shock proteins (hsp) by macrophages during this phase. Another study similarly found a late response, characterized as 10 d.p.i., of γδ T cells in mice infected with influenza A virus (Carding et al., 1990) paralleled by an increasing number of macrophages. Zhao et al. (2009) have shown that during the later phase of MDV infection, there is an up-regulation of hsp production by macrophages and these mycobacterial antigens enable γδ T cell activation (Born et al., 1990). Hsp have been shown to bind to viral oncoproteins and, more specifically, interact with the MDV oncoprotein meq (Zhao et al., 2009) allowing them to be present during the transformation phase of MDV infection when meq is most abundant. Therefore, γδ T cells may be responding to increased levels of hsp during this phase of pathogenesis.

A completely opposite trend was observed in cecal tonsils of MDV-infected chickens whereby a significant reduction in the number of γδ T cells was observed during the early cytolytic and transformation phases of infection. Transient atrophy of cecal tonsils and hence lymphoid cells has been documented during the first two weeks of infection (Heidari, Fitzgerald, and Zhang, 2014), which would encompass the early cytolytic and latency phases. The study mentioned used a bacterial artificial chromosome
(BAC)-cloned very virulent strain of MDV. Therefore, as suggested by the authors, it is possible that a wild type very virulent strain, such as RB1B used in the present study, may cause irreversible atrophy of the cecal tonsils by depletion of lymphocytes and permanent destruction of germinal follicular centers. This is further supported by the observation that BAC-cloned MDV viruses cannot elicit the same level of disease incidence as a wild type strain of MDV (Smith et al., 2011). This may explain the continued reduction in the number of γδ T cells in cecal tonsils at 21 d.p.i. in our study.

It has long been debated whether γδ T cells are considered cells of the innate or adaptive immune system. The present study has demonstrated their ability to produce IFN-γ early following MDV infection (4 d.p.i.) as well as their ability to bridge the gap between the two arms of the immune system by creating what we hypothesize as a Th1 environment for induction of CD4+ T cell responses. It has long been thought that NK cells or NK T cells are responsible for causing polarization of naïve T cells into Th1 cells; however, γδ T cells may also be involved in this process through early expression of IFN-γ. Additionally, their expression of TLR3 at 4 d.p.i. is important in alerting and initiating the host immune response. Expression of this pattern recognition receptor (PRR), TLR3, is immediately reduced in the subsequent phases of infection. This is likely a result of p53 inhibition, a tumour suppressor on the TLR3 promoter, by enhanced meq expression at 10 d.p.i. (Hu et al., 2016; Deng et al., 2010). TLR3 is also highly expressed in the lungs of MDV-infected chickens during the early cytolytic phase of infection (Abdul-Careem et al., 2009a). However, in our study, expression of γδ T cells was not enhanced until latency in the lungs of infected birds.
Although this is a novel study in regard to examining the role of $\gamma\delta$ T cells following infection with MDV, there were some limitations to this study. The first constraint we experienced in our in vivo model was the obligation to terminate the experiments at 21 d.p.i.. Infected chickens generally begin showing clinical signs such as paralysis by the transformation phase of MDV infection. As a result, the study could not carry on past 21 d.p.i.. However, it would be interesting to further determine the role of $\gamma\delta$ T cells past this phase. As MDV usually causes death of the chicken, it is likely that the population of $\gamma\delta$ T cells would be depleted soon after the transformation phase has begun. Additionally, neither experiment included the late cytolytic phase of infection, which is generally around 14-21 d.p.i.. This is the phase of reactivation whereby the virus is no longer in dormancy; therefore, this study missed a large gap between latency and transformation where $\gamma\delta$ T cells may have had other important roles. Another limitation was the delivery method of MDV infection. The natural route of infection is through the respiratory system; however, in the present studies, we infected chickens intra-abdominally. This may also be a reason for the late activation of $\gamma\delta$ T cells seen in the lungs, as the virus did not initially enter through this route; however, MDV genome has been shown to be present in the lungs of birds infected intra-abdominally (Butter et al., 2007). Although others in our group have developed a method for an aerosol-based infection model, the exact dose of virus received by individual birds cannot be monitored (Abdul-Careem et al., 2009b). Therefore, intra-abdominal infection is deemed the most appropriate means of virus administration. Finally, purified $\gamma\delta$ T cells had very little RNA following RNA extraction. As a result, the maximum amount of RNA we were able to use for cDNA synthesis was 50 ng, which is lower than the amount conventionally used.
It is possible that any low expressing genes would not be amplified with this concentration of cDNA; however, all genes were run for a sufficient number of cycles until amplification was seen in this study. In order to assess the purity of RNA, 260/230 ratios were measured. These ratios were generally quite low, which may be indicative of contamination and could have ultimately caused problems with PCR amplification. However, when 50 ng of cDNA was tested for expression of β-actin, it was deemed appropriate as the amplification curve appeared by 19 cycles.

These studies are preliminary in determining the role that γδ T cells play in chicken immune responses to viruses, such as MDV. Although a majority of the γδ T cell research focuses on mice and humans, it is critical that chickens are also evaluated because this species has such a high representation of these cells in blood and tissues. Future work should focus on further understanding the immunological role of γδ T cells in response to viral pathogens as these cells may be indispensable in clearing the viruses. Other pro-inflammatory, anti-inflammatory, and regulatory cytokines should additionally be investigated in order to further support the ideas discussed in this study, as we only chose two cytokines to encompass each group of cytokine. Furthermore, expression of other PRRs, such as melanoma differentiation-associated protein 5 (MDA5) should be measured as it has been shown to be up-regulated following MDV infection (Feng et al., 2013). It should also be determined whether pathogen-associated molecular patterns (PAMPs) associated with viruses, such as CpG and double-stranded RNA (dsRNA), can activate γδ T cells. Other tissues should also be examined for the presence of γδ T cells. The FFE is a site of utmost importance as this is where the virus is shed into the environment. If γδ T cells are shown to have a role in the FFE, perhaps the virus could be
prevented from forming fully enveloped particles at this site and ultimately block shedding to other susceptible birds. Finally, preliminary studies from our lab have suggested the possibility of MDV infection in $\gamma\delta$ T cells. The sorted cells were tested for expression of gB, a surface glycoprotein gene of MDV. It was shown that $\gamma\delta$ T cells expressed gB at 4, 10, and 21 d.p.i. with decreasing presence as MDV pathogenesis progressed. Therefore, it is important to further investigate this possibility and determine confidently whether $\gamma\delta$ T cells become infected with MDV.

As chicken immunology studies face certain constraints due to limited availability of reagents, it is sometimes difficult to explore mechanistic aspects of immune processes. However, if none of these limitations existed, there would be two interesting studies to conduct. First, knocking out $\gamma\delta$ T cells through the use of antibodies or gene editing methods would allow for further understanding of their role in MDV infection. Second, as we found increased IFN-$\gamma$ expression at 4 and 21 d.p.i., depleting its expression would allow us to, again, further elucidate its importance against MDV infection. As an example, in a study by Nishimura et al., 2004 in mice infected with HSV2 $\gamma\delta$ T cells were depleted. This study allowed investigators to determine the importance of $\gamma\delta$ T cells as nearly all mice died following infection and there was decreased IFN-$\gamma$ expression.

In conclusion, the findings in this thesis begin to elucidate the role of $\gamma\delta$ T cells in immunity against MDV. It appears that $\gamma\delta$ T cells play an important role throughout all phases of MDV infection. Through expression of TLR3 and IFN-$\gamma$ during the early cytolytic phase of infection, these cells mount an initial immune response. The increased number of $\gamma\delta$ T cells in the lungs and spleen during latency and transformation, respectively, suggests an important role in defense against MDV. Additionally the co-
expression of IFN-\(\gamma\) and IL-10 during transformation suggests that IL-10 may play a non-regulatory role, ultimately allowing the two cytokines to play a joint role. The results from these studies suggest that \(\gamma\delta\) T cells are an indispensable part of the immune response to MDV.
4.2. References


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