IMMUNE MODULATION BY MAREK'S DISEASE VIRUS

PhD

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ABSTRACT

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The emergence of highly pathogenic Marek's Disease Virus (MDV) strains, defined by acute onset of Marek's Disease (MD), has led to renewed emphasis for understanding host-pathogen interaction. Little is known about the mechanism of MDV replication due to its highly cell associated nature. An association between lipid metabolite remodelling and MDV infection in MD-susceptible lines of chickens based on atherosclerotic plaque formation has been previously established. Immunosuppressive lipid can impair immune system cell function; limit activation, infiltration into active sites of virus replication and modulate recognition of infected cells. Therefore the correlates of immune protection are unknown as antigen specific T cell responses against MDV have yet to be reported. Our primary objective was to use a pathway interference approach to study lipid biosynthesis. We demonstrate that infection with MDV leads to induction of fatty acid synthesis that can contribute towards eicosanoid synthesis in a COX-2 dependent manner. Specifically we identified induction of prostaglandin E_2 (PGE_2) biosynthesis in MDV infected CEFs and demonstrate a dependence for replication. Eicosanoids are well characterised for their ability to modulate immune system cell function including T cells. The second objective was to evaluate *in vivo* T cell responses or cell mediated immunity against MDV in both the MD-resistant (B21) and MD-susceptible (B19) lines of chicken. We demonstrate detection of a T cell response against MDV with the eliciting response directed against viral MEQ and pp38 in chickens that were either challenged (RB1B), vaccine (CVI988/RISPENS)-challenged (RB1B) or vaccine-boosted but not mock infected. $CD4^+TCRv\beta1^+$ cells were the main responding T cell subset recognising the identified immunodominant pp38 epitopes ($pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ and $pp38_{171\text{-}186}$) in all the MD-resistant (B21) and MD-susceptible (B19) lines of chicken. Induction of transcripts for cytokines (IL-2, IL-4 and IL-10) and $CD4^+$ T cell proliferation was observed in a peptide specific manner. However, challenge impaired the ability of T cell to degranulate based on cell surface translocation of CD107a; although peptide specific induction for transcript of perforin and granzyme B can be detected. Taken together we demonstrate that T cell responses against MDV are limited and may be linked to lipid induced biosynthesis.

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CONTENT/INDEX

AUTHOR'S DECLARATION

Declaration: I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.'

Signature: Nitish Boodhoo

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1: Introduction

CHAPTER 2: 1,25(OH)$_2$ Vitamin D3 modulates avian T lymphocyte functions without inducing CTL unresponsiveness

CHAPTER 3: Targeted induction of *de novo* Fatty acid synthesis enhances MDV replication in a COX-2/PGE$_{2\alpha}$ dependent mechanism through EP2 and EP4 receptor engagements

CHAPTER 4: T cell responses to Marek's Disease virus in resistant and susceptible line of chickens

ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microliter
1α,25 $(OH)_2D_3$	1α, 25-Dihydroxy Vitamin D_3
25$(OH)D_3$	25-Hydroxy Vitamin D_3
7-AAD	7-Actinomycin D
Aa	Amino acid
Ab	Antibody
AEC	3-Amino-9-Etheylcarbazole
APC	Allophycocyanin
APC	Antigen presenting cells
ATP	Adenosine triphosphate
B CELL	Bursal derived cells
bp	Base pair
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
CCHFV	Crimean-Congo haemorrhagic fever virus
CCL27	Chemokine ligand 27
CCR10	Chemokine receptor 10
CD107A	Cluster differentiation 107a
CD11B	Cluster differentiation 11B
CD16	Cluster differentiation 16
CD27	Cluster differentiation 27
CD3	Cluster differentiation 3
CD4	Cluster differentiation 4
CD56	Cluster differentiation 56
CD8	Cluster differentiation 8
CD80	Cluster differentiation 80
cDNA	Complementary deoxyribonucleic acid
CEF	Chicken embryonic fibroblast

CFSE	Carboxyfluorescein succinimidyl ester
CIAV	Chicken infectious anaemia virus
CMI	Cell mediated immunity
Con A	Concanavalin A
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T-Lymphocyte associated antigen-4
CYP27A1	Mitochondrial cytochrome P450
CYP27B1	Renal cytochrome P450
DENV	Dengue virus
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy ribonucleotide triphosphate
doa	Day of age
dpi	Days post infection
DTT	Dithiothreitol
E 199	Earle's salt 199
EBV	Epstein Barr virus
ERK	Extracellular signal regulated kinase
FACS	Fluorescence-activated cell sorter
FCS	Foetal calf serum
FFE	Feather-follicle epithelium
Fig	Figure
FITC	Fluorescein isothiocyanate
GaHV-2	Gallid Herpesvirus 2
GaHV-3	Gallid herpesvirus 3
gB	Glycoprotein B
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
hr	Hour
HRP	Horse radish peroxidase
HSV-1	Herpes simplex virus-1
HVT	Herpes virus of turkey

IBDV	Infectious bursal disease virus
ICP4	Infected cell polypeptide 4
IE	Immediate early
IFN-γ	Interferon gamma
IL	Interleukin
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
iNOS	Inducible nitric oxide synthase
ION	Ionomycin
IR_L	Internal repeat long
IR_S	Internal repeat short
LY-4	Lymphocyte antigen-4
M	Molar
mAB	Monoclonal antibody
MAPK	Mitogen activated phosphorylated kinase
MD	Marek's disease
MDV	Marek's disease virus
MeHV-1	Mellagrid herpes virus of turkey-1
MEQ	Marek's EcoRI-Q
Mg	Milligram
MHC I	Major histocompatibility 1
MHC II	Major histocompatibility II
min	Minute
ml	Millilitre
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
ng	Nanogram
NK CELL	Natural killer cells
nM	Nanomolar

NO	Nitric oxide
^{o}C	Degree Celsius
OD	Optical density
OIE	Organization for animal health
ORF	Open reading frame
PBMC	Polymorph blood mononuclear cells
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PDL-1	Programmed death ligand-1
PE	Phycoerythrin
pep	Peptide
PFU	Plaque forming unit
PHA	Phytohemagglutinin
PMHC	Peptide MHC
pp38	Phosphoprotein 38
RPM	Revolution per minute
RPMI	Roswell park memorial institute medium
RSV	Respiratory syncytial virus
RT	Room temperature
SD	Standard deviation
SFU	Spot forming unit
SPF	Specific pathogen free
T cell	Thymocyte derived cells
TCR	Thymocyte derived cell receptor
$TCRVB_1$	T cell receptor variable beta 2
$TCRVB_2$	T cell receptor variable beta 2
T_{eff}	effector T cells
T_{h1}	T helper 1 cells
T_{h2}	T helper 2 cells
T_m	memory T cells
TR_L	Terminal repeat long
TR_S	Terminal repeat short
U	Units
U_L	Unique long

U_S	Unique short
UV-B	Ultraviolet-b
VDR	Vitamin D receptor
$VDR^{-/-}$	Vitamin d receptor knockout
Vit D	Vitamin D
VZV	Varicella zoster virus
αβ T cell	alpha beta T cell
γδ T cell	gamma delta T cell
δ	delta

CHAPTER INDEX

Chapter 1: Introduction

Modified from published review paper

Marek's disease in chickens: a review with focus on immunology

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1.1 Introduction: Epidemiology

Since the initial recognition of a previously unrecognized disease in 1907, Marek's Disease (MD) has evolved into a highly pathogenic infection. Marek's Disease Virus (MDV) or Gallid herpesvirus 2 (GaHV-2), an oncogenic *alphaherpesvirinae*, targets avian species (*Gallus domesticus*) and is defined as the etiologic agent for MD. Chickens act as the primary zoonotic pool for spread of MDV (1). The virus is widely prevalent in commercial stocks and affects young birds. MD has been shown to occur worldwide according to data from the world Organization for animal Health (OIE) but is not a notifiable disease (Figure 1). It is classified as a disease with socio-economic and public health importance, estimated at more than $1 billion annually, in countries which have established international trades for animals and animal by-products.

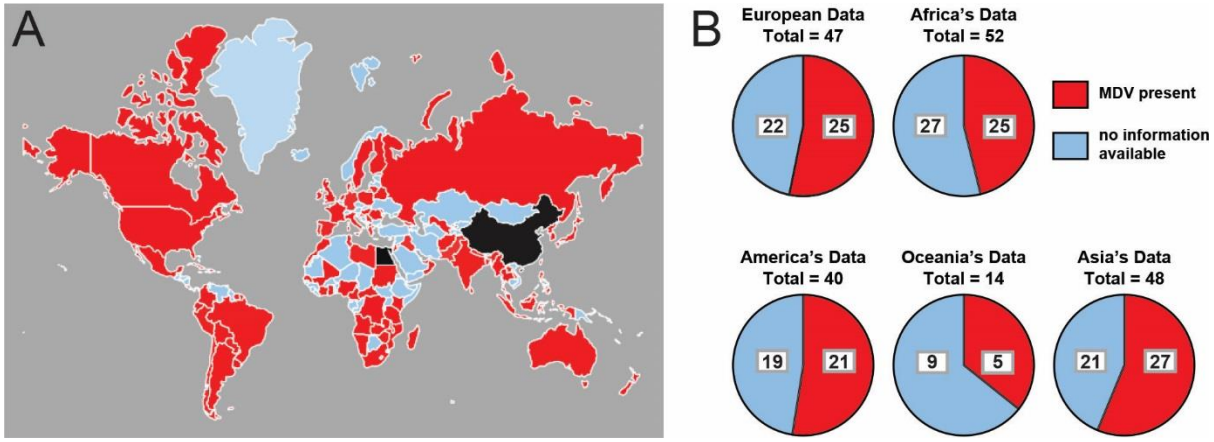


Figure 1: Worldwide map depicting distribution of MDV whereby evidence for presence has been submitted to the OIE.
Distribution data was obtained from the World Organization for Animal Health (OIE) distributed through the World Animal Health Information Database (WAHIS) interface (http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail) and summarized above based on absence (blue) and presence of disease with reported cases (red) before and after 2009. **(A)** World map depicting countries positive for MDV was constructed using the imapbuilder software (http://www.imapbuilder.net/). Both China and Egypt (black) are endemic areas for MDV with outbreaks reported on a yearly basis **(B)** Pie chart demonstrates the number of countries that have reported MDV cases to the OIE based on their geographical location (continent). Data is summarized based on absence (blue), and presence of disease with reported cases (red).

Global vaccination efforts have been efficacious in preventing disease and reduced losses associated with culling of birds. Live virus vaccines, used since the 1970, remain

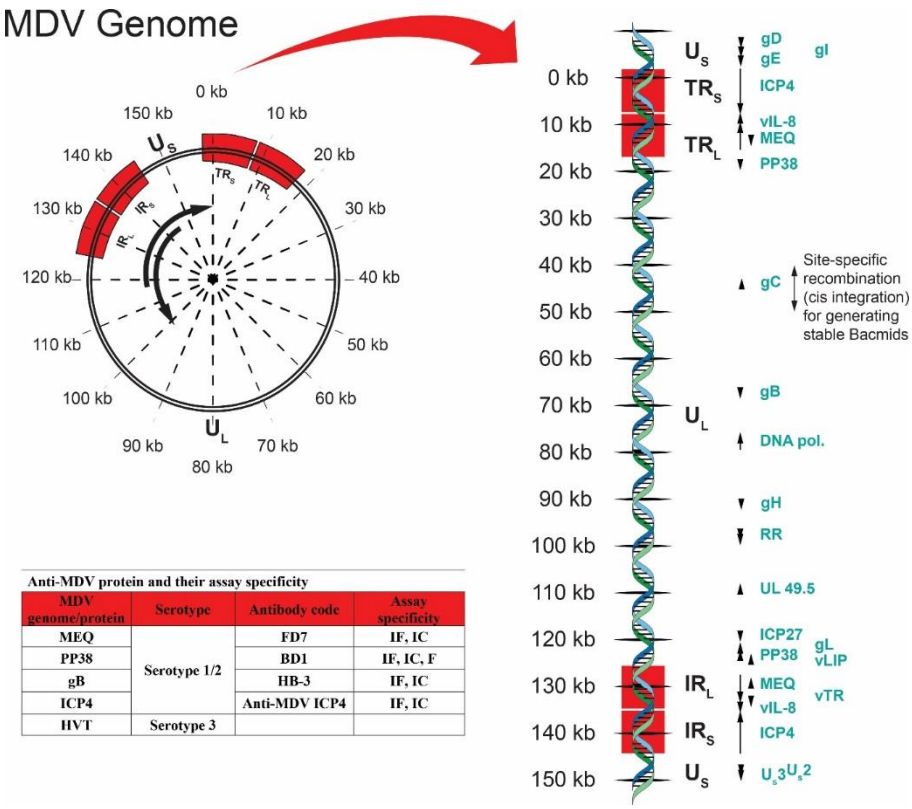
the basis of disease control programmes (2). These are usually administered to day-old chicks at hatching to provide protection against viral challenge from poultry operations. It is the first oncogenic disease against which a vaccine has been developed. However, day old, specific pathogen free (spf), birds become infected when introduced into poultry production facilities delineating an insufficiency in implanting biosecurity measures. Virus shed from feather follicle epithelium can linger in the environment for months as dust and dander (3). This has resulted in infected broilers which are active shedders for the duration of their production cycle.

The emergence of highly virulent pathotypes , increasing reports of vaccine escape and visibly notable areas of the world that are becoming endemic with reported cases of viral outbreaks has led to renewed effort to study the dynamics that promote viral virulence at the interface of host-pathogen interaction (4) and eliciting immune responses against infection (immune responsiveness) which can be used to estimate vaccine efficacy.

1.2 Marek's Disease Virus

Characterized after its human orthologue (Herpes simplex virus, HSV-1), MDV is an *alphaherpesvirus*, predominantly infects avian species. MDV was first isolated in 1967 from different parts of the world and subsequently classified into three distinct serotypes. MDV is an avian double-stranded DNA herpesvirus that belongs to the Mardivirus genus in which three closely related but distinct serotypes have been grouped. Four pathotype of GaHV-2 are currently recognized; mild (m), virulent (v), very virulent (vv) and very virulent + (vv+). The Gallid Herpes Virus type 3 (GaHV-3) serotype consists of naturally a non-pathogenic strain used in combination with Mellagrid Herpes Virus 1 (MeHV-1; serotype 3) as either bivalent or trivalent vaccine formulation. Its genome (Figure 2) is approximately 175 kb long consisting of 70 open reading frames (ORF). The MDV genome consists of specific segments namely two unique sequences, unique long (U_L) and unique short (U_S), encoding the core genes enclosed by the inverted repeat sequences repeat short (R_S) and repeat long (R_L) (5). All herpes viruses have evolved immune evasive strategies associated with immunosuppression or triggering alternative arms of the immune system. Herpes virus infection is life-long thus an intricate balance occurs between host and invading

microbe. The exact mechanism by which MDV replicates in the nucleus is not well known but it is thought to be similar to other *alphaherpesviruses* (6). The Infectious particle is an icosahedral capsid that is comprised of more than 30 different proteins assembled according to a complex architecture of central capsid containing the viral genome, a tegument protein layer and a lipid bilayer anchored with glycoproteins.



Anti-MDV protein and their assay specificity

MDV genome/protein	Serotype	Antibody code	Assay specificity
MEQ	Serotype 1/2	FD7	IF, IC
PP38		BD1	IF, IC, F
gB		HB-3	IF, IC
ICP4		Anti-MDV ICP4	IF, IC
HVT	Serotype 3		

Figure 2: Organization of the MDV genome and illustration of key viral proteins identified

The MDV genome, based on the deposited and annotated sequence (5), when packaged into a fully infectious virion is a linear double stranded DNA. The MDV genome is defined by 4 main segments; U_L (unique long), U_s (unique short), IR (Internal Repeat long; IR_L and Internal Repeat short; IR_s) and TR (Terminal Repeat Long; TR_L and Terminal Repeat Short; TR_S). Infection and replication results in circularisation

1.2.1 GaHV-2: Serotype 1

A large diversity exist within serotype 1 with strains ranging from mild to highly oncogenic (listed below). The emergence of these pathotypes is thought to be result of continuous evolution imposed by vaccine stresses. The vaccine CV1988/RISPENS is currently used as the industry gold standard and is highly efficacious in preventing death as well as T cell neoplastic transformation. Its mechanism of action is currently

unknown. Virulent MDV strains tested *in vivo* replicate earlier, faster and induce a higher viraemia titre (7). Furthermore, the classification is based on the ability of each pathotype to induce disease severity scored by: survival rate, immunosuppression, viraemia and rate of viral shedding, neuropathy, induction of $CD4^+$ T cells transformation and lymphoma dissemination and changes in immune organs weight in relationship to body weight.

Mild (m)	HPRS-B14, Cu2 and Conn-A,
Virulent (v)	HPRS-16, JM and GA,
Very virulent (vv)	RB1B, ALA-B and MD5,
Very virulent plus (vv+)	610A and 648A,
Avirulent strains	58Ap80C, CV1988/RISPENS and Md11

1.2.2 GaHV-3: Serotype 2

GaHV-3 strains ((listed below) have been utilized in vaccine formulations against virulent strains of MDV because they are non-pathogenic in chickens. Vaccination results in a lifelong infection and virus replication which is thought to stimulate immune cell. It is still utilized worldwide due to its high efficacy and relative ease of production which makes it a cheap vaccine to produce.

Avirulent strains	SB-1, HPRS-24 and 301 B/1

1.2.3 MeHV-1: Serotype 3

The Mellagrid Herpesvirus 1 (MeHV-1) was first isolated from turkeys where it is pathogenic. However it's use in chickens due to its sequence similarity with GaHV-2 meant that it has been used worldwide as a vaccine strain. The Herpes Virus of Turkey (HVT) strain is commercially available worldwide. Implementation of bacterial artificial chromosome (BAC) technology has meant that recombinant technique have been applied to modify HVT into a multivalent vaccine strain against other chicken pathogens. Chicken do become carriers but don't show any symptoms.

Avirulent strains	HVT-FC126 and HPRS-26

1.3 Pathogenesis of Marek's Disease

1.3.1 Marek's Disease

MDV, the causative agent of MD in chicken, results in transformation of $CD4^+$ T cells. The natural route of infection is defined by inhalation of airborne cell-free virus particles within the contaminated dust and dander (Figure 3), shed from infected host produced in terminally differentiated feather follicle epithelium (8), into a naïve respiratory track (9). MD pathogenesis has four phases in the susceptible birds; an early cytolytic phase within 2 to 7 days post infection (dpi) which delineates as semi productive lytic viral replication in lymphocytes. This is followed by a latency phase that occurs between 7 and 10 dpi in $CD4^+$ T cell subset that result in systemic viral dissemination. Cutaneous viral infection can occur as early as 4 dpi and eventually results in fully productive viral replication and shedding (10). MDV reactivation in $CD4^+$ T cells initiates a late cytolytic and immunosuppressive phase starting around 18 dpi. Finally a proliferative phase around 28 dpi (11, 12) is characterized by formation of visceral tumours that originate from $CD4^+$ T cells lymphoma. There is no transmission from chicken to eggs (vertical transmission) but the birds are usually infected in early stage after hatching (horizontal transmission). The presence of maternal antibody against MD can protect the neonatal chicks, and with the development of functional immune system a degree of resistant to MD is developed (13). However, husbandry-related stress or concurrent infection with other immunosuppressive pathogens such as chicken infectious anaemia virus (CIAV), Reovirus and Infectious bursal disease virus (IBDV), significantly enhances susceptibility to MDV (14). Another important factor in the susceptibility to MD is the genetic background of chicken lines which is to some extent associated with the major histocompatibility complex (MHC). MDV-associated lymphoma can only develope in genetically susceptible chickens; however the virus can replicate and shedding still occurs in both susceptible and resistant chicken lines.

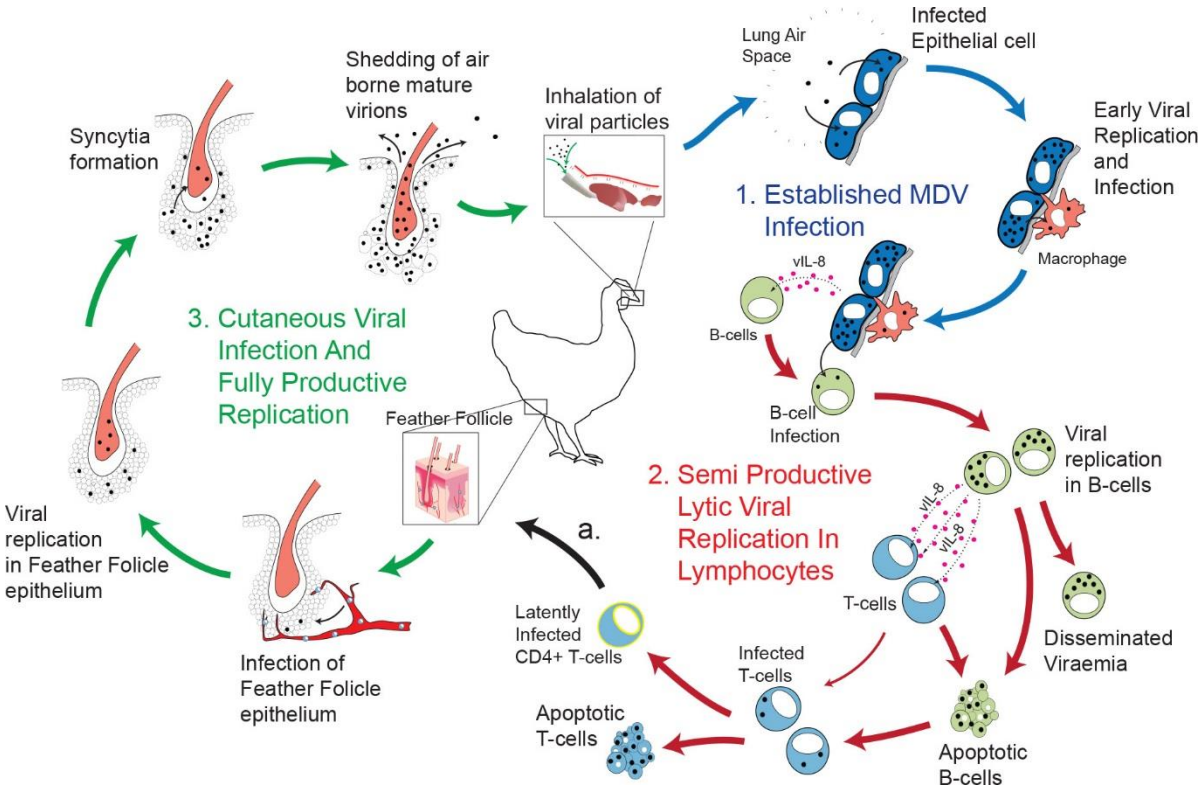


Figure 3: Model of Marek's Disease Virus (MDV) infectious life cycle in resistant birds.

MDV infection of naive host occurs via inhalation of dust or skin dander encapsulated viral particles into the respiratory tract **1.** Primary infection occurs when virus particle breaks mucosal tolerance in the lungs, site of entry into the epithelial cells. Local viral replication establishes infection and initiates viral immediate-early gene, viral Interleukin-8 (vIL-8), transcription and translation. Inflammatory responses in the underlying tissue recruit innate immune system cells which result in uptake of infectious virus particle by macrophages. Infiltration of lymphocytes via action of vIL-8 follows resulting in MDV infection of B cells. **2.** Viral replication in B cells initiates Semi Production Lytic Viral Infection and disease progression. MDV infected B cells secret vIL-8 that acts as a chemotactic factor for and gains access to T cells. This specific lymphotropism (B cells and T cells) enables systemic disseminated viraemia. Viral replication causes apoptosis of B and T lymphocytes in a hallmark of immunosuppression. MDV integrates specifically into the genome of $CD4^+$ T cells enabling escape from immune detection and initiates Latent Viral Infection. Early latently infected and activated $CD4^+$ T cells have not been phenotypically characterised by cell surface markers **a.** Early latently infected and activated $CD4^+$ T cells migrate to cutaneous sites of replication namely feather follicle. **3.** Infection of feather follicle epithelium enables fully productive viral replication. Viral replication results in syncytia formation. Infection of feather epithelium leads to secretion of mature virion in skin danders and dust that act as the major source of infectious materials. Horizontal transmission is the only recognized form for environmental persistence and infection in field conditions. Systemic infection and neoplastic transformation of $CD4^+$ T cells in susceptible birds is further discussed (Figure 4).

1.3.2 Establishment of primary infection

It is speculated that lungs epithelial cells are one of the primary target cells for MDV infection. MDV antigens, with well-defined expression during cytolytic and latent phase of replication, have been detected at significant levels at various time points in lung epithelial cells *in ovo* (15), and *in vivo* (16) suggesting an establishment of successful infection. The later was performed via an aerosol method which simulates natural infection as a respiratory disease (9). Viral replication in the lungs could be detected as early as 1 dpi. Purchase *et al.* (17) were among the first to demonstrate a novel route for high replication kinetics of infectious MDV antigens in lungs epithelial cells of chicks inoculated via intra-abdominal route. However when they repeated the experiment, a lower immunofluorescence was detected at 5 dpi compared to 7 dpi. The route of administration, whether intra-abdominal or intra-tracheal might affect viral replication as well as systemic dissemination that results in MD (18). In addition, infection of lung resident Antigen Presenting Cells (APCs), such as macrophages, is thought to result in subsequent transport to primary and secondary lymphoid organs such as thymus, bursa of fabricius, and spleen (19). Although it is unclear whether macrophages and lung epithelial cells get infected simultaneously or rather infected lung epithelial cells may play a role in transmitting viral particles to macrophages. It is evident that post MDV infection, immune responsiveness leads to macrophage infiltration although viral replication is unaffected (16). It is also believed that presence of MDV particles in the lung, during the earliest infection, stimulates secretion of cytokines and chemotactic factors that help attract B cells to site of infection (20, 21). One of the defined chemokine is a viral interleukin-8 (vIL-8) which is similar to CXCL13 and is involved in recruiting immune systems cells to site of viral replication (22) and is defined as a homologue to the host interleukin-8 (IL-8) gene. IL-8 has a well-defined role as a chemotactic molecule for T cell (21, 23) and B cells (21). Immune cells recruited to the lung such as B cells can be detected as early as 2 dpi (24).

1.3.3 Semi productive lytic viral replication

MDV has a specific tropism for immune system cells and preferentially infects lymphocytes; B cells and T cells (αβ and γδ). Infection of B cells may occur in the lung and viral replication in B cells is defined as semi-productive lytic viral replication. Lytic activity due to viral replication has been linked to phosphoprotein 38 (pp38) activities (25, 26). PP38 role as an early immediate gene is defined only in lymphocytes,

specifically B cells and T cells (27, 28). It has been shown that an MDV rMd5Deltapp38 deletion mutant for pp38 lacked the ability to induce cytolytic activity characterized by B cell apoptosis (29). This is in accordance with the notion that recruitment of lymphocytes such as B cells to site of viral replication is a key step for transmission of virus and dissemination. Deletion mutants of vIL-8 (RB1BvIL-8ΔsmGFP) when tested *in vivo* showed a reduced capacity to successfully infect lymphocytes and induce lytic infection (22). A lack of IL-8 therefore result in impaired ability to recruit B cells and as well as an observable reduction in cytolytic activity due to reduced viral titer and dissemination by lymphocytes. Infection with wild-type MDV restored lytic activity and viral dissemination to primary lymphoid organs (27). Viral lytic activity in B cells results in a drastic downfall in the overall antibody production and ability to fight against an infection. Consequently, the infected chicken suffers from immunosuppression, making it more susceptible to MD and other infections (30). During the early cytolytic phase, transcriptional modification and epigenetic changes (DNA methylation, histone post-translational modifications and non-coding RNAs), along with post-transcriptional and post-translational modifications, regulate viral replication cycle and subsequent expression of cellular and viral genes. Either way, it's been postulated that disseminated viremia to various organs in a cell associated manner results in systemic infection. Infected B cells may also be able to produce vIL-8 mRNA which would have chemotactic activity for T cells. Infected B cells therefore are able to recruit T cells which would allow for transmission of MDV virus particle from infected B cells to activated T cells. Viral replication in T cells also leads to cytolysis associated with pp38 activity. The RB1BvIL-8ΔsmGFP deletion mutant lacked the ability to attract B cells and subsequently impaired its ability to infect T cells albeit at a lower frequency (22). Furthermore, MDV preferentially targets $CD4^+$ T cell subsets and infection results in viral latency and immune evasion (21).

1.3.4 Immune evasion and latency

Hereafter, MDV enters the latency phase (Figure 3) such that it can no longer be detected by the host immune system while it continues to replicate inside the lymphocytes (12). One of the important immune evasion strategies include down regulation of MHC class I molecules on infected lymphocytes. A specific gene, encoding for viral RNA telomerase (vTR) subunit, has been reported to be present only in the oncogenic MDV pathotypes, but not in their non-oncogenic counterparts. In

addition, further study has confirmed about 88% identity of the vTR gene with the chicken terminal repeat (cTR) gene of the host. This would mean that vTR gene is also complement to host Telomerase reverse transcriptase (TERT) for cTR. Consequently, MDV and other herpes virus integrate at the ends of host's chromosomes, preferably at TR sequences. It is, therefore, thought that vTR subunit might, in fact, have a significant role in maintaining the viral oncogenicity through telomeric elongations at the host's chromosomal ends thereby inhibiting programmed cell death trough telomere shortening (31). However, it has recently been shown that both oncogenic and vaccinal strains of MDV have the ability to integrate into DNA, suggesting that integration alone is not sufficient for MDV-induced transformation (32). Chromosomal insertions of *alphaherpesvirus* DNA segments, including those from HSV and equine herpes virus types 1 (EHV-1) and EHV-3, have been associated with their oncogenesis, because many of the cells carrying integrated viral DNA displayed a transformed phenotype (31). Consistent with this, Delecluse *et al.* (33) has demonstrated evidences of MDV genome integration into host's chromosomes for all lymphoma established, MDV cell lines. Additionally, expression of MDV-MEQ gene is essential for MDV-induced neoplastic transformation of latently infected cells (Figure 4). MEQ expressed specifically by oncogenic GaHV-2 has transcriptional activities that lends to modulation of host genomic activity. In relation to this, Lupiani *et al.* (34) conducted a study using a recombinant Md5 pathotype, attenuated by deletion of MEQ-gene. As expected, MDV did infect and replicate in the lymphoid organs and feather follicles; but there was no tumour induction**,** implicating that the integrating viral genome must requires a MEQ gene for induction and maintenance of oncogenic properties. Reactivation of MDV from latency and tumorigenic transformation of latently infected lymphocytes, mainly occurs in $CD4^+$ T cells (35)

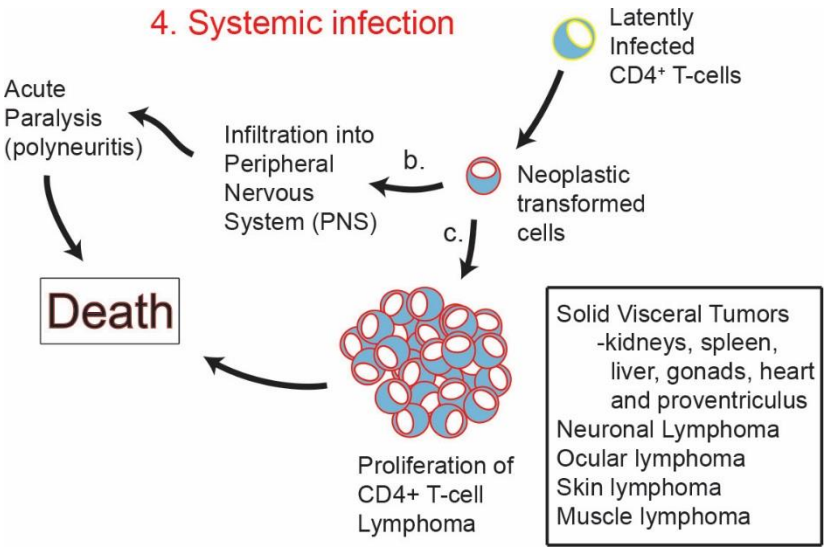


Figure 4: Model of MDV Neoplastic transformed $CD4^+$ T cells and subsequent disseminated Systemic Infection in susceptible (B19) birds.
4. Fully latent MDV-Transformed $CD4^+$ T cells proliferate in all sites where immune systems cells are involved in primary and secondary line of defence. Early latently activated $CD4^+$ T cells undergo neoplastic transformation due to transcriptional and transrepressional activity of viral oncogenic proteins such as MEQ. Pathogenesis arise in cases where vaccination failure is suspected (suboptimal dosage/titre, damage to vaccine or confirmed immunological vaccine failure), vaccination is not performed (backyard birds). **B.** Fully latent neoplastic MDV-Transformed $CD4^+$ T cells infiltrate and establish a reservoir of MDV genome in peripheral nerve fibres interspace. These cells have a $CD4^+CD25^+$ T_{reg} phenotype although additional cell surface markers have yet to be determined. Expression of viral neurovirulence factor, phosphoprotein 14 (pp14), promotes neuropathy and cell survival. Neuropathy (Polyneuritis) is presented as transient or acute paralysis of legs, wings, neck, with vision impairment and weight loss depending on MDV-1 virulence factor. Birds infected with serotype-1 eventually succumb to death from paralysis. **C.** Reactivation from latency enables a second phase of replication whereby viral oncogenic protein MEQ acts on T cell signaling pathways causing uncontrolled cellular proliferation leading to disseminated lymphoma formation in visceral organs, peripheral and central nervous system, musculoskeletal systems, skin and eyes. Severe lymphoma eventually causes death in birds. Highly pathogenic viruses (serotype-1, vv+ MDV) kill birds before they reach the lymphoproliferative phase of the disease.

Another important factor in the susceptibility to MD is the genetic background of established chicken lines which is, to some extent, associated with the MHC. MDV-associated $CD4^+$ T cell lymphoma can only be developed in genetically susceptible (B19) lines of chicken; however, the virus can replicate and be shed from both susceptible (B19) and resistant (B21) lines of chicken. It is believed that only a few subsets of $CD4^+$ T lymphocytes undergo transformation, and thus are the origin of

lymphoma (36). This may explain why in most cases of MD tumour cells obtained from different anatomical sites, such as liver, kidneys, gonads, skin and muscles, all have similar CDR3 length profile suggesting that tumour cells are monoclonal (37). In most lymphoma cells, the virus is in the latent phase and does not produce viral particles and only 0.1% of tumour cells are in the lytic phase (38). Omar *et al.* (25) have revealed an association between the genetic background of chickens and their resistance to MDV; in relation to slight variations on MDV associated-MHC presentations between resistant (B21) and susceptible (B19) lines of chicken. These studies showed evidences of T cell mediated immune responses against the MDV antigen, ICP4, only in the resistant (B21) lines of chicken; but not in the susceptible (B19) ones. In contrast, both B21 and B19 lines of chicken have been reported to show T cell immune responses against the other three MDV antigens: gB, MEQ and pp38. Nevertheless, these studies suggest that T cell mediated responses in combination with MHC play an important role in genetic resistance against MD.

1.3.5 Cutaneous infection, replication and shedding

Viral genomes are detectable by quantitative PCR in blood cells and feather tips of birds infected with oncogenic or vaccinal strains (10). Similar to other herpes viruses, MDV has a tendency to be transported towards cutaneous sites such as skin, and feather follicles. MDV is shed into the environment via scales and feather debris, which is a major source of contamination (39, 40). It is possible that T cells transport the virus to feather follicles, but the role of other immune cells in transporting the virus has not yet been ruled out. Infiltration of $CD4^+$ and $CD8^+$ T cells and expression of pro-inflammatory cytokines into the skin of birds infected with a highly virulent virus (like RB1B) or by a vaccine strain of the virus (like CVI988/RISPENS or HVT) suggest that the immunity against the virus is ineffective at blocking virus replication and shedding (41, 42). Cell-free MDV is only produced in feather follicle epithelial cells, and it is believed that MDV relies exclusively on cell-to-cell transmission (43, 44). However, in a recent report, this notion has been challenged by demonstrating that in a cell-blebbing phenomenon and cell apoptotic corps clearance mechanism, MDV can be transmitted in a cell-free condition (45). MEQ expressing tumour tissues can also be found in the skin of infected birds and neoplastic cutaneous lesions in the scaleless chickens indicate that feather follicles are not necessary for skin tumour development. Finally, the data indicate that inoculation with supernatant fluid from homogenized and sonicated skin

samples of MDV-infected scaleless chickens induces MD in susceptible birds, suggesting that skin epithelial cells not associated with feather follicles also harbour infectious viral particles (46). The process of apoptotic corps clearance may well be related to its specific tropism for adaptive immune cells which leads to immunosuppression. It is still unclear whether either phenomenon also occurs *in vivo* during the early hours of infection or vaccination as well as the respective cells involved. Furthermore, vaccinated and challenge birds don't show clinical signs of immunosuppression therefore an inherent resistance to cellular apoptosis is observed. This may have important implications in our understanding of MDV pathogenesis and development of next generation MDV vaccines. MDV can be detected in the feather follicles at around 11-14 dpi, using standard biochemical methods, and 6-7 dpi, using sensitive methods such as qPCR. In cutaneous sites, fully productive infection and replication is re-activated in feather-follicle epithelium and enveloped infectious viral particles in a cell free form are released. MDV can be found in the epithelium of feather follicles infected chickens more frequently than other tissues, both, in terms of incidence and levels of viral antigen expression (47). The virus replicates as enveloped, cell free MDVs in the feather follicles epithelium of infected chickens. These cell-free MDVs are highly infectious and are easily released into the poultry dust or litter. They have a very protective envelope, allowing them to survive for months in poultry thus facilitating horizontal transmission and infection of naïve animals in following production cycles (48).

MDV infection and its global presence could also be a product of natural reservoirs located in backyard and migratory birds (Figure 5) such as Common teal, White-fronted goose, Pintail, European wigeon, and Mallard (49) and more recently in Roulroul partridges (50). The presence of MDV in wild and migratory birds has been well documented since the early 1980. So it is of no great surprise that new reports emerge as these viruses cause disease in newly identified host. Further monitoring is required to understand the importance of wild birds as reservoirs along migratory routes for pathogenic serotypes.

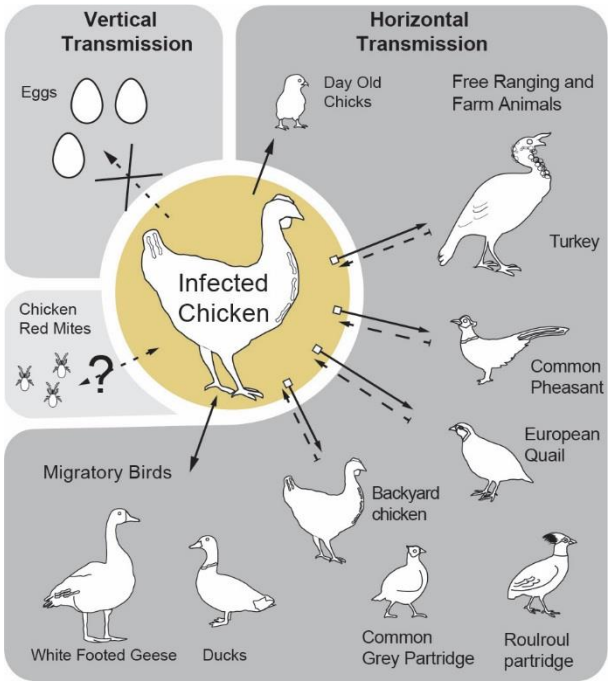


Figure 5: Model for Horizontal and Vertical transmission of MDV between avian species

MDV serotypes can infect several different avian species globally. It has been confirmed that MDV cannot be transmitted horizontally from layer hens to eggs but day old chicks become infected in broiler production housing systems from shedders. Several avian species have been grouped based on literature: Free ranging and farm animals and Migratory birds. Chicken red mites have also been identified as carriers of avian pathogens. Free ranging and farm animals consist of turkeys, common pheasant, common grey partridge and European quail. Migratory birds that have been confirmed positive are ducks and white footed geese. Although these birds become infected and are carriers, what has not been identified is their role in viral virulence factors as well as ability to infect broiler production system. Furthermore, the presence of chicken red mites has yet to be confirmed in broiler production system which could contribute to viral persistence.

1.3.6 Infection of immune cells

Based on the current model of MDV pathogenesis, phagocytic cells such as macrophages and dendritic cells in the respiratory tract become infected either directly or after an initial round of viral replication. The virus can also replicate in macrophages and induce cytolysis in the infected phagocytic cells as demonstrated by high levels of cell death in splenic macrophages expressing three herpesvirus kinetic classes: ICP4 (immediate early), pp38 (early) and gB (late). The level of infection in macrophages isolated from MDV-infected birds depends on the virulence of MDV. The *in vivo* results demonstrate that, at 4-6 days post infection, more virulent viruses (C12/130) induce 3-10 times higher percentages of $pp38^{+}$ macrophages compared to that infected

with less virulent MDV. The results also show that pp38^{+} macrophages are prone to cell death. Interestingly, MDV antigens could only be detected in MDV infected macrophages, but not in uninfected macrophages that have phagocytosed MDV infected cells (51). Consistent with this view, Abdul-Careem *et al* (16) have shown an up-regulation in nitric oxide synthase (NOS) gene in lung macrophages of MDV infected chickens. This results in production of nitric oxide (NO) reported to have anti-microbial activity against many viruses including MDV for both *in vivo* and *in vitro* conditions (19). Likewise, a similar distribution profiles of MDV antigen have been observed in MDV infected B lymphocytes during the early cytolytic phase. Replication of MDV in the lung induces host innate immune response as demonstrated by up-regulation of Toll-like receptors (*e.g.* TLR 3 and TLR 7), pro-inflammatory cytokines (e.g. IL-1β and IL-8) and iNOS genes as well as infiltration of macrophages (16). Host immune response to MDV in the lungs is ineffective in controlling virus replication in the lungs epithelium (16, 19, 24).

Generally, the infection of mammalian immune cells such as macrophages and B cells with herpes viruses down-regulates the expression of both MHC class I and II molecules consequently, evading hosts' cell mediated immunity (52). It has been shown that MDV infection down-regulates surface expression of MHC (B complex) class I (BF) glycoproteins during active but not latent infection of chicken cells (53). Further studies demonstrated that MDV012 and MDV pUL.49.5 genes (Table 1) are involved in down-regulation of MHC class I molecules by interfering with Transporter Associated with Antigen Processing (TAP) function. However, the effects of MHC class I down-regulation in the pathogenesis of the disease is unknown and recombinant viruses lacking the cytoplasmic tail of pUL49.5 exhibit almost similar pathogenicity as wild type virus in both the resistant (B21) and susceptible (B19) lines of chicken (54, 55). NK cells and more importantly cytotoxic T lymphocytes (CTL) which monitor cell surface MHC class I molecules and viral peptide complex respectively may play a major role in host defence against infection. Contrary to MHC class I, MDV infection up-regulates MHC class II molecules on chicken APC's such as macrophages (56, 57), and this may contribute to virus spread within the infected host by increasing the interaction between infected macrophages and activated CD4^{+} T cells. Since MDV requires cell-to-cell contact for virus spread and productive infection of T cells and other immune cells, this up-regulation of MHC class II molecules might, in fact, be

essential in the pathogenesis of Marek's Disease within the infected host by increasing the interaction between infected macrophages and activated $CD4^+$ T cells (53, 56). HIV infection can also induce activation of innate response and up-regulation of MHC class II molecules which can lead to immuno-pathology (58, 59). It is still unclear whether MHC class II up-regulation observed after MDV infection can induce immunopathology which is clearly manifested in MDV pathogenesis. In the infected cells, MDV virus expresses a viral antigen with high amino acid sequence homology to CXCL13, termed viral IL-8 (vIL-8) (60). CXCL13 exerts its chemotactic effects by interacting with chemokine receptor CXCR5 and is a major regulator of trafficking for B cells and subsets of T cells. This viral chemokine has the ability to recruit immune cells to the site of infection (22). The deletion of vIL-8 from open reading frames severely affects MDV pathogenesis and tumour incidence (22, 61, 62). vIL-8 induces chemotaxis of B cells and regulatory T cells ($CD4^+CD25^+$ T cells), and these cells are targets for both lytic and latently infected cells, thus demonstrating a virus specific tropism (62). Within 24 hours after infection, the virus can be detected in the bursa of Fabricius, spleen, and thymus (11, 12). It is believed that macrophages and dendritic cells can disseminate the virus from the lungs to B cells and $CD4^+$ T cells in these lymphoid organs. Splenic B cells play an important role in the replication of MDV, and the high titre of circulating virus is due to the replication of the virus within B cells, but in the absence of B cells, the virus replicates in other immune cells and MD can still be developed. In the cytolytic phase of the disease, large number of splenic B cells undergoes apoptosis and cell death. Depletion of B cells and $CD4^+$ T lymphocytes in lymphoid tissues, such as cecal tonsils (CTs), of susceptible chicken lines within 5 day post infection may contribute to immunosuppression observed in late cytolytic phase of the disease. The depletion of B and T cells in the CTs of the resistant line of chicken was minimal at 5 dpi, which also recovered by 21 dpi (63). Virus replication in B and T cells reaches its peak between 3 and 7 dpi. B lymphocytes constitute the majority (around 90%) of cytolytically infected (MDV-antigen positive) cells, while $CD4^+$ and $CD8^+$ T lymphocytes represent only 3 and 8%, respectively (64, 65). MDV can be transmitted directly to T cells from the infected macrophages or dendritic cells; however, the transfer of virus from B cells to T cells is also conceivable.

Table 1: MDV genes and their respective products involved in immune modulation and pathogenesis

MDV Gene (Protein)	Function	Antigenic potential	Infection Stage	Reference
MDV003/078 (vIL-8)	Viral chemotactic (CXCL-) protein involved in recruiting immune systems cells to site of viral replication	--	LYTIC REPLICATION	(22, 61)
MDV010 (vLIP)	Shares homology to host lipase enzyme and forms covalent bonds with lipids	--		(66)
MDV011/012	Immune evasion protein that down regulates cell surface MHC I expression	--		(54)
MDV ORF012	Phosphoprotein required for viral growth both *in-vivo* and *in-vitro*	--		(67)
MDV040 (gB)	*Hypothetical function:* Virion membrane protein that heterodimerizes with other glycoproteins facilitating viral fusion with host membrane	Yes		(25, 26)
MDV052/053 UL39/40 (RR)	Viral Ribonucleotide Reductase (RR) is essential for replication both *in-vivo* and *in-vitro*	--		(68)
MDV064 (U_L49.5)	Partially reduces MHC I expression by interacting with TAP protein	--		(55)
MDV073 (pp38)	Early protein expressed during cytolytic infection and phosphorylated by U_s3p	Yes		(25-28)
MDV092 (U_s3p)	Serine/threonine protein kinase that phosphorylates pp38	--		(69)
MDV084/100 (ICP4)	Viral gene transactivation function	Yes		(25, 26)
MDV001a (vTR)	Required for integration of viral genome into host DNA for immune evasion, neoplastic transformation and viraemia	--	LATENCY	(70)
MDV0 (pp14)	Neurovirulence factor required for PNS neuropathy (acute or transient paralysis)	--		(71)
MDV062 (VP22)	Tegument protein essential for viral replication and modulates host cell cycle	--		(72)
MDV057 (gC/UL44)	Type 1 transmembrane protein required for horizontal transmission/shedding from feather follicle epithelium	--	FEATHER FOLLICLE SHEDDING	(73)
MDV005/076 (MEQ)	Viral oncogenic protein involved in T cell neoplastic transformation which forms homodimers and heterodimers with specific intracellular signalling proteins that modulates host cell cycle	Yes	NEAOPLASTIC TRANSFORMATION	(25, 26, 34)
MDV029 (pUL17)	Co-localizes with VP5 and VP13/14 tegument protein and essential for *in-vivo* viral growth, capsid maturation and DNA packaging.	--		(74)

Cytolytic infection of B and T cells is semi-productive, which is defined by their inability to express certain viral structural components. No cell-free virus is produced by the infected T and B lymphocytes and only non-enveloped intra-nuclear particles are detected (14). The precise mechanism as to how *in vivo* MDV spreads from cell to cell has not been elucidated. However, it is assumed that MDV glycoproteins (g) B, gC and gD, similarly observed in most herpes virus, are likely to interact with host's cell surface receptors; thereby, forming an intracellular bridge between infected and uninfected cells which might, in turn, contribute to cell associated viral spread in MD. Similarly, co-existence of MDV gH and gL have been reported to be vital for GaHV-2 cell to cell viral spread. The primary peptide of gL has a high affinity for specific region of gH, and therefore binds to it giving rise to a complex, hetero-oligomer structure that anchors itself onto the cell surface of infected host cell, promoting MDV proteins (gp) cell surface expression (75). In support, Schumacher *et al.* (76) demonstrated that deletions of gE (20DeltagE) or gI (20DeltagI) were essential in restricting viral spread and plaque formation, although viral replication was not abolished. Transfecting gE or gI did not support viral spread indicating that gE and gI could work in synergistic manner to aid viral spread. Furthermore, It is possible that MDV mainly replicates within B and T cells by mitosis of infected cells rather than production of virions. Thus, expression of all viral genes are not necessary for replication *in vivo*. By not producing all the MDV viral antigens that may be highly immunogenic, the virus can escape immune control. This notion is supported by the fact that the resting T cells, with low proliferative abilities, are less susceptible to MDV infections than the activated lymphocytes. T cell activation may increase the expression of surface molecules that is engaged in virus entry, thus enhancing the occurrence of MDV infection in these cells.

Similar to MDV, the infection of human $CD4^+$ T cells by Human T-cell leukaemia virus type 1 (HTLV-1) is increased in activated $CD4^+$ T cells. This is reflected on up-regulation of heparan sulfate proteoglycans (HSPGs), a receptor for HTLV-1, on activated $CD4^+$ T cells (77). No specific receptor for MDV entry into chicken $CD4^+$ T cells has yet been identified. It is conceivable that MDV infection induces cytolysis/ inflammatory responses and T cell activation, which leads to T cell infection and viral replication via mitosis by passing the virus to daughter lymphocytes.

The underlying genetic variation within different chicken lines may play a key role in the pathogenesis and prognosis of the MDV infection. The percentages of T cells that become cytolytically infected in the lymphoid organs are less than 2% in MD-susceptible chickens and 0.2% in the resistant chickens (14, 64). In support, Omar *et al*. (25) have demonstrated evidences of T cell mediated immune responses against the MDV antigen, ICP4, only in resistant (B21) lines of chicken; but not in susceptible (B19) ones. In contrast, both B21 and B19 lines of chicken have been reported to show T cell immune responses against MDV antigens: gB, MEQ and pp38. Nevertheless, these studies suggest that the role of T cell mediated immunity, and differences in T cell receptor repertoire generation in conjuncture with other inherent genetic resistance mechanism against MD cannot be ruled out. In latent phase of infection, MDV antigens cannot be detected in the lymphoid tissues and there is no production of infectious viruses. It is difficult to distinguish latent infection from transformation phase, as both represent non-productive infections. Following *in vitro* reactivation of MDV from virus antigen-negative lymphocytes, the majority of latently infected lymphocytes were identified as T cells with only 3% being B cells (14). MEQ antigen from MDV plays a crucial role in maintaining latency by blocking apoptosis of latently infected $CD4^+$ T cells. One of the pronounced differences in gene expression profile between MDV-resistant and susceptible lines of chicken after MDV infection are genes that are associated with apoptosis (78). In addition to MEQ, it has been shown that microRNA miR-M3, an MDV-encoded miRNA, abrogates apoptosis by directly targeting Smad2, a critical component in the transforming growth factor (TGF)-β (79) providing an environment beneficial for latency and oncogenesis. miR-M4, an orthologue of the oncogenic miR-155, was shown to have a direct effect on inducing MDV-induced T cell lymphoma, as viruses deleted in miR-M4 or having mutations in the seed region failed to induce lymphoma (80). Viral miR-M4 exerts its effects by reducing the levels of latent TGF-β binding protein-1, which is involved in the maturation of TGF-β. This leads to a reduction in the levels of active Smad2/3 and release of the inhibition of the c-Myc promoter, resulting in a rise in c-Myc transcription. The production of viral protein MEQ allows the formation of complexes with c-Myc (81), which is associated with transformation. In the susceptible chickens, a second wave of semi-productive infection and cytolysis are observed between 14 and 21 dpi (11). This late cytolytic phase is associated with immunosuppression, atrophy of lymphoid tissues such as thymus, bursa of fabricius, cecal tonsils and infiltration of mononuclear cells and heterophils (11, 82).

Virus is probably transferred to the skin by latently infected $CD4^+$ T cells and infects skin and feather follicles in a yet unknown mechanism. Syncytia formation in skin epithelium may be a product of viral protein which facilitates and pools greater resources for viral replication. The involvement of other immune system cells such as macrophages and dendritic cells to transport the virus to the skin cannot be excluded. Replication of MDV starts at 7 dpi, well before tumour development. Therefore, it is possible that early infected $CD4^+$ T cells, but not necessarily latent or transformed $CD4^+$ T cells, transport the virus into the skin at this time.

1.4 The immune response to MDV

1.4.1 Innate immunity:

While effective immunity against human *alphaherpesviruses* relies on both innate and adaptive mechanisms, the innate immune response has been shown to be of paramount importance (83). Less is known about the role of innate immunity in the control of MDV in chickens.

1.4.1.1 Interferons:

Type I IFNs belong to a family of cytokines that attracted much attention owing to their protective role against viral infection. IFNs are widely expressed cytokines that possess strong antiviral and immunomodulatory properties. The IFN family can be classified into three main types of cytokines: type I, type II and type III IFNs. IFN-α and IFN-β belongs to type I IFN family, while the type II IFN family includes only one cytokine: IFN-γ, which also exhibits antiviral activities (84). The third type of IFNs is the IFN-λ family. In mammals, plasmacytoid dendritic cells (pDCs), monocytes, epithelial cells and fibroblasts are the main producers of type I IFNs (85), while type II IFNs are predominantly produced by NK cells and activated T cells. In spite of the fact that chicken type I IFNs are shown to inhibit viral infection both *in vivo* and in vitro, chicken pDCs have not been identified.

Chickens become infected with MDV via the respiratory system by inhaling infected dust. MDV is taken up by phagocytic cells such as macrophages or dendritic cells within the respiratory system. Chicken lung has a different anatomical structure than the mammalian counterpart consisting of air sacs; and due to narrower pulmonary

capillaries than in mammals there are fewer airway resident macrophages (86). Therefore, it is likely that MDV has to cross lung epithelial lining before being transported by phagocytic cells to lymphoid tissues. In the respiratory system, the virus can be recognized by toll-like receptors (TLRs), such as TLR21 (recognizing unmethylated CpG DNA), leading to the initiation of protein signalling cascaded which stimulates the expression of type I interferons (α and β), shown to be involved in antiviral defence. In fact, an increase in the expression of TLR receptors in the lungs of MDV-infected birds (35), IFN-α expression in the blood of susceptible chickens (87) and interferon regulatory factors (IRF)-1 and IRF-3 in MDV-infected chicken embryonic fibroblasts cells (CEF) have been reported (88, 89). The role of IFNs in the control of MDV replication has been confirmed in an *in vitro* model showing that chIFNs reduces plaque formation and expression of pp38 and gB in the infected cells (90). The protective role of IFNs is also indicated by the results demonstrating the differential expression patters of IRF-3 and IFN-β genes in resistant and susceptible lines of chicken (91). Similarly, oral administration of IFN-α are shown to reduce MDV viral replication *in vivo* (92).

In addition to their direct effects on viral replication, type I interferons may also activate other immune system cells such as Natural killer (NK) cells and increase their cytotoxic function (93). However, it has been suggested that chicken NK cell cytotoxicity is not increased after oral administration of recombinant chicken IFN-α or inoculation of recombinant MDV expressing chicken IFN-α. In chickens inoculated with rMDV (IFN-α), NK cell cytotoxicity was not enhanced over control chickens at 4 and 7 days dpi. Furthermore, at 4 dpi, chickens inoculated with R2/23 actually had decreased NK cell cytotoxic activity. Therefore, it is concluded that chIFN-α, given at high doses orally in the drinking water or via expression in a recombinant MDV vector, does not increase NK cell cytotoxicity as originally hypothesized (92). The suppression of immune responses by oral administration of IFN-α is not a novel phenomenon as similar observations are reported in murine model where, bone marrow functions were suppressed by administration of murine IFN-α orally or subcutaneously (94). In fact, it has been shown that type I IFNs modulate the function of both innate and adaptive immune cells including DCs and T cells in the gut and suppress the intestinal inflammation. Taken together, it is believed that type I IFN response by MDV-infected cells promotes the activation of immune cells and inhibits MDV replication and

dissemination. Considering the complexity of MDV and co-evolution of the virus with the host's type I IFNs response, it is very likely that there is a complex relationship between MDV and host response. The exact role of type I IFNs in the pathogenesis of MDV in chickens is poorly understood due to lack of immunological reagents, complexity of MDV infection and the cell-type specific effect of type I IFNs. However, it is possible that type I IFNs may be involved in promoting latency infection of MDV as has been observed in other *alphaherpesviruses* (95).

The role of type II IFNs, IFN-γ, is discussed in more detail in adaptive immunity section. IFN-γ is induced during MDV infection and shown to have inhibitory effects on MDV replication by inducing nitric oxide production (90) and the administration of IFN-γ with MDV vaccine positively influenced vaccine-induced protective immunity *in vivo* (96). There is no information on the role of type III IFNs on viral infections in chickens.

1.4.1.2 Macrophages

There is very little information on the type and function of immune system cells involved in the initiation of immune responses against MDV in the respiratory system of chicken. However, it is believed that chicken professional APC's such as macrophages and dendritic cells play an important role in the development of adaptive immunity against MDV. In addition to their ability to present MDV antigens in association with MHC class I and II molecules to initiate adaptive immunity, macrophages can also be directly involved in inhibition of MDV replication and development of MD. Macrophages isolated from B21 chickens have higher phagocytic activity than B19 chickens to MDV (97). Moreover, macrophages obtained from MDV-infected chickens inhibited viral replication *in vitro* more efficiently than macrophages isolated from non-infected chickens (98). Further confirmation on the role of macrophages in MDV infection is obtained from the results demonstrating that depletion of macrophages from splenocytes increases MDV replication (97), while stimulating macrophages *in vivo* reduces the incidence of MD (99). Taken together, the evidence presented here from several studies confirms that macrophages play a pivotal role in control of MDV replication and MDV-derived tumour incidence. One of the mechanisms involved in the inhibitory function of macrophages on Marek's disease is their ability to produce inducible nitric oxide (iNOS). The production of NO has been

reported in the spleen, brain and lungs of MDV-infected chickens (10, 100-102) and NO is shown to inhibit MDV replication with highest level of NO production detected in serum and spleen of resistant chickens compared to susceptible chickens (102, 103). Further experiments demonstrated that the inhibition of iNOS in chickens increases viral load, suggesting that NO plays an important role in the control of MDV replication *in vivo* (102). Another function of macrophages is their ability to kill tumour cells and it is believed that activated chicken macrophages have the ability to lyse MDV-derived tumour cells *in vitro* (104). In contrast to activated and fully functional macrophages, tumour associated macrophages (TAMS) represent key regulators of the complex interplay between the immune response and cancer. TAMS produce tumour growth promoting factors and induce immunosuppression by releasing immuno-modulatory factors (105). Macrophages isolated from tumour tissues of MDV-infected chickens demonstrate similar functional abilities as TAMS and have been shown to suppress T cell proliferation *in vitro*. The development of immuno-regulatory macrophages in MDV-infected chickens correlates with transient immunosuppression observed during primary cytolytic phase of infection (106), suggesting that TAMS may be involved in MDV-induced immuno-suppression.

1.4.1.3 Natural Killer cells:

NK cells represent important effectors of the innate immunity and can respond to stimuli and produce anti-viral cytokines such as IFN-γ. In addition, these cells have ability to recognize virus- infected cells/ tumour cells via ligation of cell death receptors and the release of granules. NK cells from MDV-resistant chickens have higher cytotoxic capability than the MDV-susceptible chickens, suggesting that these cells may be involved in determining resistance to MDV. This hypothesis is also supported by the results demonstrating that MDV-infected chickens have higher NK cell activity than the cells isolated from the non-infected birds and; this activity lasted longer in the resistant chickens than the susceptible chickens (107). The exact role of NK cells in providing vaccine-induced protection against MDV is still unknown, however, there are some evidences demonstrating that vaccination against MDV increases the functional abilities of NK cells. This may explain how MDV vaccine can provide protection in vaccinated chicks as early as 3 days post vaccination (107). NK cells also play a fundamental role as antitumor senses through downregulation of cell surface markers such as MHC I. A notable characteristic of herpes viral infection and specifically MDV is down regulation

of MHC I cell surface translocation. Further to that, anti-tumor activity has not yet been demonstrated in an MDV resistant model independent of genetic factors that predispose resistance to neoplastic transformation of $CD4^+$ T cells. Taken together, it has been suggested that NK cells may play an important role in controlling MDV infection (107-109). Using recently identified markers such as CD56 and CHIR-AB1 (110) for identification of chicken NK cells, researchers will be able to elucidate the role of these important cells in providing protection against MDV.

1.4.2 Adaptive immunity

The key components of adaptive immunity are B and T lymphocytes which specifically recognize antigens and generate memory response. B cells are involved in humoral immune response, whereas T cells are involved in cell-mediated immune response.

1.4.2.1 Humoral immunity

As MDV is a cell-associated herpes virus and is strictly intracellular, antibodies should not have a major role in the protective immunity against MDV infection. However, antibodies against several MDV glycoproteins including gB, gE and gI have been detected in MDV-infected chicken (111, 112). The role of these antibodies in providing protective immunity against MDV has not been clarified. However, there is some evidence suggesting that anti-gB neutralizing antibody may have a protective role against MDV, perhaps via blocking virus entry into the host cells or antibody dependent cell-mediated cytotoxicity (ADCC) of infected cells. The role of antibody response in the control of MDV infection is also confirmed by the fact that the presence of maternal antibody delays the development of clinical signs and tumour. However, the presence of maternal antibody can interfere with live replicating vaccines against MDV by neutralizing the vaccinal virus (14). Therefore vertical transmission of immunological factors such as maternal antibodies can be a limiting factor for both generations of protective immunity as well as delaying immune responsiveness to infection of offspring from vaccinated layers. Maternal antibody and interference with vaccination has been reported in both human and veterinary medicine regardless of the types of vaccine formulations used. It has been shown that while maternal antibody can interfere with antibody response, vaccine-induced cell-mediated immune responses are largely unaffected. This has been confirmed in humans, murine models and farmed animals (113-118). With regard to the inhibitory effect of maternal antibody response, new

strategies have been developed to overcome the inhibitory effects of maternal antibody on vaccine-induced antibody response (116). Therefore, it should be possible to overcome the neutralizing effects of maternal antibody against MDV in novel vaccination strategies.

1.4.2.2 Cell-mediated immunity

Antibody and cell mediated immunity is involved in the control of infection with highly cell-associated human *alphaherpesviruses* such as VZV. Both $CD4^+$ and $CD8^+$ effector and memory T cells are shown to be essential for recovery from VZV and maintaining the latent stage of infection in the subclinical state. Generally, it is believed that (a) broad (response to several epitopes), (b) durable (memory response), and (c) multi-functional (capable of producing several Th1 type cytokines/ kill virus infected cells) T cell response is associated with the control and resolution of viral infections. MDV is also a highly cell-associated *alphaherpesviruses*, and thus it is postulated that cell-mediated immunity is crucial for the vaccine induced protection (119, 120). Since MDV exists in cell associated forms inside the host, except in feather follicles, T cell mediated immunity is thought to be more important than the antibody mediated immunity in the control of the disease in chickens (121). Studies have shown evidences demonstrating the presence of $CD8^+$ T cells against MDV antigens such as: gB, MEQ, pp38 and ICP4. However, the role of CTL in conferring long term immunity, generation of memory cells, in genetically resistant chickens is unknown (35) and the role of cell-mediated immunity in vaccine-induced protection has not been determined. No cytotoxic response against ICP22 and a weak cytotoxicity against MEQ were detected in MDV-infected chickens (14, 25). Other studies confirmed the presence of anti-*gB* and anti-*gI* $TCR\alpha\beta1^+CD8^+$ T cells with cytotoxic abilities in MDV-infected birds (122).

The presence of herpes virus specific $CD8^+$ T cell response coincides with protection from cytomegalovirus (CMV) infection and adoptive cell transfer of $CD8^+$ T cells provide protection in animal models. The importance of $CD4^+$ T cells in providing protection against herpes virus infection was also demonstrated by $CD4^+$ T cell depletion experiments in animal models. There was an inverse correlation between the number of virus-specific $CD4^+$ T cells and prolonged shedding (119, 120). Therefore, it is very likely that both $CD8^+$ and $CD4^+$ T cells are also involved in the control of MDV replication. This notion is confirmed with the early studies showing that T cells are

crucial for the control of tumour growth in HVT-vaccinated chickens. The importance of T cells in the control of tumour growth was confirmed by demonstrating that HVT vaccinated birds treated with cyclosporine, a drug shown to inhibit T cell function, developed MDV-lymphoma (123, 124). However, later studies suggested that T cells are only involved in the control of viral replication but not essential for the control of tumour growth (125). Further studies are required to confirm the exact role of $CD4^+$ and $CD8^+$ T cells in the control of MDV replication and tumour growth. The availability of $CD4^+$ and $CD8^+$ knockout chicken will provide valuable tools to study the role of these cells in vaccine-induced protective immunity against viral replication and tumour growth. MHC-determined resistance to MDV in MHC: B21/B21 birds indirectly confirms the role of T cells in control of tumour development. For MHC class I molecules, the relative level of expression varies between different MHC haplotypes and reflects the consensus hierarchy of response by different MHC haplotypes with the most susceptible chickens (MHC B19/B19) having the highest expression while the most resistant chickens (MHC: B21/B21) expressing the lowest level (126). It is believed that the differences in cell surface expression level ensure the development of optimal peripheral T cell responses against MDV. The detection of MDV-specific $CD8^+$ T cells with cytotoxic ability to recognize target cells expressing MDV antigen is technically challenging. However, Schat and co-workers developed a heterologous system to investigate cytotoxic T cell response to MDV using reticuloendothelial virus-transformed cells stably transfected with specific MDV genes. The cell lines derived from resistant (MHC: B21/B21) and susceptible (MHC: B19/B19) birds were generated and antigen-specific cytotoxicity were analysed *in vitro*. The cytotoxicity was low compared to mammalian cytotoxic T lymphocyte (CTL) assays (14). In these studies, a relatively more potent cytotoxicity was detected against target cells expressing pp38 and gB antigens. No cytotoxic response against ICP22 and a weak cytotoxicity against MEQ were detected in MDV-infected chickens (14, 25). Other studies confirmed the presence of anti-gB and gI $TCR\alpha\beta1^+CD8^+$ T cells with cytotoxic abilities in MDV-infected birds (122). Vaccination of chickens with a recombinant Fowl Pox Virus (FPV) expressing these two glycoproteins have been reported to induce protective immunity when challenged with MDV and shown to induce anti-MDV neutralizing antibodies (127, 128). Recombinant FPV expressing gB was later shown to induce cytotoxic T cell response recognizing target cells expressing gB (129). These data support the importance of anti-MDV $CD8^+$ T cells responses in the control of the disease, however,

the role of MDV-specific $CD8^+$ T cell responses in the control of viral infection or tumour growth in birds immunized with HVT or CVI988/RISPENS is still unknown. In addition, there is very little information on the magnitude and quality of MDV-specific T cells responses in resistant *vs.* susceptible chickens. The only available information suggest that anti-*ICP4* $CD8^+$ T cell responses is only detected in the resistant chickens (25), while anti-*gE* $CD8^+$ T cell response is only detected in the susceptible chickens. These results suggest that the response to ICP4, an immediate early MDV gene product, but not to gE may be an important factor in genetic resistant to MDV. It has been shown that CTL responses characterized by release of perforin and granzyme B can be induced against MDV antigens such as pp38, MEQ and ICP4 (35). Moreover, there is still no information on the presence of MDV-specific $CD4^+$ T cell responses and whether transformed $CD4^+$ T cells have antigen specificity to MDV antigens. Further studies are required to identify $CD4^+$ and $CD8^+$ T cell epitopes within MDV antigens and determine their MHC restriction. Moreover, it is important to examine the quality, broadness, durability and magnitude of the $CD4^+$ and $CD8^+$ T responses in MDV infected or vaccinated chickens and determine the correlates between T cell responses and protection.

In addition to T cell responses against MDV viral antigens, it is possible that T cells may also recognize self-antigens expressed by tumour cells. However, the nature of these self-antigens and their relevance to immunity and inhibition of tumour growth in birds is still unknown. A few decades ago, Marek's disease tumour-associated surface antigens (MATSA) was identified in the MDV-transformed T cells (130). However, it was later discovered that activated T cells can also express MATSA antigens, and this molecule is not solely expressed on transformed T cells (131). Several of these MATSA are found to be activation associated lymphocyte antigens, and one of them is identified as CD30 molecule (48). Meanwhile CD30, a co-stimulatory molecule, has been shown to have pleiotropic effects on human T cell activation, apoptosis, effector function (cytotoxicity) and regulating T cell trafficking/migration (132). It has a fundamental role across all T cell lineages such as $CD4^+$, $CD8^+$ and Th17 cells (133) and may influence T cell interaction; suppressive action in the tissue microenvironment.

Immunomodulatory processes that influence CTL functional abilities in relation to persistent viral infection and tumorigenesis may be mediated via interaction of

inhibitory receptors such as CTLA4, programmed death-1 (PD-1) and its respective ligand programmed death ligand-1 (PD-L1) (Figure 6). The evidence suggests that MDV infection up-regulates the expression of CTLA-4, PD1 and PD-L1 in chicken immune system cells. The expression level of PD-1 was increased in chickens at the early cytolytic phase of the MDV infection, while the PD-L1 expression level was increased at the latent phase. In addition, the expression levels of PD-1 and PD-L1 were increased at tumor lesions found in MDV-infected chickens (134, 135). Furthermore, T cell responsiveness may be affected by chronic antigen stimulation leading to exhaustion thus altering phenotypic characteristics such as PD-1 expression also observed during *in vitro* MDV infection. A combination of reduced MHC class I and increased PD-1 cell surface translocation provides a platform for highly efficacious immune evasion tactic. CTLA-4, a potent inhibitory receptor, expressed by $CD4^+$ T cells has also be reported during the early cytolytic phase in MDV infected birds (134). Significant differences in CTLA-4 and PD-1 expression levels, which could result in a delayed immune responsiveness (anergy) and cell death respectively, were reported between resistant and susceptible lines of chickens challenged with MDV. A similar phenotype has been reported in patients infected with human *alphaherpesviruses* such as VZV whereby $CD4^+$ T cell predominantly express CTLA-4 and PD-1 (136). Induction of an early immune unresponsiveness (anergy) and cytolysis (4-7 d.p.i.) could well be a hallmark, for *alphaherpesviruses*, to establish early semi-productive viral replication and disseminated viraemia. Blocking or down regulating CTLA-4 or PD-L1 could be therapeutically significant during early infection. Furthermore, PD-L1 expression results in immune suppression thus can be used as a marker for $CD4^+$ T cell lymphoma. PD-L1 expression in MDV challenged chickens, could limit the quality of T cells immune responsiveness to MEQ, pp38, ICP4 or gB at the immunological synapse (Figure 5). PD-1 and CTLA-4 ligation with its cognate receptor results in cross-pathway interference resulting in inhibition of RAS, ERK 1/2, AKT, JNK and PLCγ phosphorylation altering cell fate; growth/proliferation, effector function and survival. Expression of inhibitory molecules on both antigen presenting cells can lead to the generation of chicken regulatory $CD4^+$ T cells (T_{reg} cells). The role of naturally occurring and peripheral derived T_{reg} cells in modulation of anti-MDV immunity is still unknown. However, it is known that MDV infection up-regulates the expression of inhibitory molecules such as CTLA-4 and expression of inhibitory cytokines such as IL-10. The expression of regulatory molecules such as CTLA-4 and IL-10 on both $CD4^+$

and $CD8^+$ T cells are at 10 and 21 dpi and this effect was more pronounced in the MDV-susceptible lines of chicken (137).

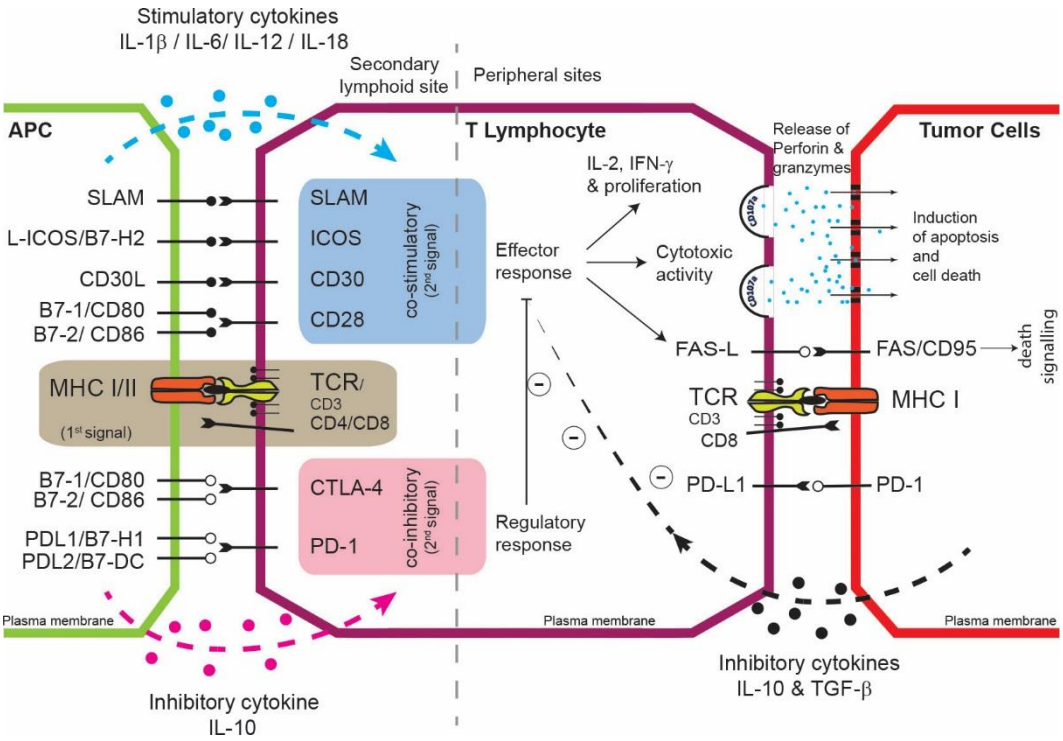


Figure 6: T cell activation and tolerance by tumour cells/ antigen presenting cells. Inhibitory or stimulatory molecules expressed on the surface of antigen presenting cells (APC) or tumour cells regulate T cell function. Moreover, stimulatory or inhibitory cytokines may drive the generation of different T cell populations (*e.g.* Th1, Th17, Treg *etc.*) with diverse functional properties. Signal one is provided to the T cell receptor of T cells by presentation of antigens via MHC class I or II molecules expressed by APCs or tumour cells, while the signal 2 is provided by co-stimulatory molecules such as B7 family. Co-stimulatory signals induce the generation of effector T cells, which can recognize and lyse target cells or produce cytokines such as IFN-γ, involved in the control of tumour growth. In contrast, inhibitory molecules deliver negative signals and suppressing the effector T cell function, and induce T cell anergy or exhaustion.

1.5 Persistence in the face of vaccination

Vaccination against MD started in the late 1960s using turkey herpesvirus (HVT) which does not induce disease in chickens. Vaccination reduced the incidence of MD by 99% and was the first successful vaccine against naturally occurring virus-induced cancer. Since then MDV has evolved, perhaps due to vaccine induced immune response, and the escape of new mutants from immune pressure. Currently only the attenuated MDV strain; CVI988/RIPENS is effective in providing protection against the very virulent

MDV. Live vaccines are administered either to day-old chicks or to the 18-day-old embryo. MDV vaccine inhibits the development of MDV-induced lymphoma but does not prevent infection and replication of pathogenic strains of MDV. Both vaccination and maternal antibodies against MDV increases viral shedding and onward transmission of hyper-virulent strains of MDV due to the survival of the host without controlling the virus shedding (138). The failure of current vaccines to induce sterile immunity can be attributed to MDV inducing latency with minimal viral replication and viral protein expression. The ideal MDV vaccine is to control both the disease and viral shedding in the infected birds. Revaccination (prime-boost) with the current cell-associated MDV vaccine (CVI988/RIPENS) improve protection against the disease and increases the magnitude of anti-MDV T cell responses as demonstrated by enhancement of anti-MDV neutralizing antibody and proliferation of $CD4^+$ and $CD8^+$ T cells (139). However, the type and quality of immunity after even revaccination with the current MDV vaccines cannot control virus shedding.

To generate more effective vaccines against MDV, we need to (A) have a better understanding of the type of immunity required for reducing viral shedding/ inducing sterile immunity (B) design a vaccine that can induce protective immunity against the virus. It is believed that the quality, broadness, durability and homing of both $CD4^+$ and $CD8^+$ T cell responses may explain why vaccines against some alpha-herpes viruses (VZV) are more effective than vaccines against other alpha-herpes viruses (HSV) (140). As yet there is little information as to whether cell-mediated immunity is induced by the MDV vaccine.

The lack of established methodology to assess cell-mediated immunity to MDV vaccine is the major obstacle to have a better understanding of how MDV vaccine actually works. Several factors including genetic background of chickens (141), presence or absence of maternal antibodies, virulence of MDV, vaccine dose (142) and concurrent infections with other immunosuppressive pathogens such as CIAV (122) can influence the efficacy of MDV vaccines. It is very likely that the induction of both innate and adaptive immune responses by vaccine strain is similar to those induced by pathogenic MDV (107, 143). There are some evidences demonstrating that a number of MDV antigens, including gB when administered as recombinant vaccine in a fowl pox vector, are immunogenic and immunity to these antigens confers protection (129). It is

postulated that vaccine-induced adaptive immunity plays a major role in providing protection against the disease. However, the types and magnitude of vaccine-induced protective immune responses to these antigens are still unknown.

Chapter 2: 1,25(OH)$_2$ Vitamin D3 modulates avian T lymphocyte functions without inducing CTL unresponsiveness

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2.1 Abstract

1,25-Dihydroxyvitamin D3 (Vitamin D) is a naturally synthesized fat soluble vitamin shown to have immunomodulatory, anti-inflammatory and cancer prevention properties in human and murine models. Here, we studied the effects of Vitamin D on the functional abilities of avian T lymphocytes using chicken Interferon (IFN)-γ ELISPOT assay, BrdU proliferation assay, Annexin V apoptosis assay and PhosFlow for detecting phosphorylated signalling molecules. The results demonstrate that Vitamin D significantly inhibited the abilities of T lymphocytes to produce IFN-γ and proliferate *in vitro* ($P \leq 0.05$), but retained their ability to undergo degranulation, which is a maker for cytotoxicity of these cells. Similarly, Vitamin D did not inhibit Extracellular signal-Regulated Kinase (ERK) 1/2 phosphorylation, a key mediator in T cell signalling, in the stimulated T lymphocytes population, while reduced ERK1/2 phosphorylation levels in the unstimulated cells. Our data provide evidence that Vitamin D has immuno-modulatory properties on chicken T lymphocytes without inducing unresponsiveness and by limiting immuno-pathology can promote protective immunity against infectious diseases of poultry.

2.2 Introduction

A vital component for competent physiological function, Vitamin D is a naturally synthesized lipid soluble vitamin classically involved in calcium and phosphorus homeostasis but recent evidence indicate a broader range of action not limited to its immune modulatory effects on mammalian leukocyte (144-146). Ultraviolet B (UV-B) irradiation of epidermal cells constitutes the primary step for photolytic conversion of 7-dehydrocholesterol to Vitamin D. Modern poultry farming practices have led to an increase in density housing with minimal ultraviolet light B (UV-B) exposure. Thus, eggs produced from indoors housed chickens have a significantly lower (3.8 µg Vitamin D/100g of dry matter) egg yolk Vitamin D_3 content compared to those housed outdoors (14.3 µg Vitamin D/100g of dry matter). The 25-hydroxyvitamin D_3 (25[OH]D_3) content of egg yolk was also influenced by sunlight exposure, although less pronounced than the vitamin D content (147). Alternatively, Vitamin D3 can be acquired in the diet or as supplements. Vitamin D3 is subsequently hydroxylated by hepatic mitochondrial cytochrome P450 (CYP27A1) into 25(OH)D_3. Finally, 25(OH)D_3 is hydroxylated by renal mitochondrial cytochrome P450 (CYP27B1) into 1α, 25-dyhydroxyvitamin D_3 (1α, 25$(OH)_2D_3$). The latter is biologically relevant and active form with endocrine actions. Macrophages have been shown to express both CYP27A1 and CYP27B1 (144, 148) enzymes required to produce 1α, 25$(OH)_2D_3$, whereas T cells can only perform the final metabolic step (149, 150). Therefore, immune system cells may be able to use Vitamin D in an autocrine and paracrine manner.

The immune modulatory functions of 1α, 25$(OH)_2D_3$ have been linked to genomic effects mediated by Vitamin D Receptor (VDR), a member of the nuclear hormone superfamily found in most immune cells (146, 151) such as macrophages (152), dendritic cells (153), B cells (151, 152, 154) and T cells (151, 152). Mice VDR-/- models have been used to demonstrate this interdependent relationship (155-157). Genetic polymorphism in the VDR gene has been associated with susceptibility to several viral infections in human; Dengue Virus (DENV; rs2228570) (158), Hepatitis B Virus (HBV; FokI C>T) (159), Respiratory Syncytial Virus (RSV; rs10735810) (160) and even in chickens; Marek's Disease Virus (MDV; S1P4) (161). In humans, Vitamin D has been shown to be effective in the prevention and control of viral diseases such as

Human Immunodeficiency Virus (HIV) (162, 163) and RSV (164). Studies into the mechanistic effects have demonstrated that Vitamin D regulates immune system cells functional abilities in an attempt to maintain immune homeostasis.

In the context of innate immunity, Vitamin D may influence the type and magnitude of antigen presenting cell responses and their retrospective ability to modulate T lymphocyte function. It has been recently demonstrated that chicken macrophages exposed to $25(OH)D_3$ have a 5-fold increase in nitric oxide production (165). Stimulating nitric oxide production enhances phagocytic activity of macrophages and induces cytostatic or cytotoxic action against viruses, bacteria, fungi and tumour cells (166). In addition, low dose Vitamin D treatment may restore human macrophage proliferative ability (167), and increase antimicrobial peptide production such as cathelicidin and β-defensin in response to stimuli (168). Vitamin D may perturb dendritic cells responsiveness to microbial stimuli, thus impeding maturation (169). Human dendritic cells and macrophages have been shown to produce less interleukin (IL)-12 when treated with high dose Vitamin D (170). This could reduce their functional capacity as antigen presenting cells (APC) required for initiation of Th1 type T cell responses.

In the context of adaptive immunity, defence against intracellular pathogens is mediated in part by $CD4^+$ and $CD8^+$ T lymphocytes. Vitamin D alters naive and effector T cell activation, and their cytokine secretion patterns (171). This pleiotropic lipid soluble vitamin may be important for potentiating induction of naive T cells via an alternative mitogen-activated protein kinase (MAPK) pathway (172). The latter is involved in establishing intracellular PLC-γ1 protein which plays a central role in classical T cell receptor (TCR) signaling pathway. However, human PBMC's stimulated with a T cell specific mitogen (PHA; phytohemagglutinin) in the presence of Vitamin D was observed to inhibit cellular proliferation which has been associated with a decrease in IL-2 production (173). Additionally, Vitamin D has been shown to inhibit IL-17 production (174) and increase IL-4, IL-5, IL-10 and IL-13 cytokines expression (175-177). T cell trafficking and homing to specific tissues may be influenced by Vitamin D (146). Vitamin D positively redirected T cell specific translocation to epidermal keratinocytes by stimulating expression of chemokine receptor 10 (CCR10),

which recognizes the chemokine CCL27 (178). This will influence the retention of T cells with specific phenotypes and effector function within the skin (179).

Vitamin D has profound effects on the function of human and murine T cells but there is an information deficit regarding its effects on the functional abilities of avian immune system cells, especially chicken T cells and their responses to pathogens. Here, we demonstrate that Vitamin D inhibits IFN-γ production and T cell proliferation without inducing program cell death. In contrast to its inhibitory effects on the inflammatory functions of T lymphocytes, Vitamin D did not inhibit CD107a expression, a degranulation and cytotoxicity marker, and is shown to be crucial for the control of intracellular pathogens. Moreover, Vitamin D did not inhibit Extracellular signal-Regulated Kinase (ERK) 1/2 signalling pathway, which is regulated in T lymphocytes by T cell receptor (TCR)-CD3 complex interacting with peptide epitopes presented by antigen presenting cells.

2.3 Materials and Methods

2.3.1 Experimental Animals:

Inbred mixed sex Rhode Island Red chickens, specific pathogen free as not gnotobiotic, were reared in filtered-air positive pressure rooms on floor pens with wood shaving at The Pirbright Institute. Birds were group housed, and had ad libitum access to water and commercial feed. The work was performed according to home office guidelines. Tissue samples were taken from animals after humane killing under schedule 1. Three week old birds were culled by cervical dislocation, and spleens were removed and collected aseptically.

2.3.2 Spleen mononuclear cell preparation

Whole spleens were kept on ice in PBS as soon as they were removed from chickens. After being rinsed in PBS, spleens were placed onto 40-μm BD cell strainers (BD Biosciences, UK), and crushed through using the flat end of a syringe plunger. Splenocytes cell suspension were prepared by layering (2:1) onto LymphoprepTM (Axis-shield PoC AS, Norway) density-gradient centrifugation, and centrifuged at 2100 rpm for 20 min to allow the separation of mononuclear cells. Mononuclear cells were

subsequently aspirated from the interface, and washed at 1500 rpm for 5 min in RPMI 1640 with penicillin (10 U/ml), and streptomycin (10 µg/ml). Mononuclear cells were suspended in complete RPMI cell culture medium; RPMI 1640 medium containing 10% foetal bovine serum (Sigma-Aldrich, Dorset, UK), penicillin (10 U/ml), and streptomycin (10 µg/ml). Cell number and viability were calculated using a haemocytometer, and trypan blue exclusion method. Mononuclear cells were suspended in complete RPMI cell culture medium at a density of 5×10^6 cells/ml and kept on ice.

1α, 25-Dihydroxyvitamin D3 (Sigma-Aldrich, Dorset, UK) was suspended in Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, Dorset, UK) at 2.5 x 10^{-4} M. Mononuclear cells were stimulated in the presence of 1α,25-Dihydroxyvitamin D3 (10^{-7} M and 10^{-8} M), or DMSO (vehicle-control) in Sterilin™ 7 mL polystyrene bijou containers (ThermoFisher Scientific, UK) and incubated (4 hrs; 41°C, 5% CO_2). Cells were harvested, washed and centrifuged at 1500 rpm for 5 min, and suspended in complete RPMI cell culture medium for use in subsequent assays.

2.3.3 Bromodeoxyuridine (BrdU) incorporation and proliferation Assay:

Detection and quantification of mononuclear cell proliferation was measured by BrdU integration (Roche, UK) into DNA. In brief, mononuclear cells were seeded in triplicates at 4.0 x 10^4 cells per well into 96-well microtiter plates and stimulated with medium alone or in the presence of Concanavalin A (ConA; 10 µg/ml). Cells were incubated (41°C, 5% CO_2) for 72 hrs with an 18 hour pulse of 10 µM BrdU. BrdU integration was detected by labelling with 100 µl anti-BrdU-POD monoclonal antibody and incubating at room temperature (RT) for 90 min. A Tetramethyl-Benzidine (TMD) substrate solution was used to develop the colour at RT for 15 min in the dark. The reaction product was inactivated with 1 M sulphuric acid. Immune complexes were detected by reading absorbance at 450 nm (OD_{450}).

2.3.4 Chicken IFN-γ ELISPOT Assay:

IFN-γ production was detected using an IFN-γ Chicken antibody pair kit (life technologies, UK). In brief, MAIPS4510 MultiScreenTM-IP 96 well plates (Millipore, UK) were coated overnight at 4°C with 2 µg/ml mouse anti-ChIFN-γ in PBS. Plates were washed twice with blocking buffer (RPMI 1640 plus 2% FCS) and incubated (1 hr; 37°C, 5% CO_2) with blocking buffer. Mononuclear cells were seeded in triplicates at

5.0 x 10^5 cells per well, stimulated with medium alone or in the presence of PMA (50 ng/ml) plus Ionomycin (ION; 1 µg/ml); (Sigma-Aldrich, Dorset, UK) and incubated (41°C, 5% CO_2) overnight. Plates were washed twice with SQ water followed by three time wash with washing buffer (PBS + 0.1% Tween 20). 1 µg/ml of anti-chicken IFN-γ biotinylated antibody in assay buffer (PBS + 0.1% Tween 20 + 1.0% BSA) was added to the plate and incubated for 1 hour in the dark at RT. Plates were washed with washing buffer and incubated for a further 1 hour with Streptavidin-HRP (1/1250) in assay buffer. A final wash with washing buffer was performed and a 3-Amino-9-ethylcarbazole (AEC) substrate solution (BD Biosciences, UK) was used to develop the colour at RT in the dark. After 20 min, the reaction was inactivated by washing in distilled water, air dried overnight and spots forming units (SFU) were counted using an automated ELISPOT reader.

2.3.5 Flow Cytometry/Phosflow:

Cytotoxicity assay: Unconjugated CD107a (LEP) and isotype control (CC63) antibodies were conjugated using the Alexa Fluor 647 labelling kit (Life Technologies, UK) according to manufacturer's recommendation prior to use. The degranulation marker, CD107a, of mononuclear cells were assessed: mononuclear cells were seeded in triplicates at 5.0 x 10^5 cells per well and stimulated with medium alone or in the presence of PMA (50 ng/ml) plus ION (1 µg/ml) with the following antibodies; LEP (CD107a-Alexa Fluor 647) or isotype control (cc63-Alexa Fluor 647) and incubated (4 hrs; 41°C, 5% CO_2). Following a wash in PBS, cells were labelled with CD3-FITC (Southern Biotech) for 15 min at 4°C and dead cells were excluded using 7-AAD-PE staining. Cells were acquired on a FACS Calibur flow cytometer and data processed by FlowJo software.

Apoptosis assay: To determine the effects of Vitamin D on cell apoptosis, mononuclear cells were stained with Annexin V-APC (BDTM Pharmigen, UK) and 7-AAD-PE (BDTM Pharmigen, UK). Apoptotic and dead cells were acquired with a FACS Calibur flow cytometer and data were analysed using FloJo software.

Phosflow signalling: Mononuclear cells were seeded in triplicates at 5.0 x 10^5 cells per well and incubated (5 and 15 min; 41°C, 5% CO_2) with medium alone or in the presence of PMA (50 ng/ml). Cells were fixed for 30 min using the fixation/permeabilization kit (eBioscience, Thermofisher, UK). Cells were incubated in Perm buffer for 10 min at 4°C, followed by staining with ERK 1/2-PE (T202/Y204) (BDTM Pharmigen, UK),

antibody for an additional 30 min at 4°C. Cells were washed twice in Perm buffer, and resuspended in FACS buffer. Cells were acquired on a FACS Calibur flow cytometer and data processed by FlowJo software.

2.3.6 Statistical Analysis

All data are presented as mean + SD. ELISpot SFU data were adjusted to 1.0 x 10^6 cells. Quantification was performed using Graph Pad Prism 6 for windows. All data points in each experiment were analysed by non-parametric Wilcoxon tests (Mann-Whitney). Results were considered statistically significant at $P < 0.05$ (*).

2.4 Results

2.4.1 Vitamin D inhibits T cell proliferation and IFN-γ production

Mononuclear chicken splenocytes were cultured in complete RPMI medium containing high (100 nM) or low (10 nM) concentration of Vitamin D for 4 hrs and their ability to proliferate *ex vivo* was examined using a BrdU-based proliferation assay. Some cells were cultured in vehicle only (DMSO) or medium only and were used as the control groups. All data points, experiment 1-3 (Figure 7A), were plotted and the results demonstrate that the cells treated with high concentration (100 nM) Vitamin D, but not low concentration (10 nM) Vitamin D, had lower proliferative ability in response to Con A (10 µg/ml) stimulation compared to the cells treated with vehicle only (DMSO; $P = 0.0002$) or medium only. The mean inhibitory percentage was calculated for both low and high levels of Vitamin D (Figure 7B). In three independent experiments performed using mononuclear cells from three different birds, Vitamin D (100 nM) inhibited proliferation by 46.2 % (experiment 1), 30.4 % (experiment 2) and 29.3 % (experiment 3), whereas Vitamin D (10 nM) inhibited proliferation by 1.76 % (experiment 1), 1.08 % (experiment 2) and 17.7 % (experiment 3) compared to the cells treated with vehicle only (Figure 7B). In experiment 3, low levels of Vitamin D also significantly reduced T cell proliferation ($P = 0.0009$). An additional proliferation assay was performed in which vitamin D-treated cells were stimulated with different concentrations of Con A (1, 5, 10, 20 µg/ml). Cells stimulated with 1 or 5 µg/ml Con A had lower proliferation levels than those stimulated with 10 or 20 µg/ml. However, there was no differences in the proliferation levels between cells stimulated with 10 and

20 µg/ml in both Vitamin D-treated and vehicle-treated cells, suggesting that Vitamin D treatment does not increase threshold of T cell activation (data not shown).

Having demonstrated that Vitamin D (100 nM) significantly inhibits T cell proliferation *ex vivo*, we analysed the influence of this vitamin on chicken T cells ability to produce IFN-γ using a chicken IFN-γ ELISpot assay. Vitamin D-treated (100 nM or 10 nM) or vehicle-treated chicken splenocytes were stimulated with a cocktail of PMA (50 ng/ml) and Ion (1 µg/ml) for 18hrs. Spots Forming Unit (SFU) at 1.0 x 10^6 cells confirmed that Vitamin D (100 nM) significantly inhibited IFN-γ production (P = 0.0002; Figure 7C) compared to the cells pre-treated with vehicle only. Low levels of Vitamin D also significantly reduced IFN-γ production compared to the vehicle only (P = 0.0073). In the five independent experiments performed, an average percentage of inhibition was calculated (Figure 7D). Vitamin D (100 nM) inhibited IFN-γ production by 25.3 % (experiment 1), 18.9 % (experiment 2), 32.6 % (experiment 3), 48.7% (experiment 4) and 43.0% (experiment 5) whereas low level of Vitamin D (10 nM) inhibited IFN-γ production by 7.6 % (experiment 1), 16.5 % (experiment 2), 25.1 % (experiment 3), 31.4% (experiment 4) and 20.4% (experiment 5) compared to the control group. In all five experiments, there was a significant difference in the ability of IFN-γ production between the cells treated with low levels of Vitamin D (10 nM) and the control groups (P = 0.0119; Figure 7D). No significant difference was observed between vehicle treated and untreated control cells, indicating that vehicle does not influence chicken T cells to produce IFN-γ.

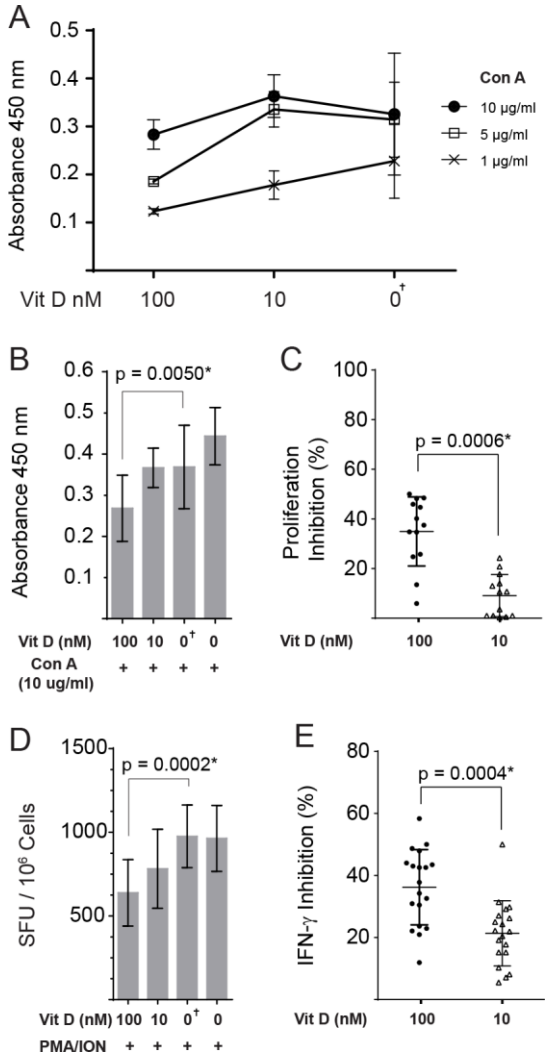

Figure 7: Vitamin D inhibits chicken T cell proliferation and IFN-γ production *ex vivo*.

Splenocytes were pre-treated for 4hrs with Vitamin D (100 nM and 10 nM) or vehicle only (DMSO). (**A**) BrdU incorporation measured by ELISA assay to quantify T cell proliferation after Concanavalin A (Con A; 10 µg/ml) stimulation. The results are presented as absorbance at OD_{450} from three independent experiments with 3-4 biological replicates in each experiment (ten replicates in total). (**B**) Represents the average percentage of inhibition for T cell proliferation induced by Vitamin D treatment in three independent experiments (Exp. 1, Exp. 2 and Exp. 3). (**C**) The frequency of IFN-γ producing mononuclear cells stimulated with a T-cell stimulation cocktail of PMA and Ion was detected using a chicken IFN-γ ELISPOT assay. The results are presented as spots forming unit (SFU) per 1.0 x 10^6 cells from five independent experiments with 3-5 biological replicates in each experiment (19 replicates in total). (**D**) Represents the average percentage of inhibition for IFN-γ production by mononuclear cells after Vitamin D_3 pre-treatment. Non-parametric Wilcoxon tests (Mann-Whitney) was used to assess normal distribution and test significance (**B, C**). The results are shown as mean ± SD (**A, C**). †(symbol) represents cells treated with Vehicle only (DMSO). ***** indicates a statistically significant difference compared to vehicle ($P < 0.05$).

2.4.2 The inhibitory effects of Vitamin D on T cells is not due to cell death or apoptosis

To examine whether the Vitamin D induced inhibition of T cell proliferation and IFN-γ production is due to Vitamin D induced-cell death or apoptosis, mononuclear splenocytes were stained with 7AAD and Annexin V post Vitamin D (100 nM) pre-treatment. The percentages of live cells ($7AAD^{-}Annexin\ V^{-}$), apoptotic cells ($7AAD^{-}$ $Annexin\ V^{+}$) and dead cells ($7AAD^{+}Annexin\ V^{+}$) were determined 4 hrs after the treatment using flow cytometry (Figure 8A). No significant difference was observed in the percentages of live, apoptotic or dead cells between Vitamin D (100 nM) and Vehicle (DMSO) pre-treatment cells (Figure 8B). The results were confirmed in four independent experiments performed on splenocytes from four different birds. To examine whether vitamin D induces T cell apoptosis after a longer term Vitamin D treatment, the percentages of apoptotic and dead cells were analysed in the treated cells 24 hrs post Vitamin D (100 nM, 10 nM) or vehicle treatment (Figure 8C). No significant differences in the levels of apoptosis or cell death were observed in these groups, suggesting that Vitamin D does not induce T cells apoptosis. However, higher levels of apoptosis were observed in the cells 24 hrs after treatment compared to those treated 4 hrs after treatment (Figure 8C). Taken together, the results demonstrate that the inhibitory effects of Vitamin D on T cell proliferation and IFN-γ production are not due to cell death or apoptosis.

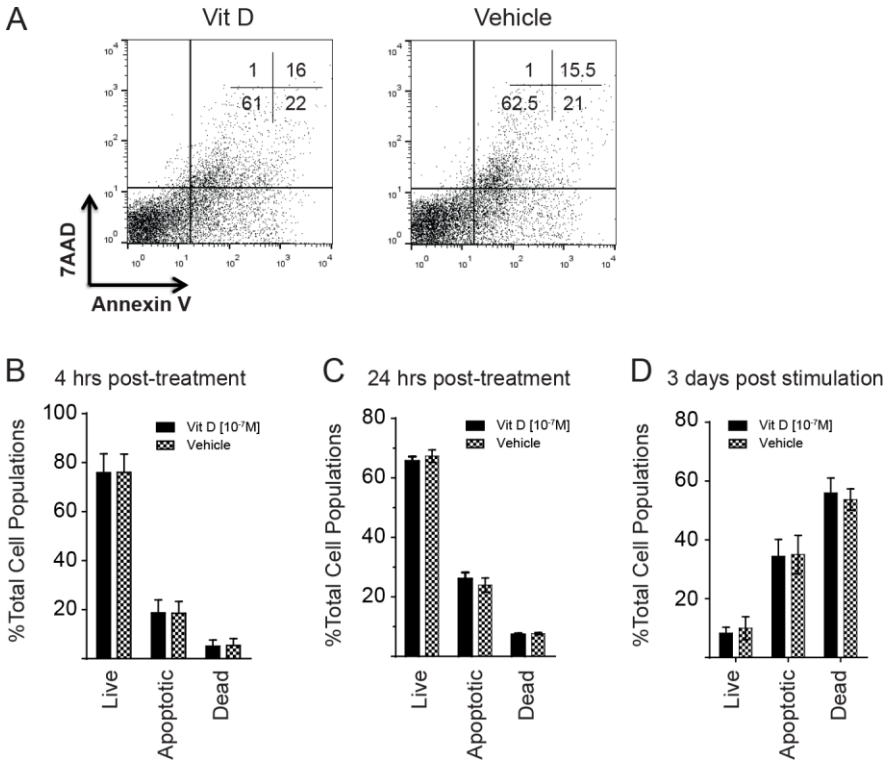

Figure 8: Vitamin D treatment does not induce cell death/apoptosis of chicken splenocytes.
Chicken splenocytes were treated with Vitamin D (100 nM) or vehicle (DMSO) for 4 hrs. The cells were stained with 7AAD (dead cell marker) and Annexin V (early apoptotic marker) and the data were analysed using flow cytometry. (**A**) Shows dot plots of the stained cells and the numbers in each quadrant represents percentage of cells. (**B**) The percentages (as mean ± SD) of live cells (Annexin V^-7AAD^- cells), apoptotic cells (Annexin V^+7AAD^- cells) and dead cells (Annexin V^+7AAD^+ cells) are shown from four independent experiments with three biological replicates in each experiment (12 biological replicates in total). (**C**) The data represent the percentage of live, apoptotic and dead cells 24 hrs post Vitamin D (100 nM) or vehicle (DMSO) treatment. (*P* = NS indicates no statistical significance).

2.4.3 Vitamin D does not inhibit $CD3^-$ or $CD3^+$ T cell degranulation

Translocation of lysosome-associated membrane protein 1 (LAMP1/CD107a) is a marker of degranulation, an important function of lymphocytes such as T cells and NK cells. We studied the effects of Vitamin D on the ability of $CD3^-$ and $CD3^+$ cells degranulation *in vitro*. Chicken mononuclear splenocytes were cultured in medium containing Vitamin D (100 nM or 10 nM) or vehicle only (DMSO) for 4 hrs, and were stimulated for an additional 4 hrs in medium with PMA (50 ng/ml) and ION (1 µg/ml). The expression of CD107a was assessed on $CD3^-$ cells and $CD3^+$ T cells using flow

cytometry (Figure 9A). The percentages of $CD3^-CD107a^+$ cells (Figure 9B) and $CD3^+CD107a^+$ T cells are shown (Figure 9C). The results demonstrate that Vitamin D (100 nM or 10 nM) has no significant effects on the cytotoxicity (degranulation) of $CD3^-$ cells or $CD3^+$ T cells *in vitro*. The results were confirmed in four independent experiments performed in triplicates.

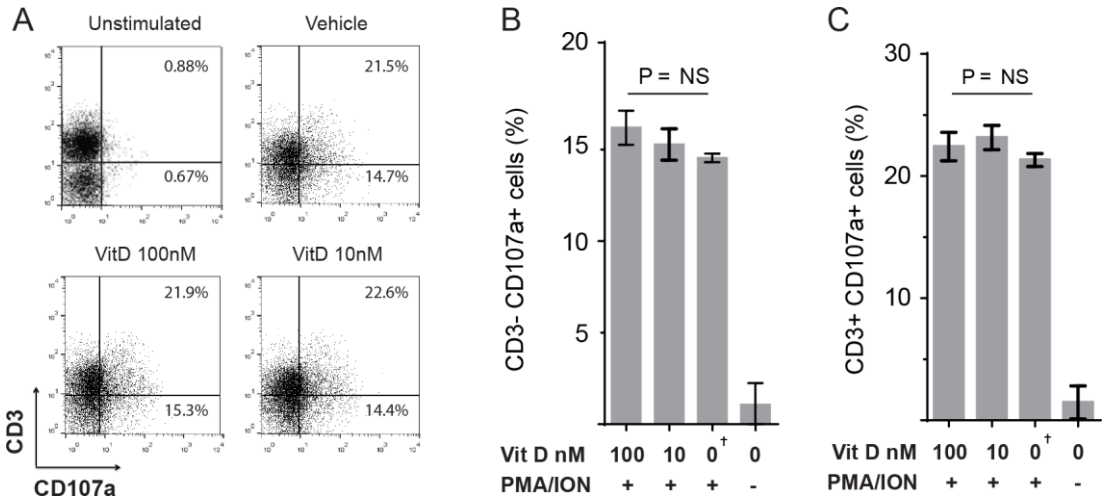

Figure 9: Degranulation of chicken T cells is not influenced by Vitamin D.
(**A**) Representative flow cytometry dot plots of spleen mononuclear cells that were cultured in medium containing Vitamin D (100 nM or 10 nM) or vehicle only (DMSO) for 4 hrs and were stimulated with a T cell stimulation cocktail of PMA and Ion for an additional 4 hrs. The expression of CD107a in $CD3^+$ and $CD3^-$ cells was analysed using flow cytometry. Upper right quadrant and lower right quadrant shows the percentages of $CD107a^+$ cells within $CD3^+$ and $CD3^-$ T cells, respectively. The bar graphs represents the percentages of $CD3^-CD107a^+$. (**B**) and $CD3^+CD107a^+$ cells (**C**) in the treated cells. The results are shown as mean ± SD of the study population of CD107a expression (*P* = NS indicates no statistical significance). † (symbol) represents the cells treated with the Vehicle only (DMSO). Similar data were obtained in four independent experiments.

2.4.4 Vitamin D limits ERK1/2 phosphorylation in unstimulated $CD3^+$ T cells

ERK1/2 signal pathway is an important regulator of T cell function. Here, we examined the effects of Vitamin D on phosphorylation of ERK1/2 (T202/y204) in unstimulated and activated $CD3^+$ T cells using Phosflow (Figure 10). Mononuclear splenocytes were cultured in medium containing Vitamin D (100 nM) or vehicle only for 4 hrs. PMA has been used in many studies for analysing human/ murine T cell signalling. In our hands, Con A cannot stimulate ERK1/2 phosphorylation and IFN-γ production by chicken T

cells. Therefore, we decided to use PMA for analysing ERK 1/2 signalling. The phosphorylation of ERK1/2 (T202/y204) was analysed in the unstimulated cells and in the cells stimulated with PMA for 5 min. The results demonstrate that Vitamin D treatment reduces ERK1/2 phosphorylation in the unstimulated $CD3^+$ T cells compared to the cells treated with vehicle only. However, there were no significant differences observed in the levels of ERK1/2 phosphorylation between Vitamin D and vehicle-treated cells after PMA stimulation (Figure 10). The results were confirmed in three independent experiments.

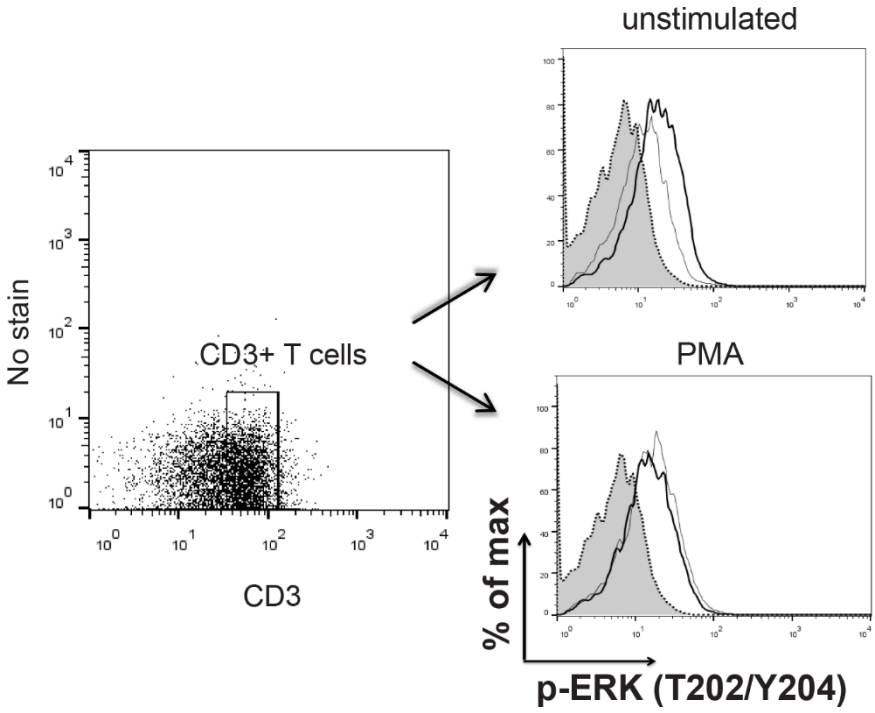

Figure 10: Vitamin D limits ERK1/2 phosphorylation in the resting chicken $CD3^+$ T cells.
Following incubation of splenocytes with Vitamin D or vehicle only for 4 hrs, phosphorylation of ERK1/2 (T202/Y204) was evaluated in $CD3^+$ T cells in the resting cells (unstimulated) or cells stimulated with PMA for 5 min using flow cytometry. The data represents flow cytometry analysis of ERK 1/2 phosphorylation in cells treated with vehicle (thick line), Vitamin D (thin line) or Isotype control (grey area) in the resting cells and PMA-stimulated T cells.

2.5 Discussion

The immuno-modulatory, anti-inflammatory and cancer prevention properties of Vitamin D are attributed to its direct effects on immune system cells, including T lymphocytes in human and animal models (164, 180-184). The results presented in this

report demonstrate that the active Vitamin D metabolite modulates avian T lymphocytes effector functions similar to the results obtained from human and murine studies (157, 172, 185). The functional heterogeneity of T lymphocytes is crucial for both limiting immuno-pathogenesis and promoting protective immune responses against pathogens. In this report, we demonstrate that Vitamin D reduces chicken T lymphocyte proliferation as well as the frequency of IFN-γ producing cells. The modulatory effects of Vitamin D were further confirmed based on down-regulation of ERK1/2 phosphorylation status that impacts subsequent downstream signal transduction pathways. Additionally, the results show that Vitamin D does not trigger programmed cell death by apoptosis, and its functional effects can only be attributed to the modulation of T lymphocyte effector function. In contrast to the inhibitory effects of Vitamin D on T cell proliferation and cytokine production, it did not have any significant effects on CD107a cell surface translocation, a marker for cytotoxic degranulation, confirming that Vitamin D does not induce general immuno-suppression of T cell functions. This notion was also confirmed with the results demonstrating that Vitamin D did not inhibit ERK1/2 phosphorylation in the activated T cells and only reduced base line ERK1/2 phosphorylation in the unstimulated cells.

T lymphocytes are the main adaptive immune cells and one of their main effector functions is their ability to proliferate in response to the stimuli or a specific antigen. Functional studies assessing Vitamin D effect on human immune system cells indicate a regulatory role as demonstrated by their ability to reduce the magnitude of T lymphocyte responses. Lemire *et al* (186) and Rigby *et al* (173) were among the first to demonstrate a dose dependent inhibition by Vitamin D on human PBMC proliferation after mitogen stimulation. Provvedini *et al (151)* provided the first evidence for the presence of a VDR in human immune system cells. However, little is known about the effect of Vitamin D on avian immune cells in health and disease and the consequent responses to the treatment. Our group and others have reported that Vitamin D may have both modulatory and stimulatory effects on chicken macrophages (168, 180, 187). In this report, we present our data demonstrating that Vitamin D modulates T lymphocyte ability to proliferate in response to the stimuli. In human, the inhibition of T lymphocyte proliferation by Vitamin D has been linked with a reduction in IL-2 cytokine production (173). Vitamin D interacts directly with its receptor (VDR), which has a VDR responsive element on the IL-2 gene, thereby repressing transcriptional

activation (188, 189), which may explain in part its mechanism of action. Adding recombinant human IL-2 to the Vitamin D-treated T lymphocytes only partially restored T cell proliferative ability (173), suggesting that Vitamin D may exert its inhibitory effects in both IL-2 dependent and independent manner. A nuclear threshold mechanism for Vitamin D to promote VDR signalling is required to repress cytokine gene expression (145, 151). On the other hand, supplementing Vitamin D restored human PBMC proliferative function in patients diagnosed as hypovitaminosis D (172). Vitamin D also exerts immuno-regulatory effects on murine T cells. For example, it has been shown that pathogenic T lymphocytes are generated in VDR-/- knockout mice due to overproduction of IL-2 resulting in excessive proliferation (190). Moreover, in a multiple sclerosis (MS) model, T lymphocyte proliferation was inhibited via the addition of exogenous Vitamin D (181). In addition, it has been demonstrated that Vitamin D reduces T lymphocyte effector function by generating dendritic cells that have lower ability to produce IL-12 (170, 185). In this report, we show for the first time that Vitamin D is able to control the rate of avian T cell proliferation similar to what had been previously reported on human and murine T cells. It has previously been suggested that the effect of Vitamin D upon proliferation of human T cells varies according to the strength of the stimulation and particularly affected by a lack of co-stimulation (191). Based on the results obtained from Vitamin D-treated cells stimulated with different concentration of the stimuli, we have demonstrated that Vitamin D does not increases the threshold of avian T cell stimulation, however further studies are required to determine the role of co-stimulatory molecules in activation of Vitamin D-treated avian T cells.

To our knowledge, this is the first report demonstrating that primary T lymphocytes treated with Vitamin D have lower ERK 1/2 (T202/Y204) phosphorylation levels at baseline in non-stimulated cells. ERK1/2 phosphorylation level was not affected in the stimulated T lymphocytes, suggesting that Vitamin D only reduces the baseline in the treated cells but these cells are fully responsive to the stimuli, confirming that Vitamin D may modulate avian immune responses but does not induce immuno-suppression or immune-unresponsiveness. Studying the effects of Vitamin D on human γδ T lymphocytes demonstrated a reduction in ERK 1/2 phosphorylation (192), suggesting that Vitamin D exerts anti-inflammatory effects by modulating the overactive immune system cells. Our data confirm these findings in avian T lymphocytes by studying ERK

1/2 in unstimulated and stimulated cells. ERK1/2 is one of the foremost studied Mitogen-activated protein kinase (MAPK) within RAF-MEK-ERK signalling pathway in mammalian systems (193). Integrated upstream signalling from TCR stimulation and cytokines activates the mechanism for ERK1/2 phosphorylation that transactivates transcriptional factors to regulate cellular proliferation at specific cell cycle checkpoint. In avian systems, specifically T cells, the complex MEK-ERK signalling pathway is scientifically compelling and hasn't been explored to elucidate potential implication in immune responsiveness, pathogenesis and disease control. In concordance, Buza *et al* (194) outlined the importance of understanding ERK pathway transactivation and crosstalk signalling specifically in chicken viral diseases that leads to T Lymphocyte transformation. Proteomic analysis of chicken cell lines correlates ERK 1/2 function as a key regulator of cellular proliferation, apoptosis and transformation. Pathogens that hijack cellular signalling are of particular consequence in immune evasion tactics and transformed cell lines can be considered as models for pathway interference analysis in human lymphomas studies. ERK1/2 has the intrinsic ability to regulate neighbouring pathways specifically ERK1/2 phosphorylation has direct effects that leads to an increase in functional activity of mTORC inhibitor Rheb resulting in decrease cellular proliferation (195). In contrast, ERK1/2 has been associated with downstream signalling effects such as phosphorylation of VDR (196). These finding provide a framework to elucidate the genomic effects of ERK1/2 and furthermore integrated cross pathway inhibition in avian systems to establish T cell functional competence when challenged with pathogenic microbes and/or in combination with Vitamin D deficiency (Hypovitaminosis D) status.

There is an increasing body of evidence reporting an anti-tumor role for Vitamin D by inducing apoptotic proteins in cancer cells from mice (182) or human tumours (183, 184). These finding indicate that Vitamin D catabolism could differentially modulate tumour cell fate. However, there has been no report to date of Vitamin D-induced apoptosis in primary mammalian cells, and our data demonstrate that physiological levels of Vitamin D does not induces apoptosis of primary avian immune cells, including T cells. The physiological serum Vitamin D concentrations (25(OH)D3) in both healthy chickens (147, 197) and humans has been estimated to range between 60-100 nM (198-200), while the levels of serum $1,25(OH)_2D3$ are significantly lower. It has been shown that APCs and T cells are able to convert 25(OH)D3 to $1,25(OH)_2D3$ at

physiologically relevant concentrations and respond to this in an autocrine fashion which can justify the supra-physiological concentration of $1,25(OH)_2D3$ used by several groups and including our study to analyse the effects of Vitamin D on T lymphocytes (199). Further studies are required to establish whether avian tumour cells, similar to what is observed in human and murine cancer cells, are susceptible to Vitamin D induced apoptosis. These experiments are being planned in our laboratory with several tumour cell lines as well as primary tumour cells. Taken together, these results support our findings demonstrating that the reduction in frequency of IFN-γ producing cells and proliferation after Vitamin D treatment is not due to apoptosis of T cells, confirming that Vitamin D specifically inhibit the production of this cytokine from the activated T lymphocytes. IFN-γ is an antiviral cytokine and has an important role in the generation of anti-viral innate and adaptive immunity in both mammalian and avian systems. Lymphoid cells such as NK cells, γδ T cells, $CD4^+$ T cells and $CD8^+$ T cells are able to produce IFN-γ in response to the stimulation. The expression of IFN-γ significantly influences the initiation of anti-viral adaptive immunity, including the generation of effector Th1 cells (201). In humans, Vitamin D has been used successfully in the prevention and control of viral infections (163, 164, 202). Reichel *et al* (203) demonstrated the inhibitory effects of Vitamin D on human T cells ability to produce IFN-γ in a dose dependent manner (204). In clinical settings, a reduction in IFN-γ production may reduce immunopathology observed in autoimmune and acute or chronic inflammatory diseases induced by infectious agents. Vitamin D provides a homeostatic framework required for generation of a protective immune response against infections without causing immunopathology. Adaptive immune system cells such as T and B cells have been shown to express VDR and constitutively transcribe the 1α-hydroxylase enzyme required for producing the active Vitamin D molecule (151, 154, 178, 188). Therefore Vitamin D can directly modulate T cell effector function. However, Vitamin D may also indirectly affect avian T cell function via generation of immuno-modulatory antigen presenting cells (187). *In vitro* exposure to Vitamin D can sustain suppression of T lymphocyte transcriptional activation and consequent production of IFN-γ mRNA in long-term culture systems (204), which could be of particular importance in over-activated T lymphocytes. These findings may have significant importance in avian systems and needs to be further explored. Restoring T lymphocyte functional abilities could be key in defence against pathogens as previously outlines in human PBMC proliferation (172).

Additionally, we evaluated cell surface co-mobilization of lytic granule membrane protein CD107a to study activation of cytotoxic T lymphocytes (CTLs) when treated with Vitamin D. CTLs play an import role in defence against viral infections as well as cancer. Activation of CTLs leads to degranulation and secretion of lytic granules containing perforin and granzymes. Recently, CD107a cell surface mobilization has been described as a marker for cytotoxic T cell degranulation and shown to be strongly up regulated in activated T cells (205). Human natural killer (NK) cells cytotoxic function has been characterized based on a positive association between stimulatory cytokines such as IL-2 and IFN-γ and up-regulation of CD107a to the cell surface (206). Experimental evidence suggests an increase in cell surface expression of CD107a in PBMCs of patients with autoimmune diseases. In avian system, CD107a is also a marker for cytotoxic activity of T cells (207) and NK cells (208), and has been suggested to be important in the control of viral infections such as Infectious Bronchitis Virus (IBV) by reducing viral load. Here, we demonstrate that physiological levels of Vitamin D do not alter cytotoxic abilities of lymphocytes, and the levels of degranulation in $CD3^+$ T cells (including $CD4^+$ and $CD8^+$ T cells) or $CD3^-$ lymphocytes (including NK cells) are not affected by Vitamin D treatment. It seems that IFN-γ production and degranulation are differentially regulated in avian T cells and non-T cells. Taken together, our results demonstrate that Vitamin D preferentially down-regulate the production of pro-inflammatory cytokines such as IFN-γ but does not inhibit their cytotoxic abilities, which is required for control of the infected cells or transformed cells. Degranulation and cytokine production can be differentially detected in different maturation stages of murine and human lymphocytes. For example, $CD11b^{low}CD27^{high}$ murine NK cells preferentially degranulate while $CD11b^{high}CD27^{low}$ NK cells display reduced degranulation but with maintained IFN-γ production (209, 210). Preferential function of different maturation stages of human NK cells has also been reported, in which $CD56^{bright}CD16^-$ NK cells (immature NK cells) are abundant cytokine producers, while they are less effective in degranulation and cytotoxicity (211). It is possible, but not proven, that Vitamin D exerts its preferential effects on $CD3^-$ lymphocyte function by modulating the maturation stages of these cells. The effects of Vitamin D on maturation stages of immune system cells was evident from our previous results demonstrating that Vitamin D inhibits up-regulation of maturation markers such as CD86, CD80 and MHC class II on avian macrophages, while

increasing their abilities to produce nitric oxide (187). The activation stage of antigen specific $CD8^+$ T cells can also influence the preferential functional abilities of CTLs. Fully differentiated CTLs can undergo degranulation without producing IFN-γ, while many effector memory $CD8^+$ T cells produced IFN-γ but are not able to degranulate in response to stimuli (156). There is no information on the effects of Vitamin D on the activation stages of murine or human T cells. However, the results presented in this report indicate that Vitamin D-treated avian T lymphocytes may have functional abilities as fully differentiated T cells, with reduced frequency of IFN-γ secretion, but retain their ability to undergo degranulation.

Respiratory infections are the major problems in poultry farms, with many possible causes including viral, bacterial and fungal. In humans, the peak incidence of respiratory tract infection coincides with the time of the year when there is insufficient UV-B light to produce Vitamin D resulting in low serum Vitamin D levels in the population (212, 213). Based on these aforementioned epidemiological assessments, indoor housed chickens may be at greater risk for respiratory infection and this might be explained by many mechanisms including variation in the levels of Vitamin D (147). It is now believed that the effects of Vitamin D on innate and adaptive immunity may play an important role in controlling seasonal respiratory infections (214).

In conclusion, we demonstrated that Vitamin D can modulate the function of avian T lymphocytes by reducing T cell proliferation, cytokine production and a reduction in phosphorylation of non-stimulated T lymphocytes. However, Vitamin D treatment did not alter the ability of T lymphocyte to undergo degranulation and did not suppress ERK1/2 phosphorylation in response to the stimuli. This may be due to differential effects of vitamin D on the functional abilities of T lymphocytes, which can inhibit immunopathology that leads to exhaustion of T lymphocytes without inducing general immunosuppression.

Chapter 3: Targeted induction of *de novo* Fatty acid synthesis enhances MDV replication in a COX-2/$PGE_{2\alpha}$ dependent mechanism through EP2 and EP4 receptor engagements

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3.1 Abstract

Many viruses alter *de novo* Fatty Acid Synthesis which can increase the availability of energy for replication, and provide specific cellular substrate for particle assembly. Marek's disease virus (MDV), the causative agent of a deadly lymphoma in chickens, has been linked to imbalances of lipid metabolism in MDV-infected chickens. Analysis of lipid metabolism over a time course of infection with MDV in cultured primary chicken embryo fibroblasts demonstrated an upregulation of genes involved in FAS pathway, elevated levels of fatty acids, phospholipids and lipid droplet (LD) formation. The results demonstrate that virus-induced FAS pathway is crucial for LD formation and MDV infectivity as inhibition of FAS pathway significantly reduced the numbers of LD, virus titter and virus gene copy numbers. Interestingly, virus-induced FAS pathway also increased COX-2/$PGE_{2\alpha}$ pathway as demonstrated by upregulation of arachidonic acid, COX-2 gene expression and $PGE_{2\alpha}$ synthesis. Remarkably, $PGE_{2\alpha}$ restored the inhibitory effects of FAS pathway inhibitors on MDV titters indicating that the activation of COX-2/$PGE_{2\alpha}$ pathway is highly dependent on FAS pathway which supports MDV replication. We conclude that MDV uses FAS to activate COX-2/$PGE_{2\alpha}$ pathway and $PGE_{2\alpha}$ promotes MDV infectivity through EP2 and EP4 receptors engagement.

3.2 Introduction

Despite their great diversity, viruses are highly dependent on host cell factors to facilitate maximal viral replication. Elucidating the mechanisms underlying hijacking of host cell metabolism by viruses can identify key determinant factors for virus replication which consequently contribute to an altered physiological homeostasis and disease susceptibility. All herpesviruses including Marek's Disease Virus (MDV) encode metabolic enzymes in their genomes which are involved in their pathogenesis (66, 68, 215, 216). MDV is a highly oncogenic herpesvirus and the etiologic agent for Marek's disease (MD). Upon infection of the host, the virus initially replicates in B and T cells. Subsequently, the virus establishes latency predominantly in $CD4^+$ T cells, allowing the virus to persist in the host for life (217). In addition, latently infected $CD4^+$ T cell can undergo neoplastic transformation resulting in deadly lymphomas (217).

A diverse range of human enveloped viruses including human cytomegalovirus (HCMV) (218), herpes simplex virus 1 (HSV-1) (219), hepatitis C virus (HCV) (220), dengue virus (DENV) (221), Kaposi's sarcoma-associated herpesvirus (KSHV), human immunodeficiency virus (HIV) (222), vaccinia virus (VACV) (223) and West Nile virus (WNV) (224), have been shown to selectively modulate fatty acid synthesis to support virus replication. In this process, acetyl-CoA is converted to malonyl-CoA and subsequently to palmitate. The first step towards Fatty Acid Synthesis is the conversion of citric acid into acetyl-CoA by direct phosphorylation of ATP-citrate lyase (ACLY). The subsequent committed step involves the conversion of acetyl-CoA into malonyl-CoA by acetyl-coA carboxylase (ACC), a process modulated by HCMV (225) and HCV (226). ACC is the rate-limiting step and contributes to cholesterol synthesis. The final step involves the committed elongation by utilizing both acetyl-CoA and malonyl-CoA coupled to the multifunctional fatty acid synthase (FASN) to make palmitic acid. DENV (221), WNV (224), and HCV (227) have been shown to preferentially enhance FASN activity and palmitic acid synthesis.

From then on, palmitic acid contributes to several key biological functions such as fatty acid oxidation (FAO), β-oxidation in mitochondria, post-translational modification (palmitoylation) of proteins or elongation of fatty acid chains to generate a diverse repertoire of very long chain fatty acids (VLCFA). This diverse role for the utilization

of palmitic acid has been demonstrated in HCV (220), VACV (223), modified vaccinia Ankara (MVA) (228), HCMV (229, 230), KSHV (231, 232), respiratory syncytial Virus (RSV) (233), and Epstein-Barr Virus (EBV) (234). Both HCV (220) and vaccinia virus (223) are highly dependent on mitochondrial β-oxidation to support infection. On the other hand, recent data suggest that VLCFA are essential for HCMV replication (235). VLCFA contribute to biosynthesis of Arachidonic acid (AA), which can be further reduced by cyclooxygenase-1 (COX-1) and inducible COX-2 to make prostaglandin $E_{2\alpha}$ ($PGE_{2\alpha}$), a potent eicosanoid and immune modulator, modulating the functions of natural killer (NK) cells (236), macrophages (237), and T cells (238). Induction of $PGE_{2\alpha}$ biosynthesis has been observed following infections with HCMV (229, 230), KSHV (231, 232), RSV (233), Influenza A virus (IAV) (239) and MVA (228). Moreover, a direct association between induction of COX-2 activity and enhancement of HCMV (229) and KSHV (232) replication has been reported. For example, $PGE_{2\alpha}$ inhibits macrophages recruitment to the lungs, reduces type I IFN production and modulated antiviral immunity in IAV infection. Intriguingly, inhibition of COX-2/$PGE_{2\alpha}$ pathway protected mice against IAV (239) and rescued the inhibitory effects of soluble factors released by MDV-transformed T cells on T cell proliferation (240). Collectively, these studies suggest that induction of FAS has major implications on virus replication and immune evasion and dissemination in the host.

Previously, atherosclerotic plaque formation has been reported in chickens infected with pathogenic MDV (241). Vaccination with herpesvirus of turkey (HVT; MDV serotype 3) prevented the development of atherosclerotic plaques (242). Lipid analysis of the arterial smooth muscles (ASM) from MDV infected birds revealed a significant increase in non-esterified fatty acids (NEFA), cholesterol, cholesterol esters, squalene, phospholipids and triacylglycerol. Furthermore, excess lipids biosynthesis triggered cellular deposition in organelles termed lipid droplets (241, 242). Despite these intriguing observation, it remained elusive if the fatty acid metabolism is altered in MDV infected cells and how MDV causes these alterations in the host.

In the present study, we investigated if Fatty Acid Synthesis and FA derivatives are induced in MDV infected cells. We could demonstrate that MDV infection induce *de novo* FAS and upregulation of genes involved in FAS and FAO. Using small pharmacological inhibitors and/or addition of exogenous fatty acids, we could show that

FAS is essential to support MDV infection in cultured cells. Short chain FAs contributed to LCFA synthesis which can be stored in lipid droplets. However, the virus replication was not dependent on the ATP production from utilization of short chain FAs by FAO. MDV infection induced COX-2 expression and $PGE_{2\alpha}$ synthesis, and this process was dependent on activation of FAS pathway by MDV. Taken together, our results demonstrate that the major role of FAS to support MDV replication is to activate $PGE_{2\alpha}$/EP2 and $PGE_{2\alpha}$/EP4 signalling pathways.

3.3 Materials and Methods

3.3.1 Ethics Statement

Ten day old chicken embryos were culled according to the EU guidelines to generate primary chicken embryonic fibroblast cells (CEFs).

3.3.2 CEFs culture and virus preparations

CEFs were generated from mixed sex SPF Valo eggs (Valo Biomedia GmbH) incubated in a Brinsea Ova-Easy 190 incubator at 37^{o}C until 10 days *in ovo*. CEFs were seeded at a rate of 1.5 x 10^5 cells /ml in 24 well plates with growth medium (E199 supplemented with 10% TBP, 5% FCS, 2.8% SQ water, amphotericin B (0.01%), Penicillin (10 U/ml) and Streptomycin (10 µg/ml)) and incubated overnight (38.5^{o}C at 5% CO_2). Next day, 80% confluent monolayer was observed and growth medium was removed and replaced with maintenance medium (E199 supplemented with 10% TBP, 2.5% FCS, 3.5 % SQ water, amphotericin B (0.01%), Penicillin (10 U/ml) and Streptomycin (10 µg/ml). RB1B (MDV; serotype 1) and RB1B UL_{35}-GFP virus expressing GFP fused to the UL_{35} capsid protein were prepared in CEFs.

3.3.3 Reagents and antibodies

Chemicals: TOCRIS/SB 204990 (Thermo Fisher Scientific, Paisley, UK), TOFA, C75, Clofibrate, Palmitic acid, SC-236 (Sigma-Aldrich, Dorset, UK), PGD_2, PGI_2, $PGE_{2\alpha}$ (Cambridge Bioscience, Cambridge, UK), SC-51322, TG-4-155 and ER-819762 (Bio-Techne Ltd., Abingdon, UK) were all reconstituted in DMSO. TXA_2 (Cambridge Bioscience, Cambridge, UK) was reconstitute in ethanol. $PGF_{2\alpha}$ (Cambridge

Bioscience, Cambridge, UK), etomoxir and malonyl-CoA (Sigma-Aldrich, Dorset, UK) were reconstituted in E199 medium.

3.3.4 Cells and MDV Infection

Metabolomics: CEFs were either mock infected or with the very virulent RB1B strain (100 pfu per 1.5 x 10^5 cells) in triplicates and harvested at 48 and 72 hrs post infection (hpi). The cells were washed, counted and after protein quantification using Bradford assay, the samples were sent for Metabolom analysis using GCxGC-MS (Target Discovery Institute, University of Oxford). In brief, the cells were homogenized using bead beater in methanol/water (1:1), and then t-butyl methyl ether was added for phase separation. The organic phase was dried under vacuum, while methanol was added to the remaining sample and mixed in bead beater. After incubation at -80^{o}C for 1 hr, the phase separation occurred after centrifugation, and liquid layer was collected and dried under vacuum. Methoxyamine and MSTFA (1% TMSCI) were added to the dried samples and subsequently injected for analysis by GC/GC-MS. The method used in this experiment is designed to detect 155 different metabolites including lipids and amino acids. The lipid profile of mock and MDV-infected cells were analysed in biological triplicates with up to six technical replicates per biological replicate. The data were adjusted and normalized based on protein content. Virus infection did not change the size of the cells as determined by microscopy.

3.3.5 Viral plaque analysis: MDV infected CEFs were washed 1 times with PBSa, subsequently fixed for 5 min at RT (1:1 acetone:methanol; Sigma, UK) and blocked with blocking buffer (PBS + 5% FCS) for 1 hr at RT. Blocking buffer was removed and next incubated with anti-gB mAb (HB-3; purified monoclonal antibodies) for 1 hr at RT. Each well were washed 2x with PBST buffer (PBSa + 0.05% Tween) and then incubated with horse radish peroxidase-conjugated rabbit anti-mouse Ig (DAKO, UK) in blocking buffer for 1 hr at RT. After development of the plaques using AEC substrate (Thermofisher, UK) the cells were washed with super Q water and viral plaques were counted using light microscopy.

3.3.6 Determining non-toxic concentration of the inhibitors: To identify non-toxic concentrations of the chemicals, mock-infected and MDV-infected CEFs were exposed to the chemicals or vehicles and cell morphology and adherence/confluency were

monitored under light microscopy at different time points post treatment. Moreover, CEFs were trypsinized, stained with 7-actinomycin D (7-AAD; BD Bioscience, Oxford, UK) and acquired using a MACS quant flow cytometry and FloJo software for analysis of the data. Non-toxic concentrations of the inhibitors and chemicals were selected based on flow cytometry data and confluency.

3.3.7 qPCR to amplify MDV genes

DNA samples were isolated from 5×10^6 cells using the DNeasy-96 kit (Qiagen, Manchester, UK), according to the manufacturer's instructions. A master-mix was prepared: primers MEQ-FP and MEQ-RP (0.4 μM), MEQ probes (0.2 μM), *ovo* forward and reverse primers (0.4 μM), and *ovo* probe (0.2 μM, 5′Yakima Yellow-3′TAMRA, Eurogentec) as listed in Table 2 and ABsolute Blue® q-PCR Low Rox master-mix (Thermo Fisher Scientific, Paisley, UK). A standard curve generated for both MEQ (10-fold serial dilutions prepared from plasmid construct with MEQ target) and *ovo* gene (10-fold serial dilutions prepared from plasmid construct with *ovo* target) were used to normalise DNA samples and to quantify MDV genomes per 10^4 cells. All reactions were performed in triplicates to detect both MEQ and the chicken ovotransferrin (*ovo*) gene on an ABI7500® system (Applied Biosystems, Paisley, UK) using standard conditions. MDV genomes were normalised and reported as viral genome per 10^4 cells.

Table 2: List of primers used for MDV Q-PCR

Gene name	Primers		Tm (ºC)	Product size (bp)
MDV-1 MEQ	MEQ Fwd	GGTCTGGTGGTTTCCAGGTGA	60 ºC	73 bp
	MEQ Rev	GCATAGACGATGTGCTGCTGA		
	MEQ probe	AGACCCTGATGATCCGCATTGCGACT (5' FAM, 3' BHQ1)		
Chicken ovotransferrin gene (control)	Ovo Fwd	CACTGCCACTGGGCTCTGT	60 ºC	71 bp
	Ovo Rev	GCAATGGCAATAAACCTCCAA		
	Ovo probe	AGTCTGGAGAAGTCTGTGCAGCCTCCA (5' Yakima Yellow, 3' TAMRA)		

3.3.8 Real-Time Polymerase Chain Reaction (RT-PCR)

RNA extraction and cDNA: Total RNA was extracted from mock and MDV infected CEFs using TRIzol (Thermo Fisher Scientific, Paisley, UK) according to the manufacturer's protocol and treated with DNA Free DNase. Subsequently, 1 μg of purified RNA was reverse transcribed to cDNA using Superscript® III First Strand

Synthesis kit (Thermo Fisher Scientific, Paisley, UK) and oligo-dT primers according to the manufacturer's recommended protocol. The resulting cDNA was diluted 1:10 in DEPC treated water.

SYBR green RT-PCR: Quantitative real-time PCR using SYBR Green was performed on diluted cDNA using the LightCycler® 480 II (Roche Diagnostics GmbH, Mannheim, GER) as previously described. Briefly, each reaction involved a pre-incubation at 95 °C for 5 min, followed by 40 cycles of 95°C for 20 sec, 55°C–64°C (T_A as per primer) for 15 sec, and elongation at 72°C for 10 sec. Subsequent melt curve analysis was performed by heating to 95°C for 10 sec, cooling to 65°C for 1 min, and heating to 97°C. Primers sequences and accession numbers are outlined in Table 3. Relative expression levels of all genes were calculated relative to the housekeeping gene β-actin using the LightCycler® 480 Software (Roche Diagnostics GmbH, Mannheim, GER). Data represent mean of 6 biological replicates.

Table 3: List of primers used for Real-Time PCR

Gene name	Accession no	Primers		Tm (ºC)	Product size (bp)
Acetyl-CoA carboxylase (ACC)	J03541.1	Fwd	ACGTTCGAAGGGCGTACATT	60ºC	161 bp
		Rev	TACGTGGACCATCCCGTAGT		
Fatty acid synthase (FASN)	J03860.1	Fwd	CTTTGGTGGTTCGAGGTGGT	60ºC	170 bp
		Rev	CTGTGGGAACCTTGCTTGGA		
Lipoprotein lipase (LPL)	NM_205282.1	Fwd	ACTGAAACTTTTTCGCCGCTG	61ºC	128 bp
		Rev	ATTCATCTCAGCTTCGGGATCG		
Carnitine palmitoyl transferase 1a (CPT1a)	NM_001012898.1	Fwd	CTAGCCCCTCTAGCTGGCTT	61ºC	113 bp
		Rev	ACTTCTCTCAAGGGTTCGGT		
Acyl-coA dehydrogenase, Long chain (ACADL)	NM_001006511.2	Fwd	CTAAGCGGCTGACTGACATCG	61ºC	221 bp
		Rev	AATATCCGCTCCAATGCCTCC		
Prostaglandin Receptor 2 (EP2)	NM_001083365.1	Fwd	CCTTCACGATCTGCGCCTAC	60ºC	92 bp
		Rev	GGGGTTGATGGAGAGGAAGC		
Prostaglandin Receptor 3 (EP3)	NM_001040468.1	Fwd	GCTGCTGGTAACGATGCTGA	60ºC	177 bp
		Rev	CGGAGCAGCAGATAAACCCA		
Prostaglandin Receptor 4 (EP4)	NM_001081503.1	Fwd	ATGTTCCAGGGTACAGGTTTTGT	60ºC	175 bp
		Rev	GCCTAGCCTGCACGGTGTT		
Clyclooxygenase 1 (COX-1)	XM_425326	Fwd	TCAGGTGGTTCTGGGACATCA	60ºC	123 bp
		Rev	TGTAGCCGTACTGGGAGTTGAA		
Clyclooxygenase 2 (COX-2)	XM_422297	Fwd	CTGCTCCCTCCCATGTCAGA	60ºC	123 bp
		Rev	CACGTGAAGAATTCCGGTGTT		
Cytoplasmic Beta Actin	X00182	Fwd	TGCTGTGTTCCCATCTATCG	60ºC	150 bp
		Rev	TTGGTGACAATACCGTGTTCA		

3.3.9 Prostaglandin $E_{2\alpha}$ ELISA

$PGE_{2\alpha}$ was quantified using a colorimetric assay (R&D Systems, Abingdon, UK) based on competition between unlabelled $PGE_{2\alpha}$ in the sample and a fixed amount of conjugated $PGE_{2\alpha}$. The assay was performed according to assay kit manufactures recommendation. In brief, CEFs were either mock or infected with RB1B in the presence/absence of TOFA (1.54 µM) and C75 (5.9 µM) or SC-236 (0.25 µg/ml) and incubated (38.5^{o}C, 5% CO_2) for 48 or 72 hpi. The supernatants were collected and diluted (1:100) in Calibrator Diluent RD5-56. $PGE_{2\alpha}$ detection antibody was added to the wells which were pre-coated with primary anti-$PGE_{2\alpha}$ antibody and incubated for 1 hr at RT. Next, $PGE_{2\alpha}$ conjugates was added and incubated for an additional 2 hrs at RT. The wells were subsequently washed and developed by incubating substrate buffer for 30 min at RT. The assay was measured at OD_{450} nm and concentrations of $PGE_{2\alpha}$ were determined against a standard curve.

3.3.10 Determination of Oxygen consumption rate (OCR) and Extracellular acidification rate (ECAR)

CEFs were seeded at a rate of 2.0 x 10^4 cells per well in an XFp 8-well V-3 PET tissue culture mini-plate (Seahorse Bioscience, Agilent, UK) in triplicates and incubated overnight at 38.5°C. Next day, cells at 80% confluency were pre-treated with etomoxir (4.42 µM) or vehicle control and then either mock or infected with RB1B (100 pfu per 1.5 x 10^5 cells) for an additional 24 hrs. Oxygen consumption rates (OCR) was measured every 6 min using the Seahorse Bioscience XFp analyzer (Seahorse Bioscience, Agilent, UK) from 16-30 hpi at 38.5°C.

3.3.11 Oil Red O staining

Lipid droplets were stained in CEFs mock or infected with RB1B in 6 well-plates. In brief at 72 hpi, cell monolayer was washed with PBS and fixed with 4% formaldehyde for 30 min at RT. Plates were subsequently stained with Oil Red O solution for 30 min at RT followed by a wash in PBS. Plates were counterstained with hematoxylin for 3 min at RT followed by a wash with super Q water. Plates were visualized and imaged using a light microscope and the pictures were processed using Adobe Photoshop software.

3.3.12 Fluorescence confocal microscopy

CEFs were seeded in 24 well plates that contained 12 mm diameter round coverslips at a rate of 1.0 x 10^5 cells per well. At 72h post mock or infection with the pRB1B UL_{35}-GFP virus in the presence/absence of SB 204990, TOFA, C75 or etomoxir, the samples were prepared for imaging. In brief, mock or infected CEFs were fixed with 4% formaldehyde for 30 min at RT and washed twice with PBS. Cells were subsequently incubated with HCS LipidTox Red Neutral lipid stain (1:1000 in PBS; 568 nm) at RT for an additional 45 min. Cells were washed twice with PBS and nuclei were labelled with DAPI. Coverslips were mounted in vectashield mounting medium for fluorescence imaging. Cells were viewed using a Leica SP2 laser-scanning confocal microscope and optical sections recorded using either the 663 or 640 oil-immersion objective with a numerical aperture of 1.4 and 1.25, respectively. All data were collected sequentially to minimize cross-talk between fluorescent signals. The data are presented as maximum projections of z-stacks (23-25 sections; spacing 0.3 mm). Maximum projections of z-stacks were analysed using IMARIS (Bitplane Scientific Software). Ninety infected cells and 40 mock-infected cells were analysed and their LipidTox-labelled Neutral lipid containing organelles were detected with the spot function of IMARIS. Images were processed using Adobe Photoshop software.

3.3.13 Statistical Analysis

All data are presented as mean $\pm$ standard deviation (SD) from at least three independent experiments. Quantification was performed using Graph Pad Prism 7 for windows. The differences between groups, in each experiment, were analysed by non-parametric Wilcoxon tests (Mann-Whitney) or by Kruskal-Wallis test (One-way ANOVA, non-parametric). Results were considered statistically significant at $P < 0.05$ (*).

3.4 Results

3.4.1 MDV infection increases lipogenesis

To determine if MDV infection affects lipid metabolism, we performed a lipidomic analysis on mock and MDV infected primary chicken embryo fibroblasts (CEFs) at 48 and 72 hrs post infection (hpi). The analysis revealed that 15 lipid metabolites were

increased and 9 metabolites were decreased in infected cells at 72 hpi (Figure 11A). To provide a better visualization of the metabolic changes, we mapped the altered fatty acids (FA) and their derivatives onto the FAS pathways at 48 (Figure 11B i) and 72 hpi (Figure 11B ii). At 72 hpi, increased levels of palmitic acid were observed ($P = 0.01$), which are the end product of FAS pathway and are elongated to make long chain fatty acids (LCFA) such as stearic acid at 72 hpi ($P = 0.03$) in the infected cells. Stearic acid is further elongated into Oleic acid ($P = 0.01$), Nervonic acid ($P = 0.04$), Mead acid ($P = 0.0028$) and Docosahexanoic acid ($P = 0.001$). In contrast, Arachidic acid and Lignoceric acid were significantly reduced in MDV infected cells ($P = 0.001$). Alternatively, palmitic acid can be utilized for phospholipid biosynthesis. Our data demonstrate that phosphatidylethanolamine (PE) synthesis is also upregulated in the infected cells at both 48 ($P = 0.0005$) and 72 ($P = 0.001$) hpi. Similarly, arachidonic acid (AA) was also significantly increased at both 48 ($P = 0.0031$) and 72 hpi ($P = 0.0028$). Taken together, our data demonstrate that MDV infection severely impacts lipid metabolism.

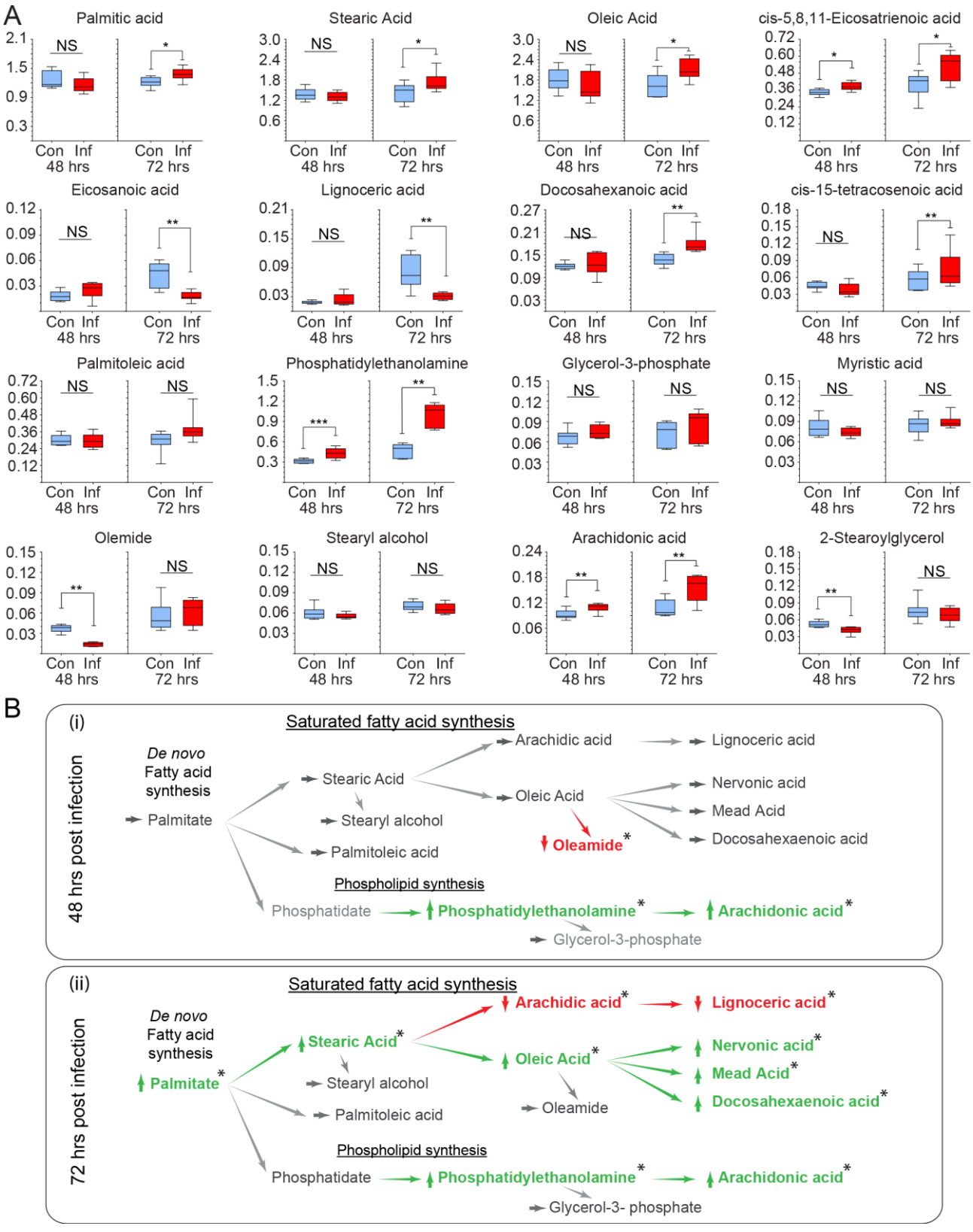

Figure 11: Induction of *de novo* Fatty acids synthesis in MDV infected CEFs. Metabolomics analysis of relative levels of lipid metabolites from mock- (control) and MDV-infected (RB1B) CEFs are shown at 48 hpi and 72 hpi. **(A)** Box and whisker plots showing minimum and maximum relative levels of named fatty acids and long chain fatty acids (LCFAs) either significantly altered or where no changes were

observed as a result of MDV infection. **(B)** Schematic summary of major metabolites outlining preferential utilization of fatty acids for LCFA and phospholipid synthesis by comparing (i) 48h and (ii) 72h post MDV infection. Arrows indicate (↑) increase, (↓) decrease or (→) no change in the respective metabolites to demonstrate flux through pathways. Non-parametric Wilcoxon tests (Mann-Whitney) was used to assess normal distribution and test significance with the results shown as mean ± SD **(A)**. ***** (P = 0.01) and ****** (P = 0.001) and *** (P = 0.0005) indicates a statistically significant difference compared to control. NS indicates no significant difference. The experiment was performed in biological triplicates with six technical replicates per biological replicates

3.4.2 Non-toxic concentration of pharmacological inhibitors were assayed

To study the metabolic pathways which are essential for MDV infection and replication, we first determined the non-toxic concentrations at which cell viability and confluency wasn't affects. CEF cell at 90% confluency were treated with the inhibitors; TOCRIS/SB-204990 (0.5, 1.0, 1.5, 2.0, 2.5, 3, 4, 5, 7, 8 and 10 µg/ml), TOFA (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10 µg/ml), C75 (0.5, 1.0, 1.5, 2.0, and 2.5 µg/ml), Palmitic acid (5, 10, 12.5, 15, 20, 25 and 50 µM), Malonyl-CoA (5, 10, 15, 20, 25 and 50 µM), Etomoxir (0.01, 0.05, 0.1, 0.5, 1.0, 1.2, 1.5, 1.7, 2.0, 5 and 10 µg/ml), Clofibrate (0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0 and 10 µg/ml), SC-236 (0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10 µg/ml), ER-819762 (1.0, 2.0, 4.0, 5.0 and 10 µM), SC-51322 (0.1, 1.0, 2.0, 4.0, 5.0 and 10 µM) and TG 4-155 (1.0, 2.0, 4.0, 5.0 and 10 µM) at the concentration here listed and incubated (38.5 ^{o}C at 5% CO_2) for 72 hrs. Cells were trypsinized at 72 hrs post treatment and labelled with 7-actinomycin-D (7-AAD) to determine percentage live cells. Here we report the concentrations at which no significant difference in cell death was detected compared to control or non-treated cells (Figure 12). Cellular confluency was observed twice a day under a normal light microscope for the duration of the treatment to confirm toxic effects as well. The concentration highlighted in black were used for the purpose of studying MDV viral infection and replication whereby no cell death as well as normal cellular growth or proliferation was observed (Figure 12).

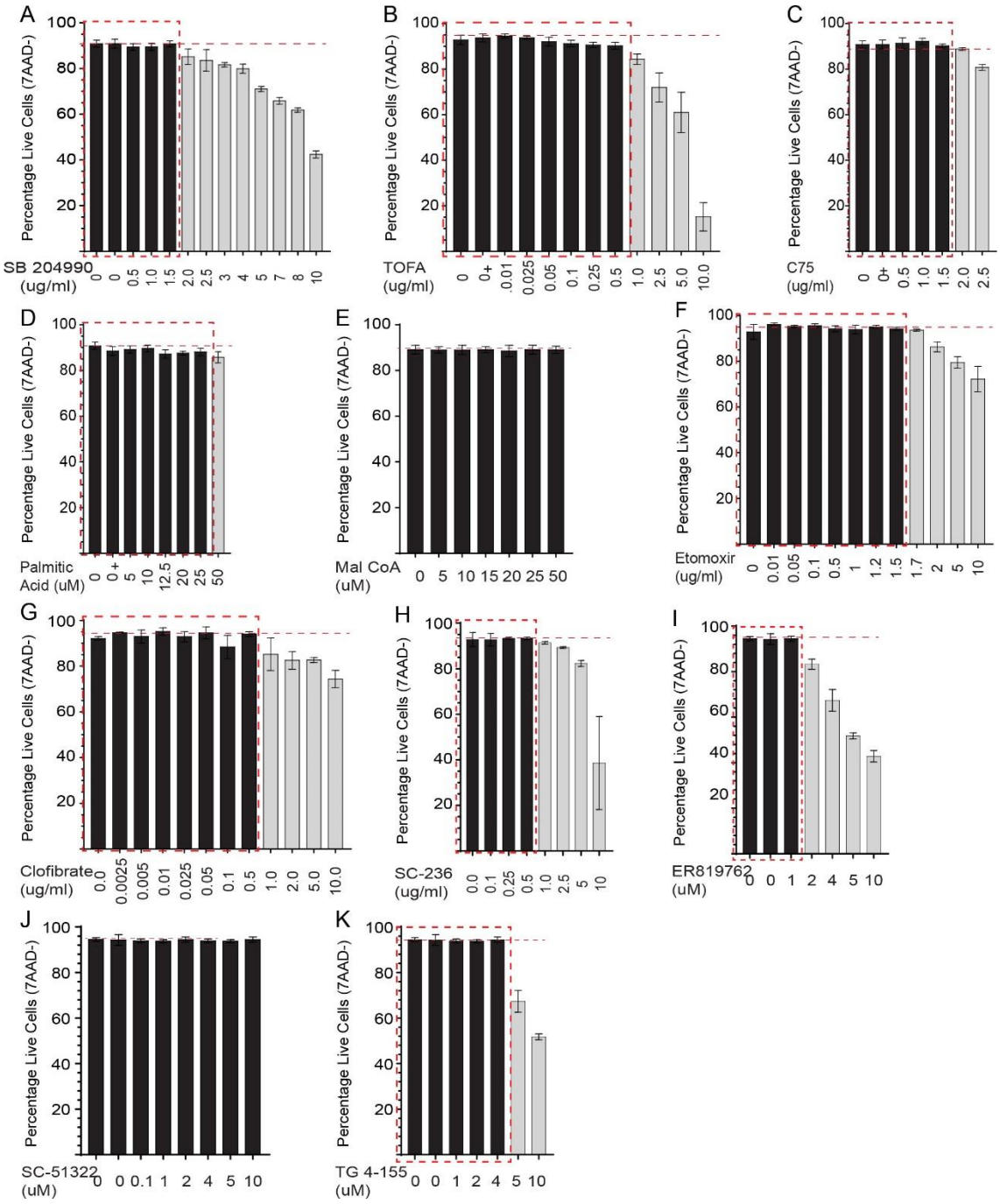

Figure 12: Non-toxic concentration of small pharmacological inhibitors
Analysis by MACS quant demonstrating percentages of 7AAD negative CEFs, representing live cells, after 72 h treatment with small pharmacological inhibitors including **(A)** Tocris (SB 204990), **(B)** TOFA, **(C)** C75, **(D)** Palmitic Acid, **(E)** Malonyl-CoA, **(F)** Etomoxir, , **(G)** Clofibrate, **(H)** SC-236, **(I)** ER-819762, **(J**) SC-51322 and **(K)** TG 4-155. Bar graphs with single bars in black represent concentrations of the inhibitors which did not induce cell death. All experiments were performed in triplicates and data is representative of 2 independent experiments.

3.4.3 MDV replication requires Fatty acid synthesis

To validate our lipidomics data and determine how these metabolic pathways are altered, we performed gene expression analyses of the cellular enzymes involved in FAS pathway using RT-PCR (Figure 13A). Both acetyl-CoA carboxylase (ACC) and Fatty acid synthase (FASN) were highly upregulated at 72 hpi (Figure 13B). To determine the role of these pathway in MDV replication, we used selective inhibitors of ACLY, ACC and FASN, SB 204990, 5-(Tetradecyloxy)-2-furoic acid (TOFA), and C75 respectively (Figure 13A). Non-toxic concentrations of the inhibitors were determined based on viability (Figure 12) and confluency of the treated CEFs. To determine the role of these pathways, CEFs were infected with MDV (RB1B - 100 pfu per 1.5 x 10^5 cells) in the presence of these pharmacological inhibitors and in the presence/absence of the downstream metabolites (malonyl-CoA and palmitic acid) for 72 hrs. Viral titre were quantified at 72 hpi. The ACLY inhibitor SB 204990 (3.85 µM) did not affect MDV replication (Figure 13C), suggesting that the conversion of citrate to Acetyl-CoA is not important for the virus. In contrast, inhibition of ACC (TOFA; 1.54 µM) and FASN (C75; 5.9 µM) significantly impaired MDV replication by 27 (Figure 13D) and 28 folds (Figure 13E), respectively. Treatment of MDV-infected CEFs with a combination of TOFA (0.77 µM) and C75 (4.5 µM) decreased ($p = 0.0043$) the transcripts of ACC (Figure 13F) and FASN (Figure 13G). To determine if the metabolites can rescue the phenotype of the inhibitors, we supplemented the cells with the respective downstream metabolites. As expected, treatment with SB 204990 and malonyl-CoA did not affect virus replication (Figure 13H). In contrast, addition of malonyl-CoA in the presence of TOFA restored MDV titre (Figure 13I). Similarly, palmitic acid restored virus replication inhibited by C75 (Figure 13K), while the fatty acid itself did not alter MDV titre (Figure 13J). To confirm our data, we assessed MDV replication upon treatment with SB 204990, TOFA and C75 by qPCR (Figure 13L). Taken together, the result demonstrate that blocking ACC and FASN decreases MDV replication and reveal that palmitic acid is a key metabolite required for MDV infection.

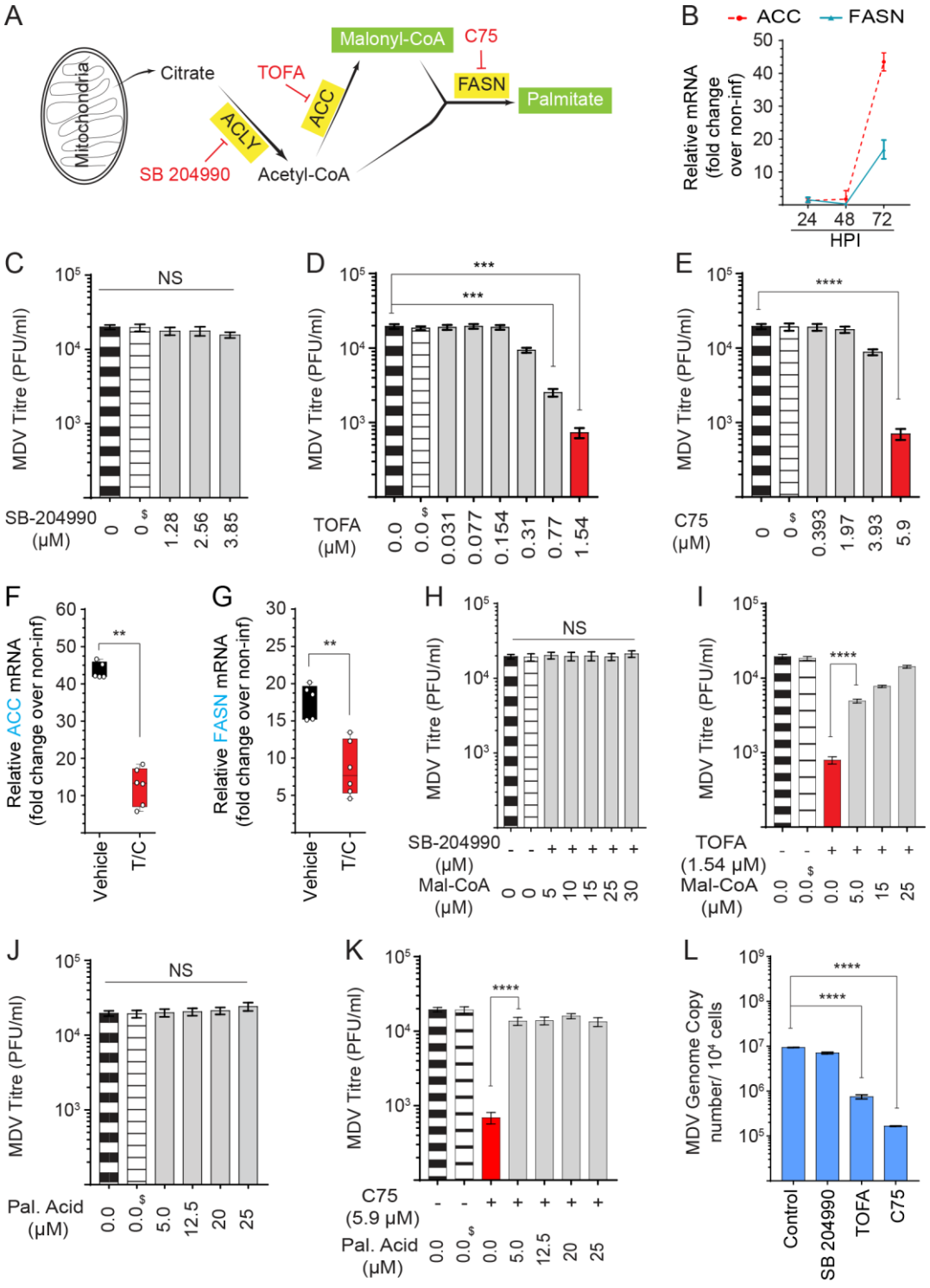

Figure 13: Fatty acid synthesis is required for MDV infection of CEFs.
A pathway interference approach to dissect the role of lipids in MDV replication. **(A)** Schematic FAS pathways highlighting the relevant pharmacological inhibitors (red) and the respective enzymes (yellow box) as well as metabolites (green box) studied within the FAS pathway. **(B)** Fold change gene expression in CEFs mock-infected or MDV-infected are shown at 24, 48 and 72 hpi. Analysis of MDV viral titer (PFU/ml) in infected CEFs in the presence of **(C)** SB 204990 (1.28, 2.56 and 3.85 µM), **(D)** TOFA

(0.03, 0.077, 0.154, 0.31, 0.77 and 1.54 µM) and **(E)** C75 (0.393, 1.97, 3.93 and 5.9 µM). Box and whisker plots demonstrating relative fold change in mRNA of **(F)** ACC and **(G)** FASN in MDV-infected CEFs cells treated with vehicle (Cont.) or TOFA+C75 (T/C) at 72 hpi. Analysis of MDV viral titre (PFU/ml) in infected CEFs in the presence of **(H)** malonyl-CoA with SB-204990, **(I)** malonyl-CoA with TOFA or **(J)** Palmitic acid (5, 12.5, 20 and 25 µM) **(K)** Palmitic acid with C75 (5.9 µM). **(L)** MDV (RB1B) genome copy number per 10^4 cells (MEQ gene with reference ovotransferrin gene) in the presence of SB 204990 (3.85 µM), TOFA (1.54 µM) or C75 (5.9 µM). *** ($p = 0.0002$) ******** ($p < 0.0001$) indicates a statistically significant difference compared to control. NS indicates no significant difference. $ symbol indicates vehicle treated cells. All viral titre experiments were performed in 6 replicates and data is representative of 3 independent experiments.

3.4.4 β-oxidation of fatty acids increases ATP synthesis in the MDV-infected cells

To determine if fatty acids oxidation increases ATP synthesis, we measured oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in MDV-infected cells over time using a Seahorse Bioscience XFp analyzer (Seahorse Bioscience). Thereby, OCR and ECAR correspond to β-oxidation of fatty acids and glycolysis for ATP production respectively. Linear regression analysis of the OCR and ECAR revealed a significant increase in MDV-infected compared to mock-infected cells (Figure 14A and 14B), suggesting that virus infection increases ATP production via both β-oxidation of fatty acid and glycolysis. At 90 min post infection, mock-infected cells had OCR rate of 75.63 pmol/min, which rose to 108.52 pmol/min by 600 min post mock-infection as the nutrient were consumed (Figure 14A). To assess if MDV replication relies on import and utilization of palmitic acid within mitochondria to generate ATP, CEFs were treated with a vehicle or etomoxir, the pharmacological inhibitor of carnitine palmitoyltransferase 1a (CPT1a) which blocks the import of long-chain fatty acids into the mitochondria. Etomoxir did not modulate β-oxidation of fatty acids in the non-infected cells (Figure 14A). In contrast, etomoxir significantly reduced the OCR in MDV-infected cells compared to the treated MDV-infected cell (Figure 14C), indicating that etomoxir can reduce β-oxidation of fatty acid for ATP production in the MDV-infected cells. Intriguingly, treatment of MDV-infected cells with etomoxir increased the ECAR rate at all time points (Figure 14D), indicating that glycolysis is activated independent of oxidative phosphorylation in the MDV-infected cells.

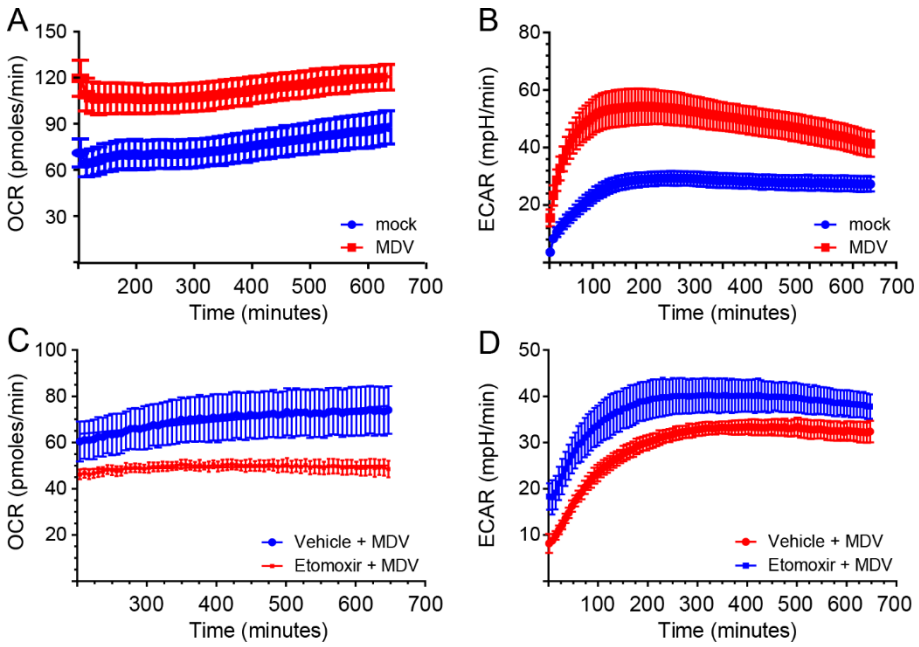

Figure 14: MDV infected cells can also utilize non-mitochondrial sources of ATP. **(A)** Oxygen consumption rate (OCR; pmoles/min) and **(B)** Extracellular acidification rate (ECAR; mpH/min) are shown for the mock-infected and MDV-infected CEFs. MDV-infected CEFs were treated with etomoxir (4.42 µM) or vehicle during the first 12 h post mock infection, and **(C)** OCR **(D)** ECAR were determined using the Seahorse XFp. All experiments were performed in triplicates and data is representative of 3 independent experiments.

3.4.5 MDV replication is independent of ATP generated by β-oxidation of fatty acids

To investigate if the ATP generated by β-oxidation are required for MDV replication, we inhibited CPT1a responsible for fatty acids transport from cytoplasm into mitochondria for β-oxidation using non-toxic concentrations of the physiological inhibitor malonyl-CoA and pharmacological inhibitor etomoxir. To activate β-oxidation, non-toxic concentrations of Clofibrate was used in this study (Figure 15A). Gene expression analysis revealed an up-regulation of CPT1a, acyl-CoA dehydrogenase long chain (ACADL) and lipoprotein lipase (LPL) in the MDV-infected CEFs using RT-PCR at 72 hpi (Figure 15B). To examine the role of β-oxidation in MDV-replication, CEFs were infected with MDV in the presence of exogenous malonyl-CoA, etomoxir or Clofibrate, and viral titre was quantified at 72 hpi. Clofibrate, a PPARα ligand which activates β-oxidation, significantly reduced MDV replication (Figure 15C). In contrast, neither malonyl-CoA (Figure 15D) nor etomoxir (Figure 15E) had any inhibitory effects on viral titre, suggesting that the ATP generated by β-oxidation is not required for MDV replication. Interestingly, even a slight increase in

viral yield was observed in the cells treated with etomoxir or malonyl-CoA. Similarly, combination of etomoxir with malonyl-CoA or palmitic acid did not reduce MDV replication but increased viral titre by 2-fold (Figure 15F). To confirm the effects of the inhibitors on virus replication, we quantified MDV genome copies by qPCR (Figure 15G). As observed in the virus titrations, only clofibrate inhibited virus replication while no effect was observed with etomoxir, confirming that ATP generation by β-oxidation is not required for MDV replication.

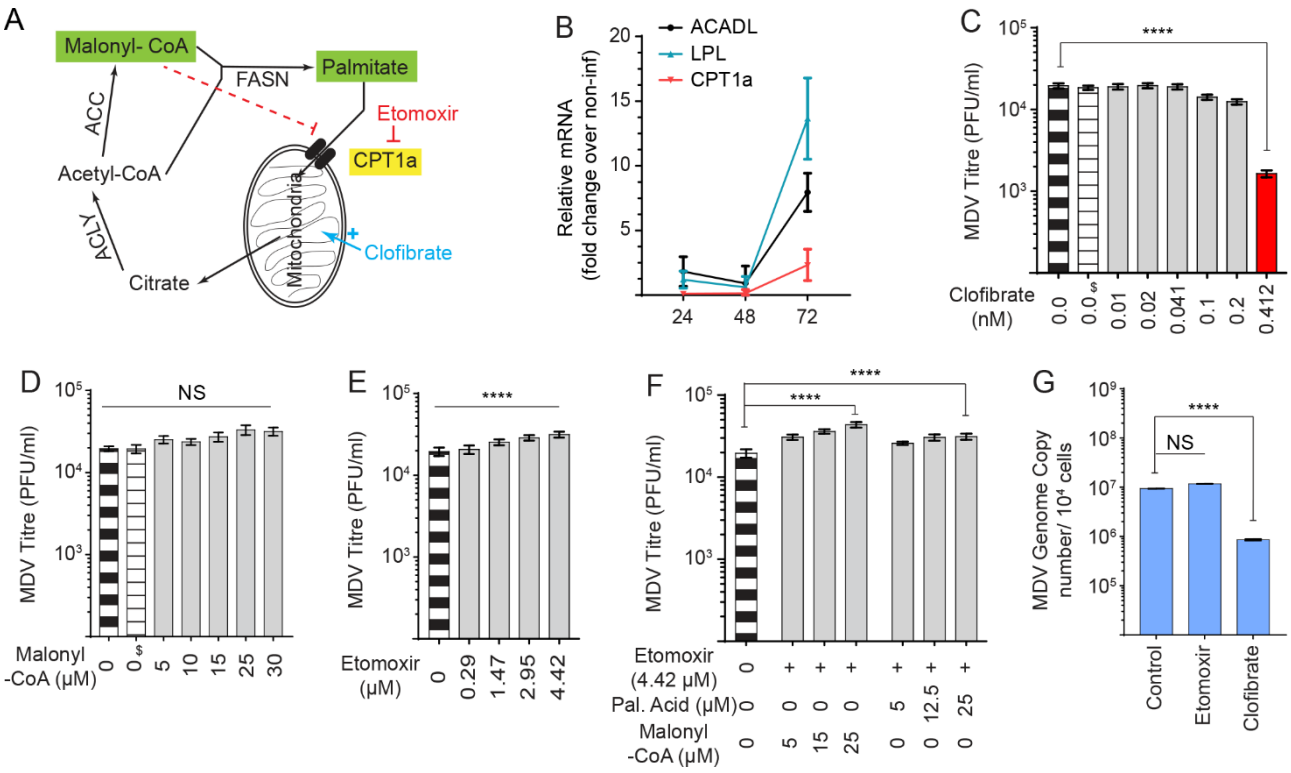

Figure 15: β-oxidation is not essential to support MDV infection of CEFs. Fatty acyl-CoA derivatives transported into the mitochondria undergo Fatty acid oxidation (FAO) to generate ATP, NADH and NADPH. **(A)** Schematic metabolic pathway outlines the relevant pharmacological molecules (red; etomoxir and blue; clofibrate) and the respective enzyme (yellow box) as well as metabolites (green box) studied within the FAO pathway. Pharmacological molecules were titrated for non-toxic levels and where no effect on cellular confluency was observed (supplementary Fig1). **(B)** Fold change gene expression comparing mock and MDV-infected CEFs at 24, 48 and 72 hpi. MDV viral titre in MDV-infected CEFs treated with **(C)** Clofibrate (0.01, 0.02, 0.04, 0.1, 0.2 and 0.41 5.9 µM), an agonist of PPAR-α, **(D)** malonyl-CoA (5, 10, 15, 25 and 30 µM) and **(E)** etomoxir (0.29, 1.47, 2.95 and 4.42 µM). **(F)** MDV-infected CEFs were treated with etomoxir (4.42 µM) with either palmitic acid (5, 12.5 and 25 µM) or malonyl-CoA (5, 15, 25 µM) and viral titre was analysed using a plaque assay. **(G)** MDV genome copy numbers per 10^4 cells (MEQ gene with reference ovotransferrin gene) were determined using qPCR in CEFs treated with etomoxir (4.42 µM) or

Clofibrate (5.9 µM). Non-parametric Wilcoxon tests (Mann-Whitney) and One-way ANOVA was used to assess normal distribution and test significance with the results shown as mean ± SD. $ indicate vehicle treated cells. ******** ($P < 0.0001$) indicates a statistically significant difference compared to control. NS indicates no significant difference. All experiments were performed in 6 replicated and data presented is representative of all 3 independent experiments.

3.4.6 MDV-induced lipogenesis results in formation of neutral lipid droplets

Lipid droplets (LD) are endoplasmic reticulum (ER) derived organelles that consists of a neutral lipids core with a phospholipid bilayer. An increase in the numbers of LD is an indication of lipogenesis. We examined if the increase in palmitic acid synthesis and excess VLCFA during MDV infection results in LD formation. Initially, Oil Red O staining was used to detect neutral lipid and hematoxylin for cell nuclei identification in mock and MDV infected CEFs at 72 hpi (Figure 16A). An accumulation of LD was observed in the MDV-infected (Figure 16Ai) compared to mock-infected cells (Figure 16Aii). To quantify the number of LD, pRB1B UL35-GFP-infected CEFs were stained with a neutral lipid dye Red LIPIDTOX (488nm), analysed by confocal microscopy and Z-stack images taken. As expected, the viral capsid protein UL35 fused to GFP was detected in the nucleus, while LD only localized within the cytoplasm (Fig 16F). A higher numbers of LD per cell were observed in MDV-infected compared to mock-infected cells ($P = 0.0001$) (Figure 16B). This was confirmed with an unbiased quantification of the LD using the IMARIS software (Figure 16C). Treatment of CEFs with SB 204990, or etomoxir had no effect on total LD numbers whereas treatment with TOFA and C75 decrease ($P = 0.0001$) the LD per cell in both mock (Figure 16D) and MDV-infected cells (Figure 16E). Altogether, these data show that MDV infection induces the accumulation and redistribution of fatty acids resulting in LD formation.

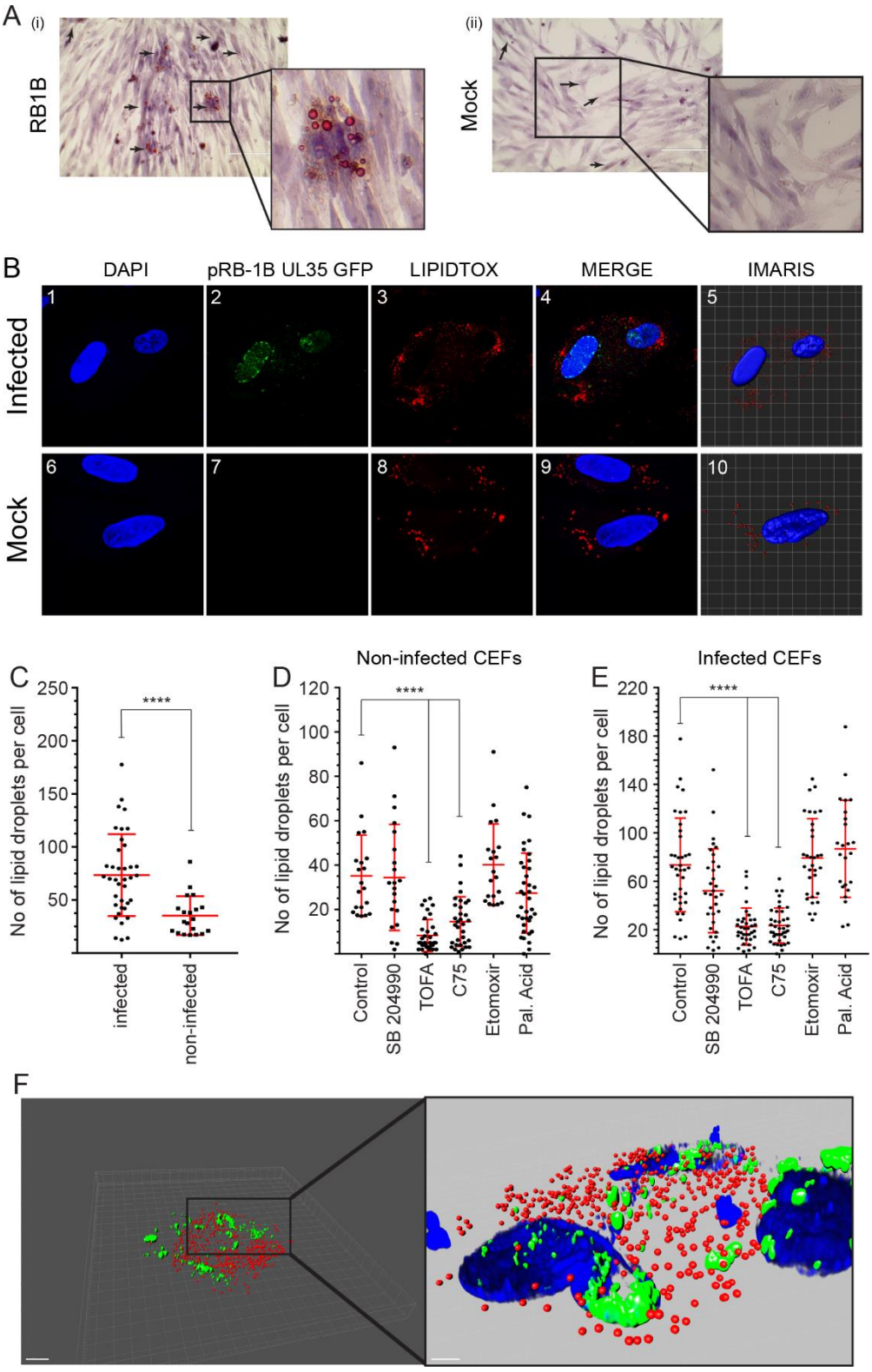

Figure 16: Excess FA are stored in lipid droplets during MDV infection
(A) Visualisation of cytoplasmic lipids in neutral lipid droplet organelles of CEFS **(i)** infected with MDV and **(ii)** mock-infected. Cell monolayer was fixed and stained with an Oil Red O staining kit and counterstained with hematoxylin and visualized using light microscopy (magnification 10X). Black arrows indicate lipid droplets. **(B)** Confocal microscopy imaging with maximum projection of Z-stacks for each channel

demonstrating nuclear and cytoplasmic distribution of pRB1B UL35-GFP virus (green) and lipid droplets (red). Mock and pRB1B UL35-GFP infected cells were fixed at 72 hpi and stained with DAPI (nuclear stain) and the neutral lipid stain LIPIDTOX-568nm. Images 5 and 10 are 3-D representative images analysed using the IMARIS software. Z-stacks were analysed using the IMARIS spot function analysis tool to quantify the relative amount of lipid droplets per cell in infected CEFs and non-infected CEFs (50-90 cells). **(C)** The numbers of lipid droplets per cell in MDV-infected and mock-infected CEFs. The numbers of lipid droplets per cell in **(D)** mock infected **(E)** MDV-infected CEFs treated with SB 204990 (3.85 µM), TOFA (1.54 µM), C75 (5.9 µM), etomoxir (4.42 µM) and palmitic acid (25 µM) are shown. **(F)** Analysis by IMARIS, using the co-localization function, to visualize neutral lipid droplet organelles (red) formation and relative position to the pRB1B UL35-GFP virus capsid protein (Green). ******** ($P < 0.0001$) indicates a statistically significant difference compared to the control. All experiments were performed in duplicates and data is representative of 3 independent experiments.

3.4.7 Virus-induced FAS pathway activates COX-2/$PGE_{2\alpha}$ pathway

As shown above, AA synthesis was elevated in the MDV infected CEFs ($P = 0.003$) at 48 and 72 hpi (Figure 11A). AA is a substrate for the production of eicosanoids, a class of lipids mediators converted by clycooxygenase-1 (COX-1) and inducible clycooxygenase-2 (COX-2). Therefore, we investigated the importance of eicosanoid synthesis in MDV infection as shown in the schematic diagram (Figure 17A). No significant difference in COX-1 gene expression levels was observed between the mock and MDV-infected cells at 72 hpi (Figure 17B). Treatment of MDV-infected CEFs with TOFA, C75 (FAS pathway inhibitors), or SC-236 (COX-2 inhibitor) also did not alter the expression of COX-1. However, COX-2 expression was significantly increased in the MDV-infected cells (Figure 17C). In addition, treatment of the infected cells with TOFA and C75 (T/C), or SC-236 decreased ($P = 0.0007$) COX-2 mRNA expression, suggesting that FAS pathway is involved in activation of COX-2. Treatment of the MDV-infected cells with non-toxic concentrations of a COX-2 inhibitor (SC-236) diminished MDV replication in a dose dependent manner. Specifically, 4-fold reduction in MDV titres was observed in CEFs treated with SC-236 ($P = 0.0022$; Figure 17D). Beyond that, we could demonstrate that $PGE_{2\alpha}$ is the only prostaglandin that can rescue the inhibitory effects of SC-236 on MDV titre (Figure 17E). In the absence of the COX-2 inhibitor, exogenous $PGE_{2\alpha}$ did not alter MDV titre (Figure 17F). A concentration as low as 0.1 µg/ml of PGE2α sufficed to rescue the inhibitory effects of SC-236 on MDV titre (Figure 17G). Strikingly, exogenous $PGE_{2\alpha}$ also restored the inhibitory effects of

TOFA and C75 on virus titre (Figure 17H), suggesting that the inhibitory effects of FAS pathway inhibitors is dependent on inhibition of $PGE_{2\alpha}$ synthesis. When CEFs were treated with low concentrations of TOFA or C75, exogenous $PGE_{2\alpha}$ fully restored MDV replication ($P = 0.0007$). However, $PGE_{2\alpha}$ only partially recovered MDV titre when high concentrations of TOFA or C75 were used (Figure 17H). To determine if $PGE_{2\alpha}$ was released from MDV infected cells, we measured $PGE_{2\alpha}$ in the supernatants of mock and MDV infected cells at 48 and 72hpi by ELISA (Figure 17I). Higher concentrations of $PGE_{2\alpha}$ were released by the MDV-infected compared to mock-infected cells at 72hpi ($P = 0.0001$). Interestingly, inhibition of FAS pathway by TOFA and C75 reduced $PGE_{2\alpha}$ release from MDV-infected cells ($P = 0.0022$), but not the mock infected cells at 72hpi. The results indicate that the treatment with TOFA and C75 only reduces MDV-induced $PGE_{2\alpha}$ synthesis, while physiological levels of $PGE_{2\alpha}$ are still produced in treated cells. In contrast, SC-236 significantly inhibited $PGE_{2\alpha}$ production from both the MDV-infected ($P = 0.0001$) and mock-infected ($P = 0.0001$) cells at 48 and 72hpi (Figure 17I). Taken together, our results suggest that virus-induced FAS pathway is critical for the increased $PGE_{2\alpha}$ synthesis in the MDV-infected cells.

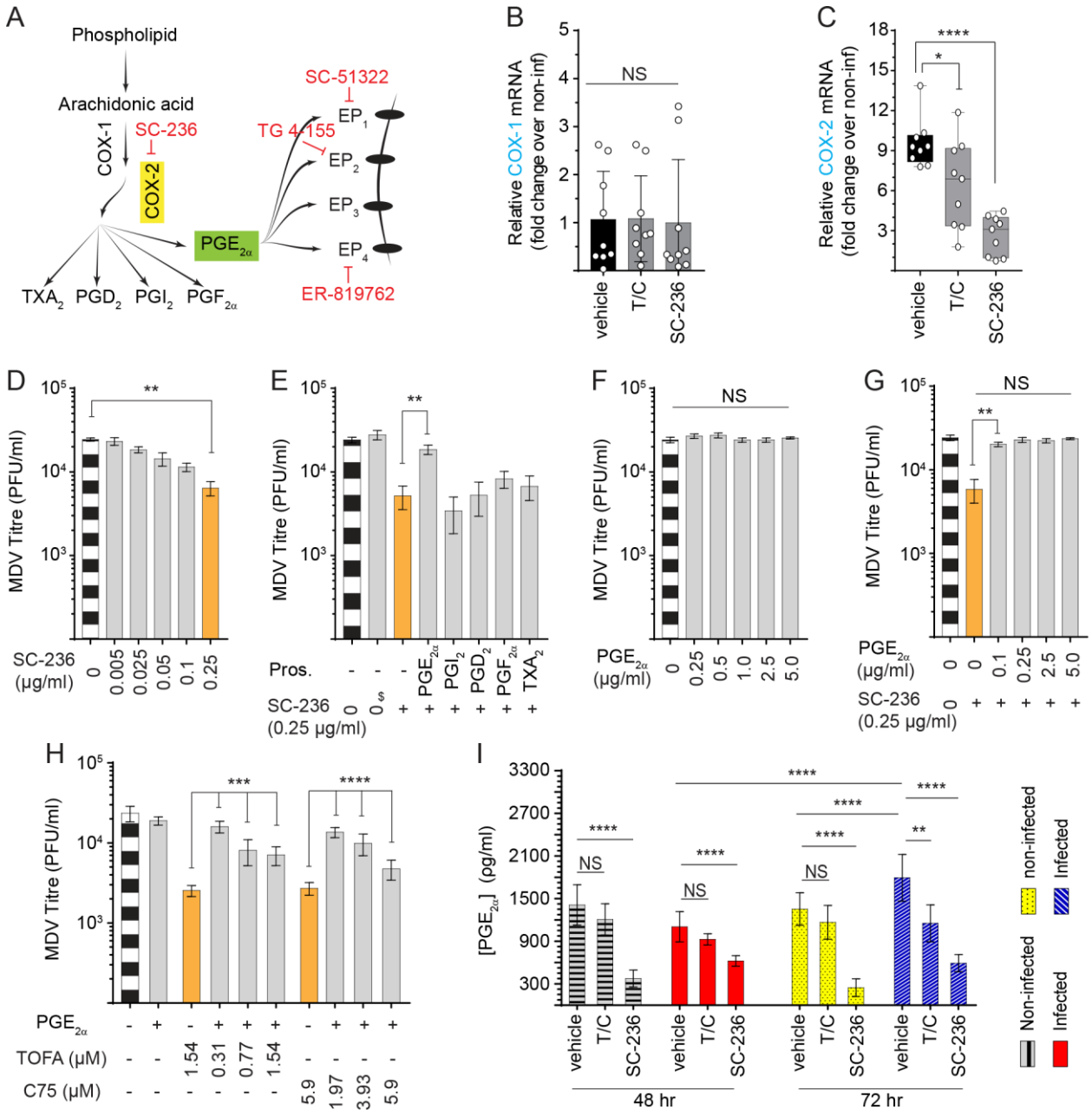

Figure 17: MDV infection modulate inducible COX-2 gene expression to produce PGE_2

(A) Schematic pathway outlines the relevant pharmacological inhibitor (red) and the respective enzyme (yellow box) as well as metabolites (green box) studied within the eicosanoid biosynthesis pathway. Fold change in expressions of **(B)** COX-1 and **(C)** COX-2 in mock-infected and MDV-infected CEFs treated with T/C (TOFA; 0.5 µg/ml in combination with C75; 1.0 µg/ml), or SC-236 (0.25 µg/ml). MDV titre (PFU/ml) in the MDV-infected CEFs in the presence of **(D)** SC-236 (0.005, 0.025, 0.05, 0.1 and 0.25 µg/ml), **(E)** SC-236 (0.25 µg/ml) in combination with $PGE_{2\alpha}$ (5.0 µg/ml), PGI_2 (0.5 µg/ml), PGD_2 (0.5 µg/ml), $PGF_{2\alpha}$ (0.5 µg/ml), and TXA_2 (0.5 µg/ml). MDV titre in CEFs treated with **(F)** different concentrations of $PGE_{2\alpha}$ (0.25, 0.5, 1.0, 2.5, and 5.0 µg/ml) **(G)** $PGE_{2\alpha}$ (5.0 µg/ml) in combination with SC-236 (0.25 µg/ml) and **(H)** $PGE_{2\alpha}$ (5.0 µg/ml) in combination with either TOFA (0.1, 0.25 and 0.5 µg/ml) or C75 (1.0, 1.2 and 1.5 µg/ml). **(I)** PGE_2 (ρg/ml) concentrations in supernatant of mock-infected and

MDV-infected CEFs were measured using an ELISA assay after 48 and 72 hpi in the presence of SC-236, T/C or no treatment. $ symbol indicates vehicle treated cells. * (*P* = 0.03), ** (*P* = 0.0022), *** (*P* = 0.0007) and **** (*P* = 0.0001) indicate statistically significant differences. NS indicates no statistical difference. Experiments were performed in 6 replicates for plaque assays and 3 replicates for Real-Time PCR and ELISA assays. The data are representative of 3 independent experiments.

3.4.8 Identification of the $PGE_{2\alpha}$ receptor(s)

Four different types of $PGE_{2\alpha}$ receptors have been identified in humans termed EP1, EP2, EP3, and EP4. Chicken EP2, EP3, and EP4 receptors have been cloned and characterized (243, 244) while EP1 has not yet been identified. Analysis of gene expressions in the MDV-infected and mock-infected CEFs demonstrated that the EP2 and EP4, but not EP3, were up-regulated upon MDV infection (Figure 18B), suggesting that $PGE_{2\alpha}$ may support MDV replication through an EP2 or/and EP4 mediated mechanisms. To examine the role of different EP receptor in MDV infection, we used receptor antagonists for EP1 (SC-51322), EP2 (TG 4-155) and EP4 (ER-819762) were utilized. As expected, the EP1 receptor antagonist (SC-51322; 0.1, 1, 5 and 10 µM) had no effect on MDV replication (Figure 18C). In contrast, TG 4-155 (2 and 4 µM) and ER-819762 (0.1, 0.5 and 1.0 µM) impaired MDV infection (*P* = 0.0001), confirming that $PGE_{2\alpha}$ support MDV replication through the EP2 and EP4 receptors (Figure 18D and 19E). Taken together, our data indicate that virus-induced FAS enhances $PGE_{2\alpha}$ synthesis and that this process is dependent on the EP2 and EP4 receptors in the MDV-infected cells.

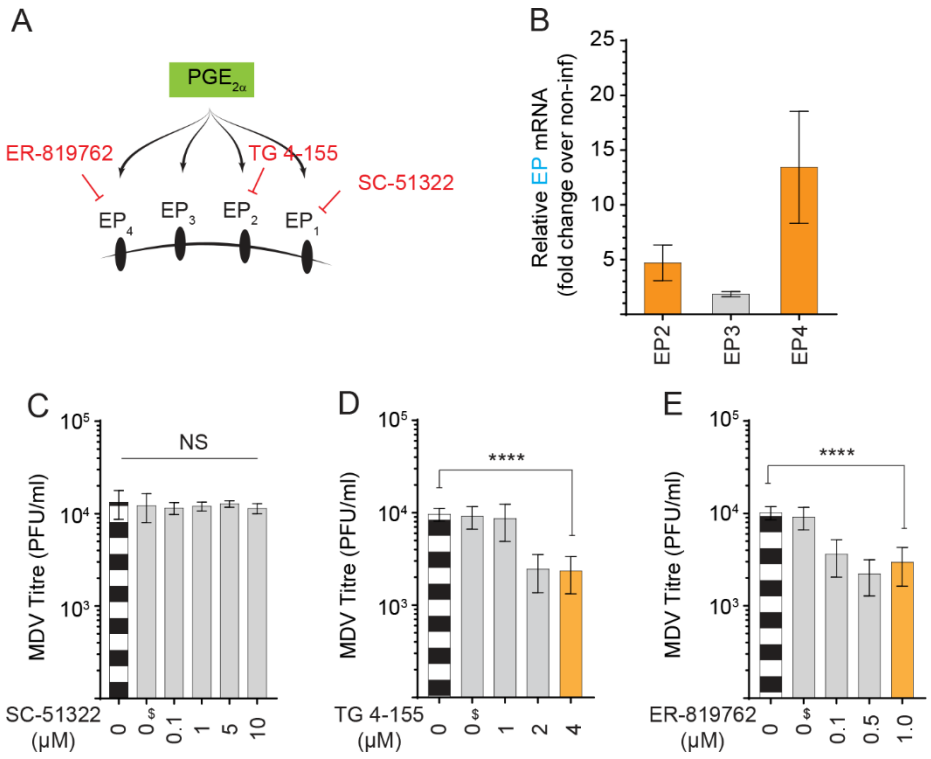

Figure 18: MDV infection is dependent on PGE_2 receptor engagement
(A) Schematic pathway outlines the relevant pharmacological inhibitor (red) and the respective metabolites (green box) studied within the eicosanoid biosynthesis pathway. **(B)** Fold change in gene expression of $PGE_{2\alpha}$ receptors EP2, EP3 and EP4 in MDV-infected CEFs at 72 hpi. MDV viral titre (PFU/ml) in MDV-infected CEFs in the presence of different concentration of $PGE_{2\alpha}$ receptor antagonists **(C)** SC-51322 (EP1 antagonist; 0.1, 1, 5 and 10 µM), **(D)** TG 4-155 (EP2 antagonist, 1, 2 and 4 µM) and **(E)** ER-819762 (EP4 antagonist; 0.1, 0.5 and 1.0 µM). $ symbol indicates vehicle treated cells. * (p = 0.03), ** (p = 0.0022), *** (*P* = 0.0007) and **** (*P* = 0.0001) indicate statistically significant differences. NS indicates no statistical difference. Experiments were performed in 6 replicates for plaque assays and 3 replicates for Real-Time PCR and ELISA assays. The data are representative of 3 independent experiments.

3.5 Discussion

Viruses have evolved strategies to target and modulate lipid signalling, synthesis and metabolism in the host cells and provide an optimal micro-environment for viral entry, replication and morphogenesis. Interestingly, even two related viruses such as HSV-1 and HCMV drive host cells to achieve distinct metabolic programs. While HCMV increased *de novo* lipid synthesis, HSV-1 enhanced the synthesis of pyrimidine nucleotides (219). Other viruses can also modulate common lipid metabolism including

upregulation of FAS, providing building blocks for different lipids. Replication of different viruses may require activation of distinct lipid pathways and source of energy.

MDV is an *alphaherpesvirus* that infects chickens and causes a deadly lymphoproliferative disease. In addition to transformation of $CD4^+$ T cells, MDV causes atherosclerosis by disturbing the lipid metabolism in the infected chickens (245), which can be inhibited by vaccine-induced immunity (241, 246). Surprisingly, little is known about the processes involved in disturbance of lipid metabolism in MDV infection. Our lipidomic analysis of MDV-infected CEFs demonstrates that MDV infection enhances FAS and promote synthesis of AA, the prostaglandin precursor. Here, we demonstrate that MDV hijacks host metabolic pathways to provide essential macromolecular synthesis to support infection and replication. *De novo* FAS generates the metabolic intermediate acetyl-CoA, malonyl-CoA and finally palmitate. Targeted inhibition studies against the enzymes involved in FAS during infection have yielded an alternative understanding of alteration of lipid metabolism in infections with HCMV (247), EBV (234), HCV (248, 249). The first step towards FAS is the conversion of citric acid into acetyl-CoA by direct phosphorylation of ACLY. Hepatitis B virus (HBV) and HCMV infections activate the expression of ACLY (250, 251), however the enzymatic activity of ACLY is not critical for virus-induced lipogenesis. Virus-infected cells can use glucose carbon for FAS by ACLY from citrate generated in mitochondria and acetyl-CoA synthetase short-chain family member 2 (ACSS2) from acetate (252). In our study, blocking ACLY activity via small pharmacological inhibitors had no effect on MDV replication and genome copies. This finding suggests that acetate can be produced in avian cells via other endogenous mechanism under rich medium conditions, which would mask the effects on viral replication. The subsequent step involves the conversion of acetyl-CoA into malonyl-CoA by acetyl-coA carboxylase (ACC) and finally elongation by utilizing both acetyl-CoA and malonyl-CoA coupled to the multifunctional fatty acid synthase (FASN) to make palmitic acid. Our results demonstrate that blocking ACC and FASN activity significantly reduces MDV replication, suggesting that MDV preferentially modulates the FAS pathway to generate a variety of lipids which contributes to several key cellular processes. Our data confirm that the inhibitory effects of FAS inhibitors on MDV replication could be overcome by the addition of palmitic acid, a metabolite downstream of FASN in the FAS pathway. This indicates that inhibition of MDV by FAS inhibitors was not simply detrimental to

the cell but was essential for the production of infectious virus. Future studies are required to examine the mechanism involved in activation of FAS by MDV which can lead to induction and accumulation of fatty acids.

Palmitic acid contributes essential carbons in synthesis of VLCFA that can be subsequently utilized in synthesis of various lipids required for membrane biosynthesis and lipid droplet formation. Our results demonstrate that VLCFA are increased in MDV infection. Alternatively, β-oxidation of fatty acids within the mitochondria can generate energy and macromolecular precursors to support cellular activity and fuel viral replication. It has been shown that the synthesis and mitochondrial import of fatty acids, in which β-oxidation generate ATP production, are essential for replication of vaccinia virus (223). This can be explained by the fact that vaccinia virus does not require glycolysis for replication and thus depends on the energy generation via β-oxidation. Our results demonstrate that β-oxidation is activated in the MDV-infected cells and palmitic acid is converted into energy in the mitochondria. However, we could demonstrate that limiting energy derived from mitochondrial β-oxidation during lytic viral infection had no detrimental effect on MDV replication. In fact, inhibition of β-oxidation rather increased virus titre, suggesting that MDV-infected cells obtain their energy from other sources. This could be explained by the facts that some herpesviruses encode metabolic enzymes to support essential functions independent of mitochondria derived energy carriers (66, 68, 215, 216). Alternatively, MDV could induces glycolysis and thus does not require energy generated via β-oxidation as observed for other herpes viruses (219, 247). Consequently, activation of lipolysis (mitochondrial β-oxidation) decreased virus titre and genome copies, confirming that β-oxidation is not beneficial for MDV replication. This indicates that the generated fatty acids are utilized for activation of other lipid pathways, which are crucial for viral replication.

It is known that elongation of palmitic acid in lipogenesis contributes to a total cellular pool of VLCFA which are essential components for the initiation of key cellular processes such as membrane lipid synthesis, generation of lipid droplets and eicosanoid synthesis (253). It has been shown that the assembly of some viruses such as HCMV are highly dependent on induction of VLCFA from FAS (235). Similarly, VLFCA contribute to IAV envelope formation in a strain dependent manner (254). Lipid droplets are classically defined as organelles with stored neutral lipids and some reports

suggest that lipid droplets are a site for replication and assembly of some viruses. However the functional relationship of lipid droplets to cellular processes are not well defined (255). Infection with a range of pathogens have been demonstrated to induce lipid droplet formation including bacteria, specifically *M. tuberculosis* (256) and *M leprae* (257), as well as protozoan infection such as *Trypanosoma cruzi* (258) in Chagas disease. Viral infection including HCV and its relationship to lipid droplet formation has been extensively documented (259). It has been demonstrated that the HCV core protein interacts with lipid droplet for virion assembly (260). Similarly, infection with HCV (260), DENV (261) and rotaviruses (262) promoted the formation of lipid droplets. Similarly, our data demonstrate that MDV infection increases lipid droplet formation; however, the exact role of lipid droplets in MDV infection is unknown. Formation of lipid droplets in MDV infection was dependent on FAS pathway as FAS inhibitors reduced the numbers of lipid droplets. Lipid droplets are a significant source of triglyceride-derived arachidonic acid (AA) which can be converted to eicosanoids such as prostaglandins. Upon stimulation, AA is released from lipid droplets and metabolised into $PGE_{2\alpha}$ by the cyclooxygenase enzymes COX-1 and COX-2. In this study, we observed that MDV promotes lipid droplet formation, and synthesis of phospholipid and AA. The upregulation of COX-2 but not COX-1 transcripts in MDV-infected cells confirms that COX-2/PGE2 pathway are induced by the virus. Some viruses such as EBV (263) promote viral replication and dissemination by supressing $PGE_{2\alpha}$ biosynthesis. In contrast, HCMV (229, 264), KSHV (232) and MVA (228) activate $PGE_{2\alpha}$ release in response to infection. Here, we demonstrate that MDV upregulate COX-2 expression and $PGE_{2\alpha}$ synthesis, which is highly dependent on the FAS pathway. To our knowledge, this is the first report demonstrating that a viral infection increases COX-2/$PGE_{2\alpha}$ pathway via induction of the FAS pathway, and demonstrate that the inhibitory effects of FAS inhibitors on virus replication could be recovered by exogenous $PGE_{2\alpha}$. The proposed model for the role of FAS and COX-2/$PGE_{2\alpha}$ pathways in MDV infection is summarized in Figure 19. Taken together, our results demonstrate that virus-induced FAS pathway enhances $PGE_{2\alpha}$ synthesis which support MDV infectivity through EP2 and EP4 receptors.

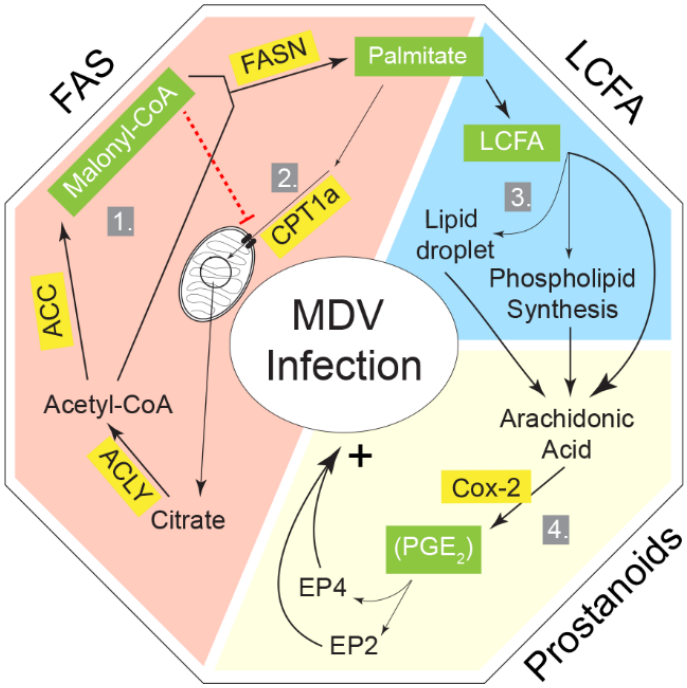

Figure 19: Schematic representation of MDV modulation of lipogenesis and Prostanoids

MDV systematically modulates cellular lipid metabolic pathways to support its replication through FAS and COX-2/PGE2 pathways. The three major phases of lipogenesis are grouped under FAS, VLCFA and Prostanoids. **1.** Infection of CEFs with MDV results in an increase of *de novo* FAS pathway, which is required for MDV replication. **2.** Palmitic acid biosynthesized in FAS pathway can be utilized in the mitochondria by FAO (β-oxidation) to regenerate energy intermediates, however this pathway is not required for virus replication. **3.** Elongation of palmitic acid results in biosynthesis of VLCFA which can be stored in lipid droplets organelles, utilized for phospholipid or eicosanoid synthesis **4.** Subsequently, $PGE_{2\alpha}$ are synthesized enzymatically from arachidonic acid. Signalling of $PGE_{2\alpha}$ is mediated through its EP2 and EP4 receptors in MDV-infected cells. Specific enzymes studied along the pathway, their relative importance for MDV infection are highlighted in yellow. Major metabolites which was identified as essential lipids are highlighted in green.

Chapter 4: T cell responses to Marek's Disease virus in resistant and susceptible line of chickens

4.1 Abstract

This study defines that pp38 is highly immunogenic and elicits a T cell reactivity in birds whether they were challenged, vaccine-challenged or vaccine-boosted. The immunodominant peptide epitope ($pp38_{5-20}$, $pp38_{161-176}$ and $pp38_{171-186}$) eliciting this syngeneic immunoreactivity pattern is identified for the first time in both MD-resistant (B21) and MD-susceptible (B19) lines of chickens. In order to identify T cell subsets which are recognizing these immunodominant peptide epitopes, we depleted $CD4^+$, $CD8^+$, $TCR_V\beta_1^+$ or $TCR_V\beta_2^+$ from splenocytes and the results shows that depletion of $CD4^+$ or $TCR_V\beta_1^+$ T cell but not $CD8^+$ or $TCR_V\beta_2^+$ T cell cells diminishes antigen specific T cell responses as measured by *ex vivo* cIFN-γ ELISPOT assay suggesting that the reacting T cells are $CD4^+$ $TCR_V\beta_1^+$ T cell in both the resistant and susceptible line of birds. Furthermore, these results indicate that $CD4^+$ and not $CD8^+$ T cells proliferate and induce upregulation of IL-2, IL-4 and IL-10. Peptide ($pp38_{5-20}$, $pp38_{161-176}$ and $pp38_{171-186}$) specific memory T cells can be identified at 4 weeks post either challenged, vaccine-challenged or vaccine-boost in both susceptible and resistant lines of birds. However, challenge resulted in a diminished of the ability of T cell degranulation as measured by expression of CD107a in both antigen-specific and antigen-non-specific manner in both susceptible and resistant lines of birds. Infection of both naïve and MDV-vaccinated birds impaired T cell degranulation, however Antigen-specific induction of Perforin and Granzyme B (GZMB) mRNA transcripts can be detected in splenocytes of both susceptible and resistant lines of birds. This is the first report on identification and characterisation of MDV specific $CD4^+$ T cell responses in susceptible and resistant lines of birds following challenge, vaccine-challenged or vaccine-boosted.

4.2 Introduction

T cells play a critical role in defence against intracellular pathogens. The $CD8^+$ (265) and $CD4^+$ αβT (266) cell subsets express T cell receptors (TCR) that recognizes 8-21 mer. Peptides. These peptides are presented in association with major histocompatibility complex (MHC) on antigen presenting cells (APC) which result in directed effector T cells cell clonal expansion: differentiation/proliferation, cytokine production (IFN-γ, IL-2, IL-4), and cytotoxic function ($CD107a^+$) (267). Cytotoxic properties of antiviral $CD8^+$ and $CD4^+$ T cells play an important role in the control and clearance of many viral infections, and the degranulation process is a necessary prerequisite to T cell cytotoxicity. Polarized T cells give rise to memory T cells that form the fundamental responding population upon subsequent infection; differentiation back to an effector phenotype. All these encompass fundamental T cells characteristics (268).

With identification of T cell targets, the antigenic epitope can be mapped with synthetic peptides libraries. Numerous T cell epitopes from various human pathogens specifically varicella zoster virus (VZV) (269), herpes simplex virus-1 (HSV-1) (270), Human cytomegalovirus (HCMV) (271), Crimean-Congo haemorrhagic fever virus (CCHFV) (272), human immunodeficiency virus (HIV), Epstein-Barr virus (EBV) (273) and *Mycobacterium tuberculosis* have been identified (274). Mice infected with *M. tuberculosis*predominantly had $CD4^+$ T cell response which were identified as the effector T cell subset. T cells are essential for both recovery and prevention of reactivation during primary VZV infection (275). Zoster vaccination on its own elicits a broad $CD4^+$ T cell response thus priming helper responses to secondary viral reactivation (269). Furthermore, T cells and not B cell functional activity as defined by cytokine production (IFN-γ and IL-2) and cytotoxic activity (Granzyme B and Perforin) are good determinants for quality of T cell responses which correlates with protective immunity in ageing human populations against VZV reactivation (276). In the MD susceptible line of chicken, infiltrating $CD8^+$ T cell have be detected in MDV induced $CD4^+$ lymphoma (37). However infiltrating CD8+ T cell are unable to perform cytolytic activity evident by the metastasis of MDV induced $CD4^+$ lymphoma. Our group has previously demonstrated in an avian influenza virus model that T cell responses can be detected against the hemagglutinin antigen (HA) of avian influenza virus. These minimal epitopes can be utilized to further characterise T cell functions; proliferation

and cytokines (IFN-γ, perforin, granzyme and IL-10) gene expression (277). T cell epitope mapping studies have yet to be performed against other avian pathogens including MDV antigens. In fact, our understanding of MDV induced T cell activation is largely based on the study performed by *Omar et al.* (25) who demonstrated $CD8^+$ T cell specific responses against MDV antigens pp38, MEQ, ICP4 and gB. Despite the potentially vast number of peptides MHC (pMHC) that could be recognized by the T cell repertoire of the host, only a small proportion of the overall viral proteome elicits a host response. Therefore, T cell clonal heterogeneity generated against MDV may well be limited and haplotype specific (25).

Therefore, the objective of this study was aimed to unravel the differential immunoreactivity pattern between two genetically defined lines of chickens, MD resistant (Line N; B21) and susceptible (Line P; B19) after either mock, challenge, vaccine-challenge or vaccine boosted. Antigen specific T cell recall responses against pp38 and MEQ was confirmed by analysing the frequency of IFN-γ producing cells in an *ex vivo* chIFN-γ ELISpot. The magnitude and breadth of antigen specific recall response was tracked (week 1, 2, 3 and 4 post inoculation) over the course of this study. The results indicate that both MEQ and pp38 are immunogenic in both B21 and B19. Interestingly, MEQ was found to be immunogenic in the B21 birds and not B19. However pp38 was highly immunogenic in both B21 and B19. Furthermore, the differential response between the animal groups indicate that vaccination not sufficient to elicit a recall response upon challenge against MEQ in B19. More importantly, recall response against pp38 can be boosted in both B21 and B19 birds and this response is directed to specific peptide pools here termed immunodominant peptide pool. These results confirm that B21 birds are able to elicit a stronger antigen recall compared to B19 birds against MEQ or pp38. This suggest that strength of response and differential immunoreactivity pattern could well provide an association for resistance or susceptibility to MD.

4.3 Material and Method

4.3.1 Experimental Animals:

Genetically defined mixed sex Line N (resistant; B21 haplotype) and Line P (susceptible; B19 haplotype) SPF chickens were reared at The Pirbright Institute or purchased from National Avian Research Facility (NARF) at University of Edinburgh. Day old chicks were grouped housed throughout the experiment in specific pathogen free as not gnotobiotic filtered-air positive pressure rooms on floor pens with wood shaving. Group housed birds had ad libitum access to water and commercial feed. All animal works were performed according to United Kingdom (UK) home office licence.

4.3.2 Virus preparation

Virus stocks were prepared as a cell associated 3rd passage virus for both the pathogenic (RB1B) and vaccine strain (CVI988-RISPENS) in chicken embryonic fibroblast cells (CEFs) and frozen (liquid nitrogen) in freezing medium (CEF growth medium with 5% DMSO). Virus were isolated from splenocytes, kindly provided by Dr.Venugopal Nair, harvested from either MD-resistant or MD-susceptible lines of chicken at 2 weeks post challenge with the vaccine strain or pathogenic strain.

4.3.3 Synthetic peptide Library

In total 66 peptides spanning the entire MEQ (GeneBank: ADN05237.1) protein and 55 peptides spanning the entire pp38 (GenBank: ABR13155.1) protein respectively was synthesized by Mimotopes (United Kingdom). Peptides were 15 residues long and overlapped by 5 residues. Peptide were dissolved in DMSO and peptide pools (1 mM) were prepared at 10 peptides per pool for a total of 7 and 6 peptide pools for MEQ and pp38 respectively. For subsequent experiments, individual pp38 peptides ($pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ and $pp38_{171\text{-}186}$) were synthesized at a purity of 95% and re-suspended to 2.25 mM using the solvent recommended by the manufacturer.

4.3.4 Experimental Design

Line N (B21; resistant) and Line P (B19; susceptible) chickens were either mock inoculated (non-infected CEF), challenged (RB1B; 1000 pfu/bird) or vaccinated (CVI988-RISPENS; 1000 pfu/bird) at 1 day of age (doa.) via intra-abdominal route and the vaccinated group were either boosted (CVI988-RISPENS; 1000 pfu/bird) or

challenged (RB1B; 1000 pfu/bird) 2 weeks later via intraabdominal route. Post-inoculations, birds were culled at different days post inoculation and organs were harvested (Figure 20).

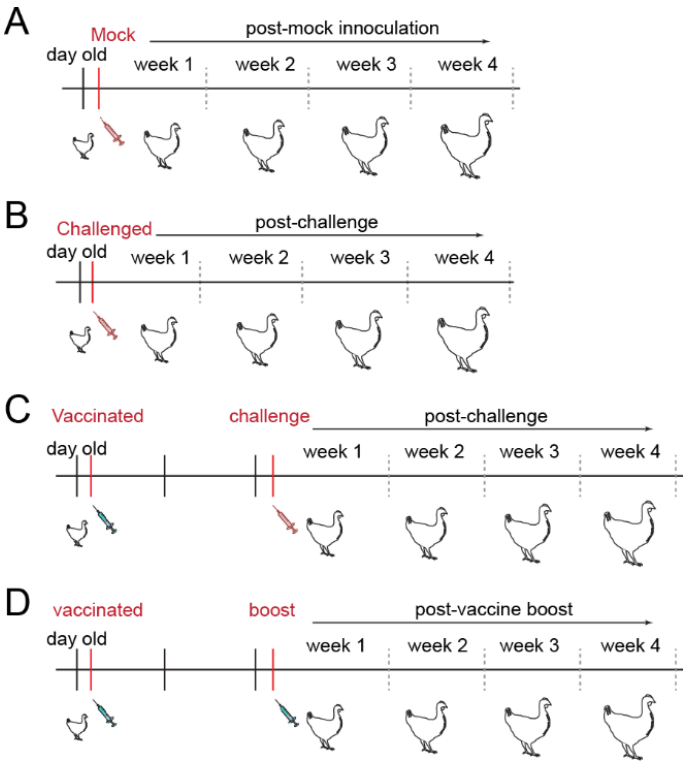

Figure 20: Avian infection models developed to study T cell mediated immune responses
Schematic representation of animal models developed to study T cell response to MDV in Line N (B21; resistant) and Line P (B19; susceptible) chickens. Day old birds from both B21 and B19 were inoculated with **(A)** Non-infected CEF **(B)** CEF infected with RB1B (1000 PFU/bird) **(C)** CEF-infected with CVI988/RISPENS (1000 PFU/bird) and 2 weeks later were challenged (RB1B; 1000 PFU/bird) or **(D)** boosted (CVI988/RISPENS; 1000 PFU/bird) and 2 weeks later Boosted (CVI988/RISPENS; 1000 PFU/bird).

4.3.5 Spleen mononuclear cell preparation

Spleens harvested from resistant (B21) and susceptible (B19) chickens were stored aseptically on ice in PBS and isolated as previously described (278). Following isolation, cells were re-suspended in complete RPMI cell culture medium; RPMI 1640 medium containing 10% foetal bovine serum (Sigma-Aldrich, Dorset, UK), penicillin (10 U/ml), and streptomycin (10 µg/ml) at a density of 5×10^6 cells/ml and kept on ice until required.

4.3.6 *In vitro* expansion of pp38 specific T cells

Mononuclear cells isolated at 1, 2, 3 and 4 weeks post inoculation were cultured (AIM-V medium plus 10% FCS) in the presence of recombinant IL-2 (rIL-2; 2 units per 100 µl), medium alone, vehicle (DMSO) or specific pp38 pool 1 and 4 peptides; each individual peptides were at a final concentration of 2 µmol/mL. At day 8, differentiated cells were harvested and used in cultured ELISPOT assay.

4.3.7 T cell epitope mapping: *ex vivo* ELISPOT Assay

4.3.7.1 T cell depletion

Approximately ten million cells were washed in buffer solution (PBS + 0.5% FCS) and incubated (4^{o}C for 15 min) in the presence of anti-CD4-PE, anti-CD8-PE, anti-TCRvβ_1-PE or anti-TCRvβ_2-PE antibody. Cells were washed (1500 rpm for 4 min) and further incubated (4^{o}C for 15 min) with 85 µl of buffer solution and 15 µl of anti-PE micro beads (MACS Micro Magnetic Beads). Micro Magnetic bead labelled cells were applied to MACS column (Miltenyi Biotec, Surrey, UK; cat no. 130042201) and the pass through collected. Depletion purity (> 95%) was confirmed by flow cytometry using a MACS quant. Cell suspension were washed again, re-suspended in complete RPMI 1640 medium at a rate of 3.3×10^6 cells/ml and stored on ice until required

4.3.7.2 T cell stimulation with putative epitopes in a Chicken IFN-γ ELISPOT assay

IFN-γ production was detected using a chicken IFN-γ antibody pair kit (life technologies, UK). In brief, MAIPS4510 MultiScreenTM-IP 96 well plates (Millipore, UK) were incubated overnight at 4°C with 2 µg/ml mouse anti-chIFN-γ (capture antibody) in PBS and next day blocked (1 hr; 37°C, 5% CO_2) with blocking buffer (RPMI 1640 plus 2% FCS). Mononuclear cells were seeded in triplicates at a rate of 3.3 x 10^5 cells per well. The cells were cultured with medium alone (control), diluent (control), PMA (50 ng/ml) plus Ionomycin (ION; 1 µg/ml) (positive control); (Sigma-Aldrich, Dorset, UK), pp38 or MEQ peptide pools (1.0 µM), $pp38_{5\text{-}20}$, $pp38161_{\text{-}176}$ and $pp38_{171\text{-}186}$ peptides or irrelevant peptide (influenza HA peptide: $H5_{246–260}$) and incubated (41°C and 5% CO_2) overnight. Next day plates were washed twice with SQ water and three time with washing buffer (PBS + 0.1% Tween 20). Plates were subsequently incubated (1 hr in the dark at RT) with detection antibody (1 µg/ml of anti-chicken IFN-γ biotinylated antibody) in assay buffer (PBS + 0.1% Tween 20 + 1.0% BSA). Following a further incubation (1 hr in the dark at RT) with Streptavidin-

HRP (1/1250) in assay buffer, plates were washed three times with washing buffer and developed in the presence of 3-Amino-9-ethylcarbazole (AEC) substrate solution (BD Biosciences, UK) at RT in the dark. Reactions were inactivated after 20 min by washing in distilled water, air dried overnight

The frequency of IFN-γ specific T cell (spots forming units; SFU) were counted using an automated ELISPOT reader. The SFU was calculated by subtracting the number of spots obtained in the non-stimulated control well from the stimulated samples. A positive control was included with each plate to validate the sensitivity of the assay

4.3.8 Flow Cytometry: CFSE proliferation, T cell cytotoxicity (CD107a)

4.3.8.1 CFSE staining

In brief, approximately 1.0 x 10^7 spleen mononuclear cells were labelled with CFSE (5 µM; eBioscience, Thermo fisher Scientific, UK). Staining was quenched by washing and further incubating (37^{o}C for 15 min) cells in complete RPMI culture medium.

4.3.8.2 In vitro T cell proliferation assay

Stained mononuclear cells were stimulated in the presence or absence of specific pp38 peptide ($pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ and $pp38_{171\text{-}186}$). At 72 hrs post stimulation cells were harvested and stained with anti-CD4-PE and anti-CD8-APC (Southern Biotech, UK) and dead cells marker (7AAD; Miltenyi Biotech Ltd, UK). Cell proliferation and frequency of specific T cell subsets were detected by monitoring changes in fluorescence intensity of CFSE-labelled cells at 72 hrs post stimulation.

4.3.8.3 T cell Cytotoxicity (CD107a) assay

Unconjugated CD107a (LEP) and isotype control (CC63) antibodies were conjugated using the Alexa Fluor 647 labelling kit (Life Technologies, UK) according to manufacturer's recommendation prior to use. Mononuclear cells were seeded in triplicates at 5.0 x10^5 cells. The degranulation marker, CD107a, of mononuclear cells were assessed by pulsing cells with pp38 peptide ($pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ and $pp38_{171\text{-}186}$), irrelevant peptide, medium alone (control), diluent (control), PMA (50 ng/ml) plus Ionomycin (ION; 1 µg/ml) (positive control); (Sigma-Aldrich, Dorset, UK) and co-cultured (4 hrs; 41°C, 5% CO_2) with the following antibodies; LEP (CD107a-Alexa Fluor 647) or isotype control (cc63-Alexa Fluor 647). Following a wash in PBS, cells were labelled with CD4 or CD8 PE (Southern Biotech, UK) for 15 min at 4°C and dead cells were excluded using 7-AAD-PE (Miltenyi Biotech Ltd, UK) staining. For all flow

cytometry assays, cells were acquired on a MACS quant and data analysed using the Flow Jo software.

4.3.9 Real Time-PCR

RNA extraction and cDNA synthesis: Total RNA was extracted from splenocytes isolated from both resistant (B21) and susceptible (B19) chickens stimulated *ex-vivo* with the putative $pp38_{5\text{-}20}$, $pp38161_{\text{-}176}$ and $pp38_{171\text{-}186}$ peptides, irrelevant peptide (influenza HA peptide: $H5_{246–260}$), and PMA; 50 ng/ml plus ION; 1 μg/ml (positive control); (Sigma-Aldrich, Dorset, UK) using TRIzol (Thermo Fisher Scientific, Paisley, UK) according to the manufacturer's protocol. Subsequently, 1 μg of DNase (Thermo Fisher Scientific, Paisley, UK) treated and purified RNA was reverse transcribed using a Superscript® III First Strand Synthesis kit (Thermo Fisher Scientific, Paisley, UK) and oligo-dT primers according to the manufacturer's recommended protocol. The resulting cDNA was diluted 1:10 in DEPC treated water for use in Real Time-PCR assay.

SYBR green Real Time-PCR: Quantitative real-time PCR using SYBR Green was performed on diluted cDNA using the LightCycler® 480 II (Roche Diagnostics GmbH, Mannheim, GER) as previously described. Each reaction involved a pre-incubation at 95°C for 5 min, followed by 40 cycles of 95°C for 20 sec, 55°C–64°C (T_A as per primer) for 15 sec, and elongation at 72°C for 10 sec. Subsequent melt curve analysis was performed by heating to 95°C for 10 sec, cooling to 65°C for 1 min, and heating to 97°C. Primers sequences and accession numbers are outlined in Table 4. Relative expression levels of all genes were calculated relative to the housekeeping gene β-actin using the LightCycler® 480 Software (Roche Diagnostics GmbH, Mannheim, GER). Data represent mean of 6 biological replicates.

Table 4 List of primers used for Real-Time PCR

Gene name	Accession no	Primers		Tm (ºC)	Product size
Interleukin 2 (IL-2)	NM_204596.1	Fwd	ACAGTGGCTATAGGAGACGA	60ºC	166
		Rev	TGTCTTGCTGGCTGTTGTGT		
Interleukin 4 (IL-4)	NM_001007079.1	Fwd	TGTGCCCACGCTGTGCTTACA	60ºC	193
		Rev	CTTGTGGCAGTGCTGGCTCTCC		
Interleukin 10 (IL-10)	XM_025143715.1	Fwd	GGGAGCTGAGGGTGAAGTTTGAGGA	60ºC	200
		Rev	CTGCTGATGACTGGTGCTGGTCTG		

Perforin	XM_004945690.3	Fwd	ATGGCGCAGGTGACAGTGA	60ºC	271
		Rev	TGGCCTGCACCGGTAATTC		
Granzyme A (GZMA)	NM_204457.1	Fwd	TGGGTGTTAACAGCTGCTCATTGC	60ºC	454
		Rev	CACCTGAATCCCCTCGACATGAGT		
Cytoplasmic Beta Actin (ACBT)	NM_205518.1	Fwd	TGCTGTGTTCCCATCTATCG	60ºC	150 bp
		Rev	TTGGTGACAATACCGTGTTCA		

4.3.10 Epitope conservation analysis

In order to identify conserved putative T cell epitopes from different serotypes, the MDV strains (GaHV-2: RB1B, CVI988-RISPNES, MD5, and GA, GaHV-3: SB-1, HPRS24, MeHV-1: HVT) pp38 (GenBank: ABR13155.1; RB1B, YP_001033989; MD5, ABF72309.1; CVI988-RISPENS, NP_066892.1; SB-1 and NP_073357.1; MeHV-1) were aligned within and with each other. Clustal W was employed for the protein sequence alignment in MEGA6.

4.3.11 Statistical Analysis

ELISPOT SFU data were adjusted to 1.0 x10^6 cells. Quantification was performed using Graph Pad Prism 6 for windows. All data were analysed by Wilcoxon and Mann Whitney non-parametric to test significance and presented as mean $\pm$ SD. Results were considered statistically significant at $P < 0.05$ (*).

An immunological response/responder was defined as a 2-fold increase in the frequency of cytokine-producing cells above control peptide/pools.

4.4 Results

4.4.1 Higher frequencies of MEQ-specific IFN-γ producing T cells in MD-resistant (B21) compared to susceptible (B19) lines of chickens

To investigate the role of cell mediated immunity against MDV in both the MD-resistant (B21) and susceptible (B19) lines of chickens, antigen specific T cell responses to a panel of MEQ peptide pools (7 pools) was analysed using *ex vivo* chicken IFN-γ ELISPOT assay from 6 MD-resistant (Figure 21A) and susceptible (Figure 21B) line of chickens at weekly intervals. MEQ specific T cell responses were detected in the MD-resistant line of chickens that were challenged, vaccine-challenged and vaccine-boosted.

Interestingly, MEQ-specific T cell responses were not detected in the MD-susceptible line of chickens that were primed-boosted. No MEQ-specific T cell response was detected in mock infected chickens. Higher frequencies of MEQ-specific IFN-γ producing T cell responses were detected in the MD-resistant (Figure 21A) compared to the susceptible (Figure 21B) line of chickens. The highest frequency of IFN-γ producing T cells to MEQ peptide pools was observed at 2 weeks post infection or vaccination in both the MD-resistant and susceptible line of chickens; challenge (mean = 49.5 SFU/10^6 cells), vaccine-challenge (mean = 347 SFU/10^6 cells), or vaccine-boost (mean = 198 SFU/10^6 cells). MEQ-specific T cell responses were reduced and no antigen specific T cell response to any of the MEQ peptide pools were detected at week 4 post either challenge, vaccine-challenge or vaccine-boost in both the MD-resistant or susceptible line of chickens. Furthermore, a robust T cell response was observed in the MD-resistant line of chickens that were either vaccine-challenge or vaccine boosted. However, our results demonstrate antigen specific T cell recall responses to MEQ peptide pools in the susceptible line of chickens that were either vaccine-challenge or vaccine boosted at all weekly intervals. However, in both MD-resistant and susceptible line of chickens which were challenged only, no detectable antigen specific recall response was observed at either week 1, 2, 3 or 4 to MEQ peptide pools. Interestingly, vaccine-boost was unable to elicit any detectable antigen specific T cell responses in the MD-susceptible lines of chicken (Figure 21B).

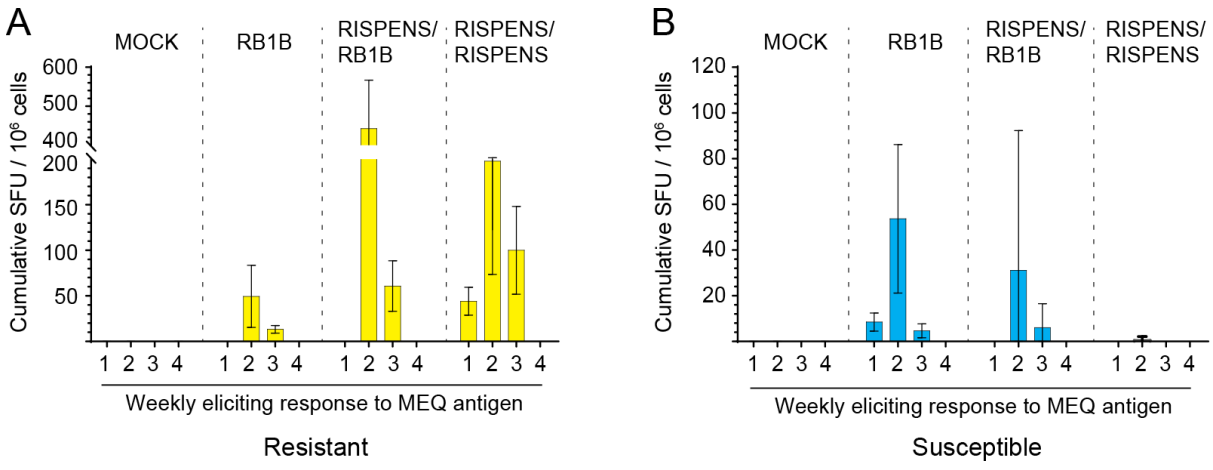

Figure 21: Differential T cell response to MEQ peptide pools.
MEQ-specific T cell responses were in an *ex vivo* chIFN-γ ELSIPOT assay. The frequency of IFN-γ producing T cells, presented as cumulative spots forming unit (SFU)

per 1.0 x10^6 cells, are graphed as weekly responses in both the MD **(A)** resistant (B21) and **(B)** susceptible (B19) line of chickens that were mock, challenged, vaccine-challenged and vaccine-boost. All experiments were performed in triplicates and results are representative of 32 chickens each from both the resistant and susceptible lines of chicken.

4.4.2 MDV pp38 is highly immunogenic in both the MD-resistant (B21) and susceptible (B19) lines of chicken

Antigen specific T cell responses against a panel of pp38 peptide pools (6 pools) were analysed from both the MD-resistant (B21) and susceptible (B19) lines of chickens. The frequency of IFN-γ (SFU/1.0x10^6 cells) producing cells was tested in an *ex vivo* chicken IFN-γ ELISPOT assay from both MD-resistant (yellow; Figure 22A) and susceptible (blue; Figure 22B) lines of chickens at weekly intervals. Our results demonstrate that pp38 peptide pools elicited potent antigen specific T cell recall responses in both the MD-resistant and susceptible lines of chickens. Antigen specific T cell eliciting responses were observed in chickens that were only challenged, vaccine-challenged and vaccine-boosted but not mock inoculated. Furthermore, our results demonstrate that pp38 peptide pools elicited a more potent T cell recall response, at least 2 folds greater, in the MD-resistant compared to the susceptible line of chickens. The highest frequency of IFN-γ producing T cell to pp38 peptide pools was observed at 2 weeks post inoculation in both the MD-resistant; challenge (mean = 169 SFU/10^6 cells), vaccine-challenge (mean = 556 SFU/10^6 cells), or vaccine-boost (mean = 482 SFU/10^6 cells) and susceptible line of chickens; challenge (mean = 140.1 SFU/10^6 cells), vaccine-challenge (mean = 274.5 SFU/10^6 cells), or vaccine-boost (mean = 214.2 SFU/10^6 cells). No antigen specific T cell recall response to any of the pp38 peptide pools were detected at week 4 post either challenge, vaccine-challenge or vaccine-boost in both the MD-resistant or susceptible lines of chicken. The highest frequency of IFN-γ producing T cell to pp38 peptide pools was observed in both the MD-resistant and susceptible lines of chicken that were vaccine-challenged. However, a lower antigen specific eliciting T cell response to pp38 was observed in the susceptible line of chicken that were vaccine-boosted compared to the MD-resistant line of chicken.

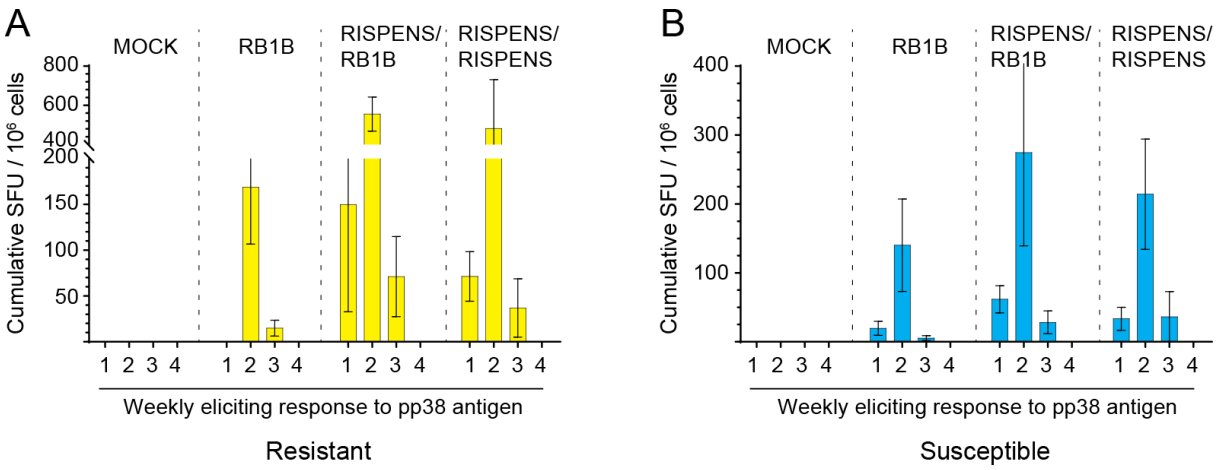

Figure 22: Differential T cell response to pp38 peptide pools.
T cell specific responses were analysed against the MDV pp38 antigen in an *ex vivo* chIFN-γ ELSIPOT assay. The frequency of IFN-γ producing T cells, presented as cumulative spots forming unit (SFU) per 1.0 x10^6 cells, are graphed as weekly responses in both the MD **(A)** resistant (B21) and **(B)** susceptible (B19) line of chickens that were mock, challenged, vaccine-challenged and vaccine-boost. All experiments were performed in triplicates and results are representative of 32 chickens each from both the MD resistant and susceptible lines of chicken.

4.4.3 Immunoreactivity pattern to pp38 peptide pools indicates the presence of an immunodominant epitope

The immunoreactivity pattern eliciting antigen specific responses against MEQ and pp38 peptide pools are here reported at two week post inoculation in both the MD-resistant and susceptible lines of chicken. The frequency of IFN-γ producing antigen specific T cell is presented as a heat map with red indicating highest SFU and white representing lowest SFU (Figure 23). The breadth of response differed among each group and lines of chicken. No detectable responses were observed in mock inoculated chickens (Figure 23Ai and v). Vaccine-challenge elicited the greatest breadth of response against MEQ and pp38 in the MD-resistant lines of chicken only (Figure 23Aiii and vii). In contrast, challenge (Figure 23Aii and vi) or vaccine-boost (Figure 23Aiv and viii) were poor at increasing the breadth of response to MEQ in both the MD-resistant and susceptible lines of chicken based. Comparing between the resistant and susceptible lines of chicken, post challenge, vaccine-challenge and vaccine-boost, a haplotype specific response was observed against pp38. The most potent T cell responses were detected to pool 4 in the MD-resistant and pools 1 and 4 in the MD-susceptible lines of chicken. Based on the immunoreactivity pattern, pp38 was defined

as immunodominant; high immunoprevelance of multiple peptide pools eliciting immunoreactivity in all chickens regardless of the grouping, while MEQ here is defined as subdominant; low immunoprevelance whereby most individuals react to dissimilar peptide pools. Therefore pp38 peptide pool 1 and 4 potentially contain an immunodominant peptide (black arrows) based on the immunoreactivity

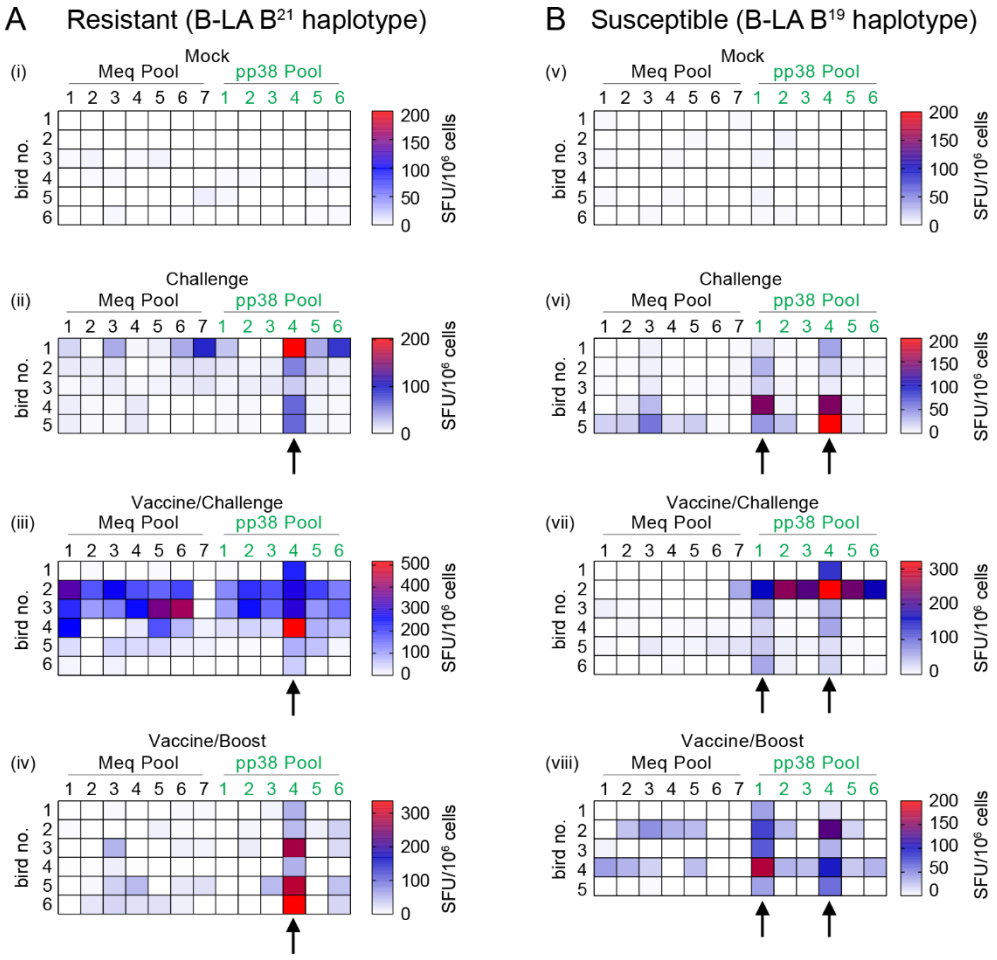

Figure 23: Differential eliciting response to MEQ and pp38 peptide pools.
T cell specific responses were analysed against the MDV MEQ and pp38 peptide pools in an *ex vivo* chIFN-γ ELISPOT assay. The frequency of IFN-γ producing T cells are presented as a heat map from both the MD **(A)** resistant (B21) and **(B)** susceptible (B19) lines of chicken at two weeks post mock (i and v), challenge (ii and vi), vaccine and challenge (iii and vii), and vaccine and boosted (iv and viii) inoculums. Columns represent specific peptide pools generated for both MEQ and pp38. Rows demonstrate the mean magnitude of chIFN-γ (spot forming units; SFU/10^6 cells) production per chickens in response to peptide pool stimulation. Black arrows indicate the peptide pools identified for further

4.4.4 Putative peptide epitope screening of pp38 library confirms the presence of an immunodominant peptide in both MD-resistant and susceptible lines of chicken

To identify the putative epitopes eliciting antigen specific T cell responses, candidate 15 mer single peptides from pp38 peptide pool 1 and 4 were screened against mononuclear cells isolated from both the MD-resistant and susceptible lines of chicken. Our approach to peptide pool deconvolution involved stimulation with smaller pools. Fine mapping of smaller pools, single peptides for discrete analysis and differentiation, was utilized to identify the minimal epitope eliciting IFN-γ production (Figure 24). Representative chIFN-γ ELISPOT assay from the MD-resistant (Figure 24A and B) and susceptible (Figure 24C and D) lines of chicken are shown. Our results demonstrate identification of 3 peptides eliciting antigen specific T cell response. Eliciting responses to the peptide epitopes were only detected in chickens that were either received vaccine strain of MDV or the oncogenic virus, while no response was detected in mock injected chickens from both the MD-resistant and susceptible lines of chicken. Specifically, two immunodominant-peptides were identified in the MD-susceptible and one immunodominant-peptide in the MD-resistant lines of chicken (Figure 24E). Candidate single peptide epitope were clearly differentiated. $pp38_{161-176}$–specific T cell responses (YADLLVEAEQAVVHS) were observed in all the MD-susceptible chickens (n=29), while $pp38_{5-20}$–specific T cell (EHEGLTASWVAPAPQ) was detected in 18 MD-susceptible out of 25 chickens. T cell response to $pp38_{171-186}$ (AVVHSVRALMLAERQ) was detected in all the MD-resistant chickens examined in this study (n=25). Interestingly, none of the immunodominant peptide epitopes, $pp38_{5-20}$, pp38161-176 or $pp38_{171-186}$, induced a cross-reactive eliciting T cell response, demonstrating that T cells are activated in a MHC restricted manner.

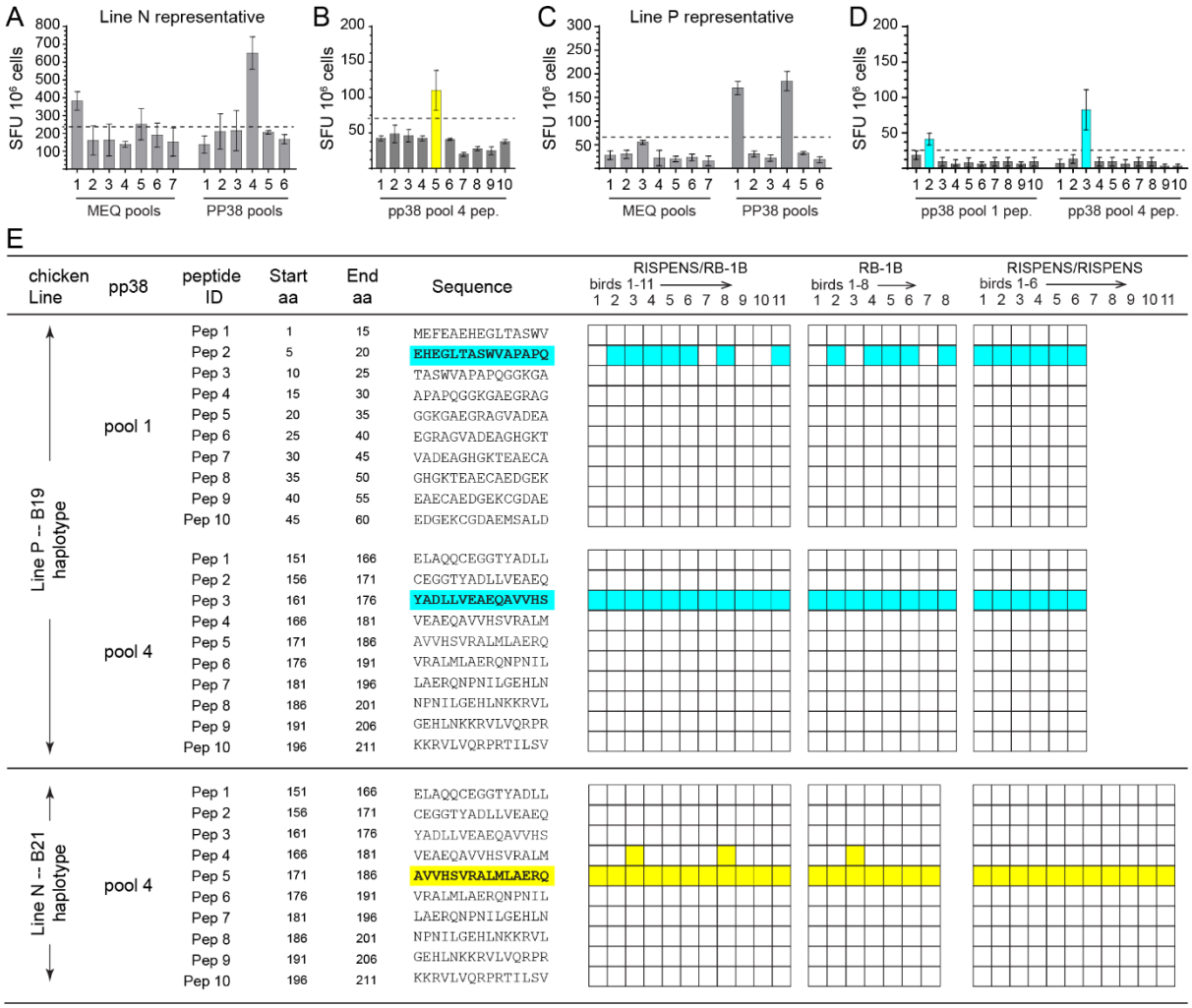

Figure 24: Deconvoluted pp38 peptide pools illustrates identification of three immunodominant peptides.

T cell specific epitope mapping was performed by confirming single peptide able to elicit antigen specific responses as analysed in an *ex vivo* chIFN-γ ELSIPOT assay. Data are presented as spot forming units (SFU/10^6 cells). Representative ELSIPOT assay as performed in the MD **(A and B)** resistant (B21) and **(C and D)** susceptible (B19) lines of chicken are presented. Each candidate peptide pool was deconvoluted with single peptide eliciting response presented from the MD **(B)** resistant (B21) lines of chicken pp38 pool 4 and **(C)** susceptible (B19) lines of chicken pp38 pool 1 and pool 4. Dotted line demonstrates cut-off value based on 2X background to identify specific single peptides. **(E)** All positive responses as single peptide reactivity are demonstrated for each chickens assayed with coloured in boxes from the MD-susceptible lines of chicken highlighted in Blue and resistant line of bird highlighted in Yellow. White boxes indicate negative responses. All experiments were performed in triplicates and results are representative of 30 chickens each from both the resistant and susceptible lines of chicken.

4.4.5 Immunodominant epitopes within pp38 antigen (pp38$_{5-20}$, pp38$_{161-176}$ and pp38$_{171-186}$) are highly conserved within the GaHV-2 strains

Here, we used an approach to determine whether the peptide sequences identified were conserved across the known MDV serotypes (Figure 25), and whether sequences were also present in other avian or non-avian pathogens. Peptide sequences, pp38$_{5-20}$, pp38$_{161-176}$ and pp38$_{171-186}$ were blasted using the NCBI SMARTBLAST tool (2017) and confirmed that these sequences are highly conserved within MDV strains. Alignments were performed using the NCBI multiple alignment tool with default settings. We present our sequence alignment based on GenBank deposited amino acid sequences for pp38 of GaHV-2 (RB1B, MD5, MD11, GA, CVI988/RISPENS), GaHV-3 (SB-1 and HPRS24) and MeHV-1 (HVT/FC-126) major strains. Blue and yellow highlighted regions correspond to the susceptible and resistant single-antigenic peptides identified respectively. Alignment of sequences demonstrate that pp38$_{5-20}$ (EHEGLTASWVAPAPQ) is conserved only within the MDV-1 (MD5; YP_001033989.1, GA; AAF66817.1, MD11; AAS01704.1, RB1B; ABR13155.1 and CVI988/RISPENS; AAB33524.1) strains. Peptide pp38$_{161-176}$ (YADLLVEAEQAVVHS) and pp38$_{171-186}$ (AVVHSVRALMLAERQ) sequences are also highly conserved (15/15 aa) across MDV-1 strains and weakly conserved (6/15 aa) with MDV-2 strains. Both pp38$_{161-176}$ and pp38$_{171-186}$ identified from the susceptible and resistant lines of chicken share a short overlapping segment of 5 amino acids long. Sequence variations were noted in SB-1 (AEI00271.1), HPRS-24 (BAB16570.1) and HVT/FC-126 (NP_073357.1) with HVT/FC-126 containing a truncated pp38 protein (Figure 27). No pp38 peptide sequence (pp38$_{5-20}$, pp38$_{161-176}$ and pp38$_{171-186}$) similarities were observed with HVT/FC-126. All together our results demonstrate the identification of peptide epitope-containing regions shared within the MDV-1 strains.

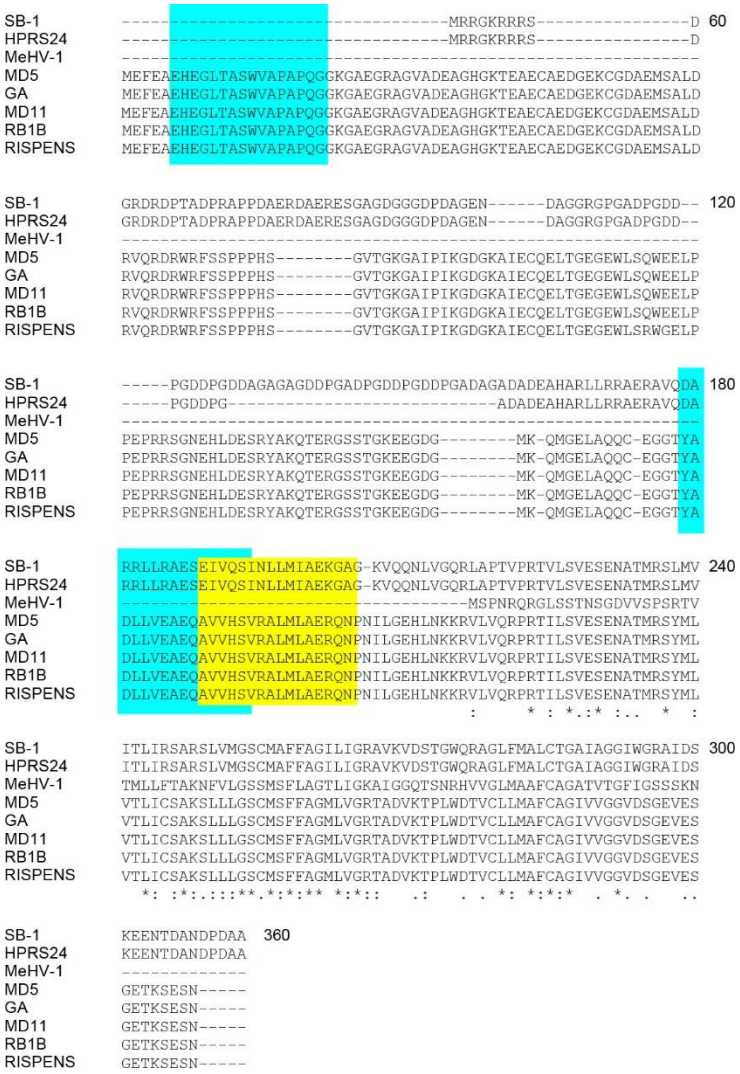

Figure 25: Alignment of MDV pp38 proteins with emphasis on peptide sequences. Specific peptide segments identified were blasted against all known avian viruses as well as MDV to assess whether these peptide sequences were conserved. **(A)** Alignment of pp38 amino acid sequences from all known MDV serotypes (MDV-1; RB1B, CVI988/RISPENS, MD-5, HPRS-24, GA, MDV-2; SB-1 and MDV-3; HVT/MeHV-1). Blue box represent B19 peptide sequence ($pp38_{5\text{-}20}$ and $pp38_{161\text{-}176}$) and Yellow box represent B21 peptide sequence ($pp38_{171\text{-}186}$) previously identified.

4.4.6 pp38 antigen is recognized by $CD4^+$ $TCR\nu\beta_1^+$ T cell subsets

The recognition of peptide specific T cell responses was analyzed using an *ex vivo* depletion assay. Our approach involved depleting mononuclear cells of specific T cell subsets, $CD4^+$, $CD8^+$, $TCR2\nu\beta_1^+$ or $TCR\nu\beta_2$ and stimulating them with the identified $pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ or $pp38_{171\text{-}186}$ epitopes in an *ex vivo* chIFN-γ ELISPOT assay from both MD-resistant (yellow; Figure 26A) and susceptible (blue; Figure 26B) lines of chickens. Our results demonstrate that depletion of $CD4^+$ (Figure 26A and C) or

TCRvβ$_1^+$ T cells (Figure 26B and D) but not CD8+ or TCRvβ$_2$ T cells diminishes the frequency of peptide specific chIFN-γ producing T cells, suggesting that the reacting T cells are CD4$^+$ TCRvβ$_1^+$ T cell subset in both the MD-resistant and susceptible lines of chickens (Figure 26).

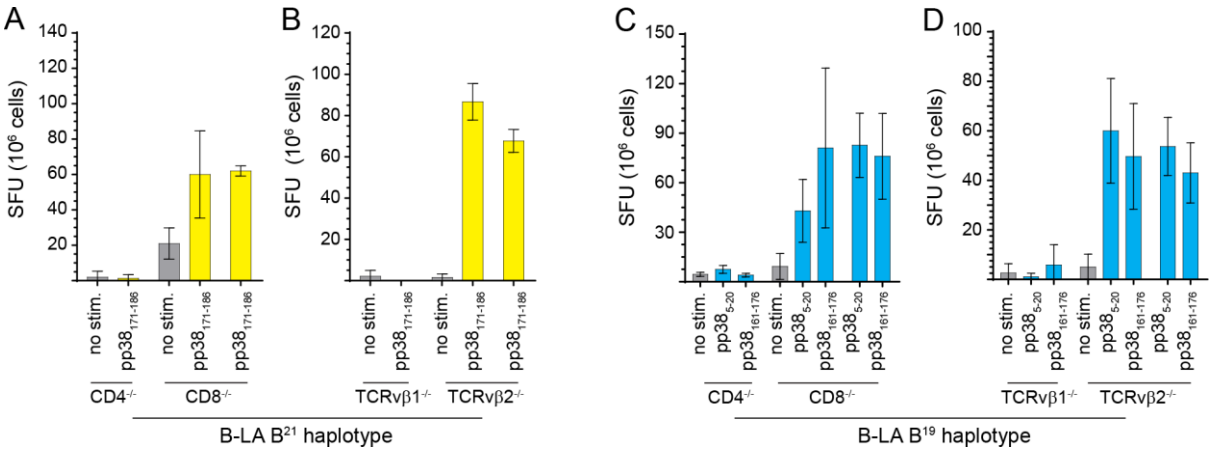

Figure 26: Peptide specific IFN-γ responses to pp38$_{5-20}$, pp38$_{161-176}$ and pp38$_{171-186}$ are restricted to CD4$^+$ TCRvβ$_1^+$ T cell subsets.
Specific T cell subsets were depleted by magnetic beads and stimulated *ex vivo* in a chicken IFN-γ ELISpot assay with the identified pp38 peptides. Depletion assay performed for both **(A and B)** the MD-resistant (B21) and **(C and D)** susceptible (B19) lines of chicken. **(A and C)** CD4$^+$, CD8$^+$, **(B and D)** TCRvβ$_1^+$ or TCRvβ$_2^+$ T cells were depleted. Data here presented as spot forming units (SFU/10^6 cells). All experiments were performed in triplicates and results are representative of 5 chickens each from both the MD-resistant and susceptible lines of chicken performed on different days.

4.4.7 Induction of Th1/Th2 cytokines expression in a peptide-specific manner

The ability of T cell to express peptide specific (pp38$_{5-20}$, pp38$_{161-176}$ and pp38$_{171-186}$) cytokines other than IFN-γ was analyzed using real-time PCR. Transcript levels of interleukin (IL)-2, IL-4 and IL-10 were analyzed over non-stimulated cells in both the MD-resistant (B21) and susceptible (B19) lines of chickens that had been challenged. Our results demonstrate peptide specific induction of IL-2, IL-4 and IL-10 which were demonstrated in five chickens from MD-resistant or susceptible lines (Figure 27). Furthermore, in a peptide specific manner, our results indicate a higher induction of IL-2 (Figure 27A) and IL-4 (Figure 27B) transcript levels in the MD-resistant (yellow; n = 5) compared to the susceptible (blue; n = 5) lines of chicken. Both pp38$_{5-20}$ and pp38$_{161-176}$ peptides were able to induce T cell specific IL-2 and IL-4 transcripts in the MD-

susceptible lines of chicken. However, similar levels of peptide-specific IL-10 transcripts (Figure 27C) were observed between the MD-resistant and susceptible lines of chicken. Taken together our results indicate that the $pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ and $pp38_{171\text{-}186}$ epitopes are able to elicit, other than IFN-γ, T cell specific cytokines such as IL-2, IL-4 and IL-10.

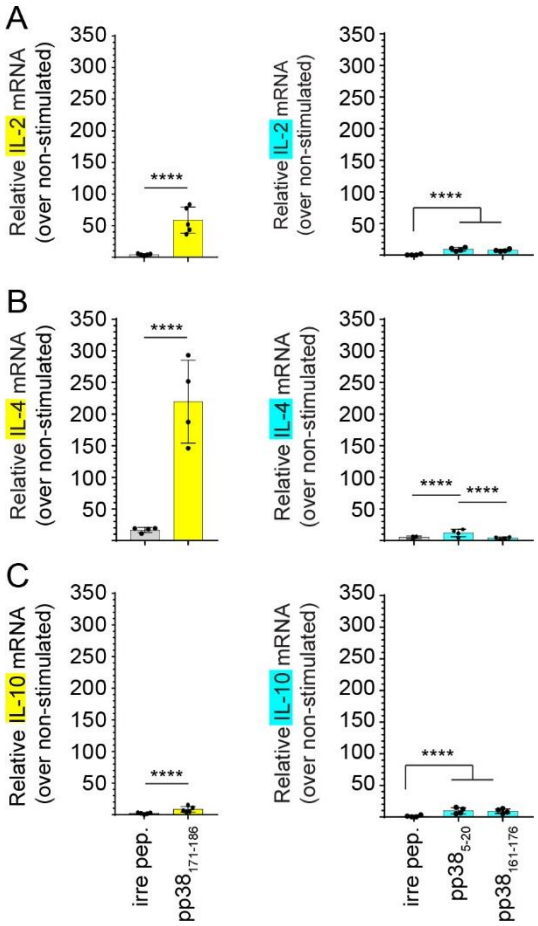

Figure 27: T cell specific cytokine mRNA expression in response to $pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ and $pp38_{171\text{-}186}$ stimulation.
Real-Time PCR was used to analysed fold change gene expression of mononuclear stimulated *ex vivo* with $pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ and $pp38_{171\text{-}186}$, peptide solvent or positive control from the MD-resistant (Yellow) and susceptible (Blue) lines of chicken for **(A)** Interleukin (IL)-2 and **(B)** IL-4 and **(C)** IL-10 and normalized to β-actin. Data is representative the mean of experiment triplicates performed from 5 individual chickens on different days. * and ** Indicates a statistically significant difference compared to control ($p < 0.05$, 0.001). Irre pep. indicates irrelevant peptide (influenza HA peptide: $H5_{246–260}$), and NS indicates no significant difference.

4.4.8 Peptide specific induction of $CD4^+$ T cells proliferation

The ability of peptide specific ($pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ and $pp38_{171\text{-}186}$) T cells to recognize the relevant peptide and proliferate in vitro was analysed using an *in vitro* CFSE based proliferation assay. The percentage of $CD4^+$ or $CD8^+$ T cells recognizing the identified peptide epitope and proliferate *in vitro* and the representative dot plot are outlined to demonstrate gating strategy (Figure 28A and C) from both the MD-resistant and susceptible lines of chickens. Concanavalin A (Con A) was utilized as positive control, medium alone was used as negative control (Cont.) and irrelevant peptide (influenza HA peptide: $H5_{246–260}$) was utilized to demonstrate T cell specific proliferation (Figure 28). Our results demonstrate that T cells from both the MD-resistant (Figure 28B) and susceptible (Figure 28D) lines of chickens proliferate in a peptide specific manner. Stimulation with the peptide epitope elicited $CD4^+$ T cells proliferation in a peptide specific manner ($P = 0.0095$), and no $CD8^+$ T cell proliferation was detected. Similarly, in the MD-susceptible lines of chicken, a significantly higher frequency of $CD4^+$ ($P = 0.0095$) and not $CD8^+$ T proliferated in a peptide specific manner. No T cell proliferation was detected in the presence of medium alone.

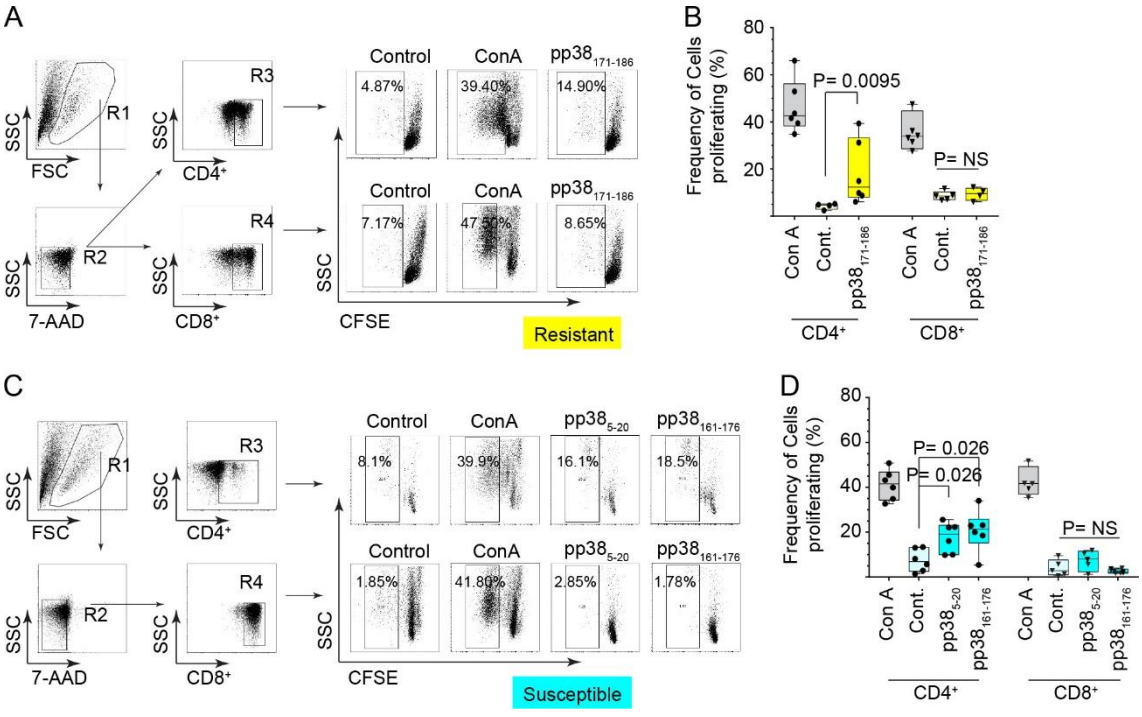

Figure 28: $CD4^+$ T cell subsets from MD resistant and susceptible lines of chickens recognize pp38 peptides and proliferate *in vitro*.
Splenocytes isolated from MDV-infected chickens were cultured in the presence of putative peptides $pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$, and $pp38_{171\text{-}186}$, Concanavalin A (Con A), or cell culture media (Cont.) and T cell proliferation was analysed using CFSE based proliferation assay. Representative dot plot and gating strategy for both **(A)** the MD-

resistant **(C)** and susceptible chicken lines are shown. The frequency of proliferating $CD4^+$ or $CD8^+$ cells are demonstrated for both **(B)** the MD-resistant and **(D)** susceptible lines of chickens. Data is representative of the mean of experiments performed from 5 individual chickens in triplicates. * and ** Indicates a statistically significant difference compared to control ($P < 0.05, 0.001$). NS indicates no significant difference.

4.4.9 MDV infection significantly impairs $CD4^+$ and $CD8^+$ T cell degranulation in the MD-resistant (B21) chicken line

Cytotoxic function are essential for antiviral activity mediated by the degranulation pathway identified by CD107a cell surface translocation. Splenocytes isolated from the MD-resistant chickens were stimulated with the identified B21 restricted immunodominant $CD4^+$ T cell epitope ($pp38_{171\text{-}186}$) or PMA/ION to investigate antigen specific and antigen non-specific T cell surface expression of CD107a, respectively (Figure 29A). Splenocytes cultured in cell culture medium alone or an irrelevant peptide (influenza HA peptide: $H5_{246–260}$) were used to determine the baseline expression of CD107a expression in $CD4^+$ and $CD8^+$ T cells using flow cytometry (Figure 29). As $pp38_{171\text{-}186}$ is identified as a B21 restricted $CD4^+$ T cell epitope, upregulation of $CD107a^+$ on $CD4^+$ T cells in the MDV infected chickens are considered as antigen specific T cell degranulation. No significant increase in MDV-derived peptide-induced cell surface translocation of CD107a was detected in either $CD4^+$ (Figure 29B) or $CD8^+$ T cells (Figure 29C) at 1-4 weeks post infection. Our results demonstrate that the percentage of $CD4^+CD1017a^+$ and $CD8^+CD107a^+$ in response to PMA/ION stimulation were significantly diminished in the MDV-infected chickens at 1-4 weeks post infection. Within 4 weeks post infection, the highest frequency of $CD4^+CD107a^+$ T cells in response to PMA/ION was observed at 2 weeks post infection (mean = 8.75%) with the lowest being detected at the first week post infection (average of 1.9%). In contrast, the highest percentage of $CD8^+CD107a^+$ T cells in response to PMA/ION was detected at 2 (mean = 6.25%) and at 4 (mean = 8.04 %) weeks post infection with the lowest percentage being detected at first week post infection (average of 0.16%). Taken together, our results suggest that infection with MDV impairs T cell degranulation.

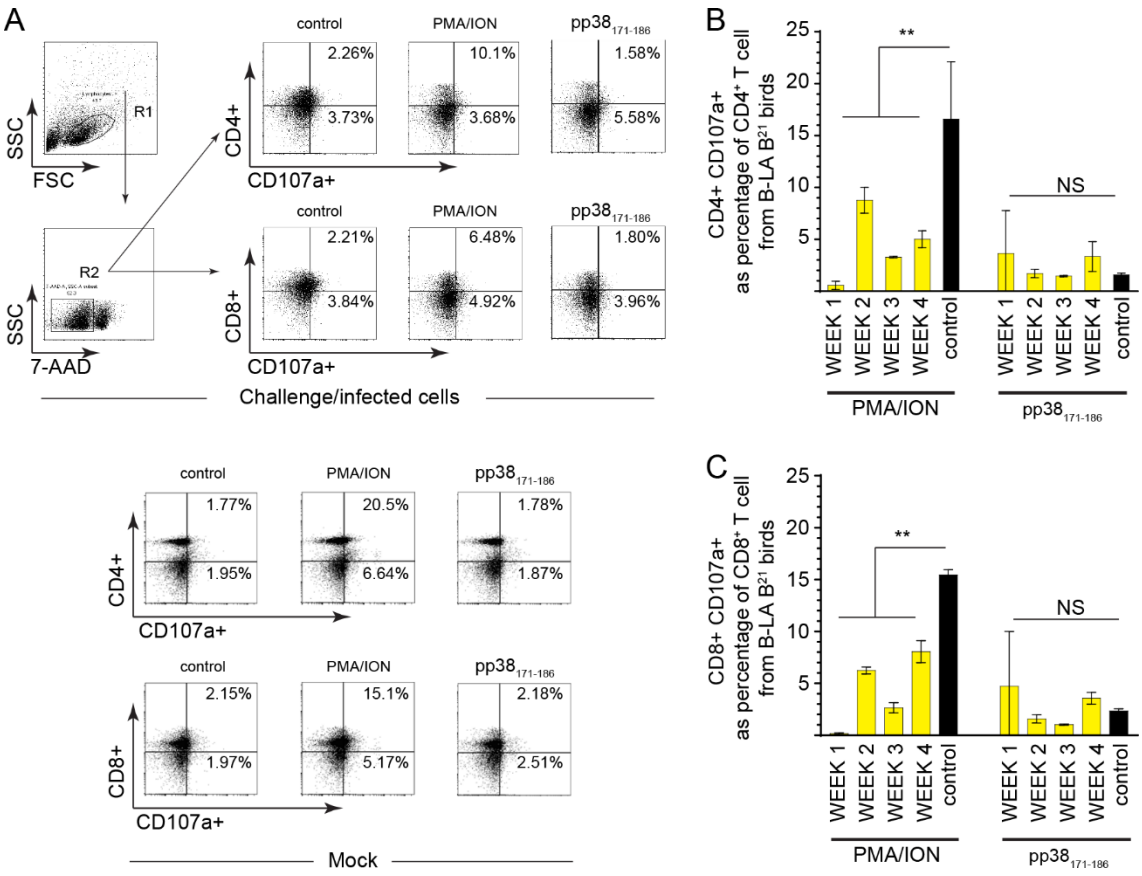

Figure 29: Impaired CD4$^+$CD107a$^+$ and CD8$^+$CD107a$^+$ T cell degranulation in the MD-resistant line of birds

Spleen mononuclear cells were isolated from B21 chickens at 2 weeks post challenge. Cells were subsequently incubated for 4 hrs in the presence of the putative immunodominant peptide (pp38$_{171-186}$), PMA (50 ng/ml) plus Ionomycin (ION; 1 µg/ml) and/or cell culture medium only. The frequencies of CD4$^+$CD107a$^+$ and CD8$^+$CD107a$^+$ T cell population were analysed using flow cytometry. **(A)** Representative dot plot and gating strategy of CD4$^+$CD107a$^+$ and CD8$^+$CD107a$^+$ are demonstrated. The frequencies of **(B)** CD4$^+$CD107a$^+$ and **(C)** CD8$^+$CD107a$^+$ are presented as a percentage of CD4$^+$ T cells. The data are representative as the mean percentage from 5 individual chickens. ****** Indicates a statistically significant difference compared to control ($p < 0.05$, 0.001). NS indicates no significant difference.

4.4.10 MDV infection impairs T cell degranulation in the MD-susceptible (B19) line of bird in antigen-specific as well antigen-non-specific manner

To further address the question of cell surface translocation of CD107a, we analyzed CD4$^+$ and CD8$^+$ T cell degranulation in MDV-infected MD-susceptible lines of chicken. Splenocytes isolated from MDV-infected and mock-infected chickens were stimulated with the identified immunodominant epitopes (pp38$_{5-20}$ and pp38$_{161-176}$) or PMA/ION to investigate the ability of T cell degranulation using CD107a detection as our readout in

antigen-specific and antigen-non-specific manner, respectively. Representative dot plot are outlined to demonstrate gating strategy (Figure 30A). Splenocytes cultured in cell culture medium alone or an irrelevant peptide (influenza HA peptide: $H5_{246–260}$) were used to determine the baseline expression of CD107a expression in $CD4^+$ and $CD8^+$ T cells using flow cytometry (Figure 30). As $pp38_{15\text{-}20}$ and $pp38_{161\text{-}176}$ is identified as a B19 restricted $CD4^+$ T cell epitope, upregulation of $CD107a^+$ on $CD4^+$ T cells in the MDV infected birds were considered as antigen specific T cell degranulation. Our results demonstrate no significant increase in peptide-specific induction of cell surface translocation of CD107a in either $CD4^+$ (Figure 30B) or $CD8^+$ T cells (Figure 30C) at 1-4 weeks post infection. Non-antigen-specific activation of T cell cells with PMA/ION demonstrate that T cells isolated from MDV-infected chickens have lower expression of CD107a compared to that observed in mock-infected chickens. Surprisingly, cell surface translocation of CD107a in $CD8^+$ T cell was completely abrogated at week 3 (mean = 0.78%) and 4 (mean 0.77%) post challenge (Figure 30C) while $CD4^+$ T cell still retained partial ability to express CD107a (Figure 30B). Cell surface translocation of CD107a in $CD8^+$ T cell was diminished but detectable at week 1 (mean = 2.53%) and 2 (mean = 6.33%) post infection albeit still significantly lower compared to that observed in the mock-infected chickens. Taken together, our results demonstrate that the overall reduction in T cell degranulation are severely impaired and this failure is more pronounced in the MD-susceptible chickens compared to that observed in the MD-resistant lines of chicken.

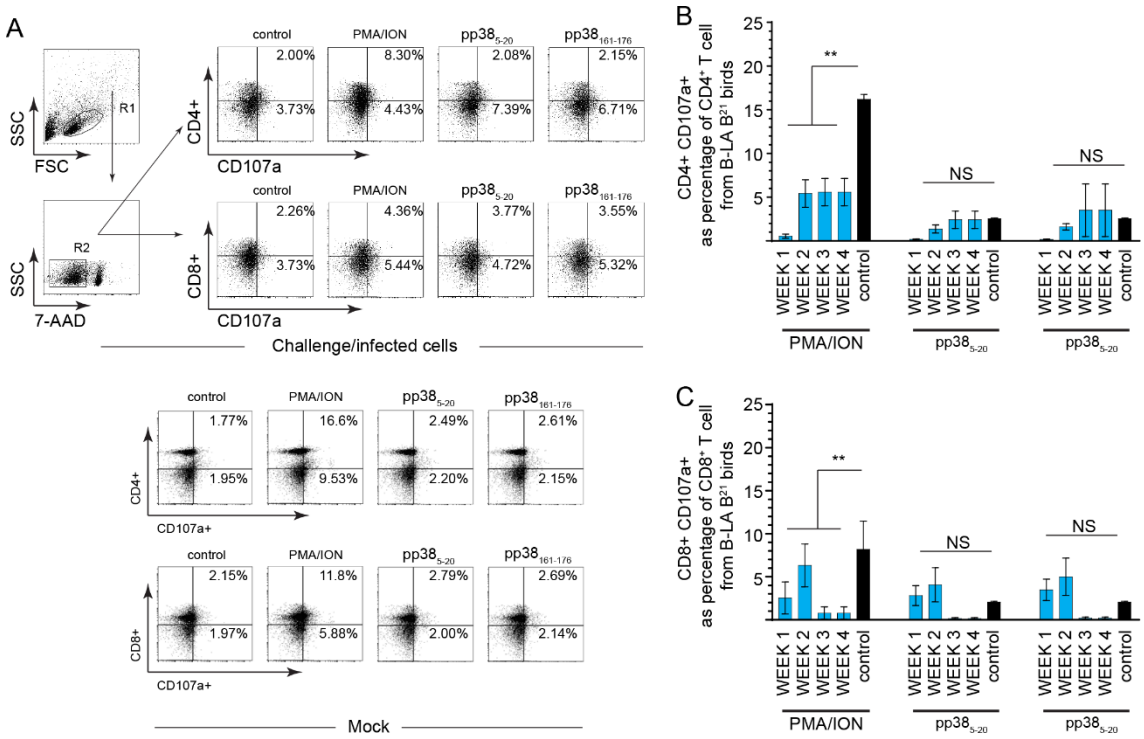

Figure 30: MDV infection impairs T cell degranulation in the MD-susceptible line of birds

Spleen mononuclear cells were isolated from B19 birds post challenge and were subsequently incubated for 4 hrs in the presence of the putative immunodominant peptide ($pp38_{171\text{-}186}$), PMA (50 ng/ml) plus Ionomycin (ION; 1 μg/ml) or cell culture medium only. The frequencies of $CD4^+CD107a^+$ and $CD8^+CD107a^+$ T cell population were analysed using flow cytometry. **(A)** Representative dot plot and gating strategy are demonstrated. The frequencies of **(B)** $CD4^+CD107a^+$ and **(C)** $CD8^+CD107a^+$ are presented as a percentage of $CD4^+$ T cells. The data are representative as the mean percentage from 5 individual chickens. ****** Indicates a statistically significant difference compared to control ($P < 0.05, 0.001$). NS indicates no significant difference.

4.4.11 Vaccination is ineffective at inhibiting MDV-induced T cell degranulation in both MD-resistant and susceptible lines of birds

We have shown that MDV infection impairs T cell degranulation in naïve chickens within both the MD-resistant and susceptible lines of chicken. To examine the effects of MDV infection on T cell degranulation in vaccinated lines of chicken, vaccinated MD-resistant and susceptible l lines of chicken were subsequently challenged two weeks after vaccination. Splenocytes were stimulated with B19-restrcited immunodominant epitopes and surface CD107a expression was analysed using flow cytometry. To examine antigen non-specific T cell degranulation, CD107a expression in response to PMA/ION was also analysed at week 2 post challenge in the MD resistant (B21) lines of chicken. Representative dot plot are outlined to demonstrate gating strategy (Figure

31A) in both the MD-resistant and susceptible lines of chicken. Medium alone and an irrelevant peptide was used for controls (Figure 31). The results demonstrate that vaccination prior to challenge had no effect in restoring the ability of either $CD4^+$ or $CD8^+$ T cell degranulation in both the MD-resistant (Figure 31A and C) and susceptible (Figure 31B and D) lines of chicken. Again, in the vaccine-challenge group, no peptide specific induction of degranulation was observed.

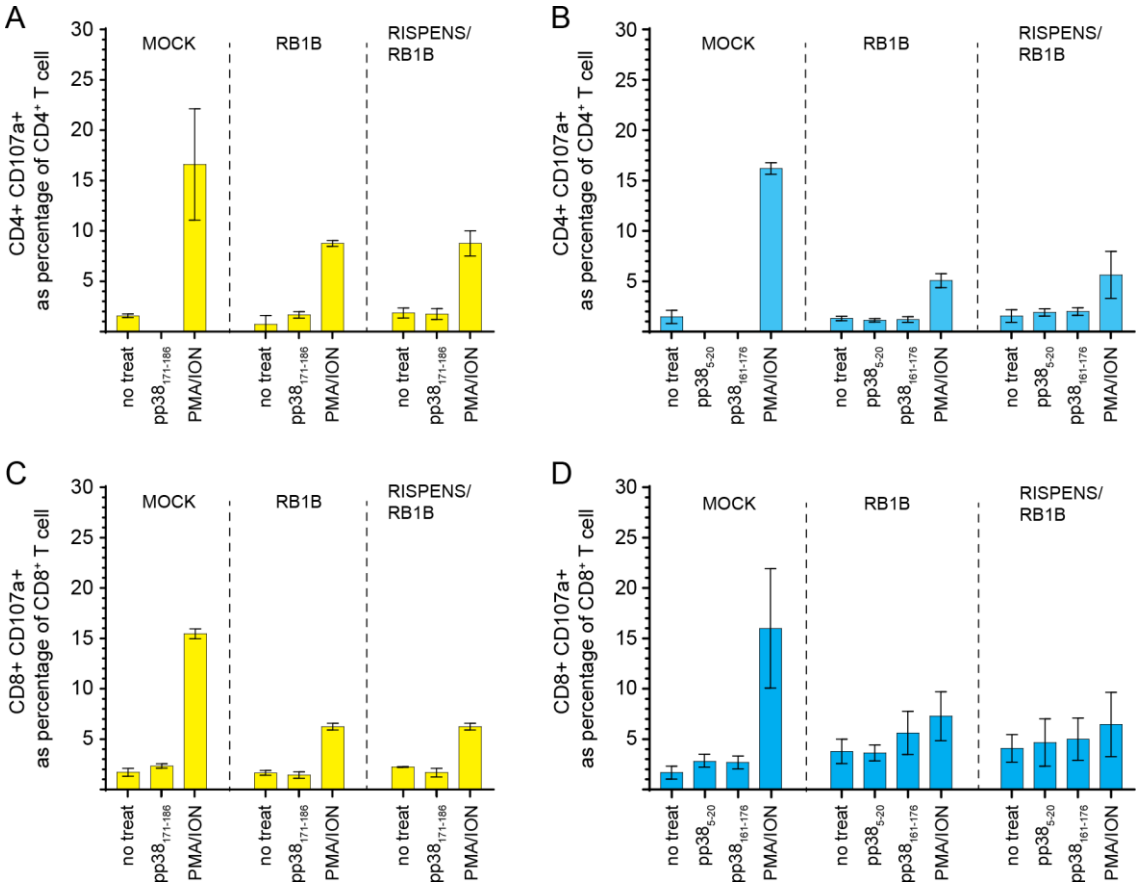

Figure 31: MDV infection also impairs T cell degranulation in vaccinated chickens Spleen mononuclear cells were isolated from both MD-resistant and susceptible lines of chicken 2 weeks after mock-infection, MDV-infection or vaccine-challenge. Cells were subsequently incubated in the presence of the putative immunodominant peptides ($pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$, and $pp38_{171\text{-}186}$) or PMA (50 ng/ml) plus Ionomycin (ION; 1 μg/ml) for 4 hrs and expression of surface $CD107a^+$ was determined using flow cytometry. The frequency of **(A and B)** $CD4^+CD107a^+$ and **(C and D)** $CD8^+CD107a^+$ are presented as a percentage of $CD4^+$ or $CD8^+$ T cells for both the MD-resistant **(A and C)** and MD susceptible **(B and D)** lines of chicken. The data are representative as the mean percentage from 5 individual chickens.

4.4.12 Antigen specific Granzyme B and Perforin expression in MDV infected birds

Cytotoxic abilities of $CD8^+$ and $CD4^+$ T cells utilize the perforin/granzyme cytotoxic pathway to kill virally-infected cells and tumours. We had observed that antigen-specific and antigen non-specific T cell degranulation is impaired in MDV infected chickens. Here, we examined whether T cells from MDV infected birds are able to upregulate Granzyme B and Perforin expressions in both antigen specific and antigen non-specific manner. Splenocytes isolated from MDV infected chickens were stimulated with $pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ or $pp38_{171\text{-}186}$, and the expression of perforin and Granzyme B were analysed using real-time PCR. Transcript levels of perforin and Granzyme B were also analyzed following stimulation of the cells with PMA/ION in an antigen non-specific manner in both the MD-resistant and susceptible lines of chicken 2 weeks post MDV infection. The results demonstrate a peptide specific induction of perforin and Granzyme B in both the MD-resistant and susceptible lines of chicken (Figure 32). In contrast, stimulation of the cells with an irrelevant peptide did not induce upregulation of perforin (Figure 32B and D) and Granzyme B (Figure 32A and C) expressions in both the MD-resistant and susceptible lines of chicken. Furthermore, our results demonstrate a higher induction of perforin (Figure 32B and D) and Granzyme B (Figure 32A and C) transcript levels in the MD-resistant (yellow; n = 5) compared to the MD-susceptible (blue; n = 5) lines of chicken. Taken together our results indicate that the $pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ and $pp38_{171\text{-}186}$ epitopes are able to elicit upregulation of perforin and Granzyme B transcripts.

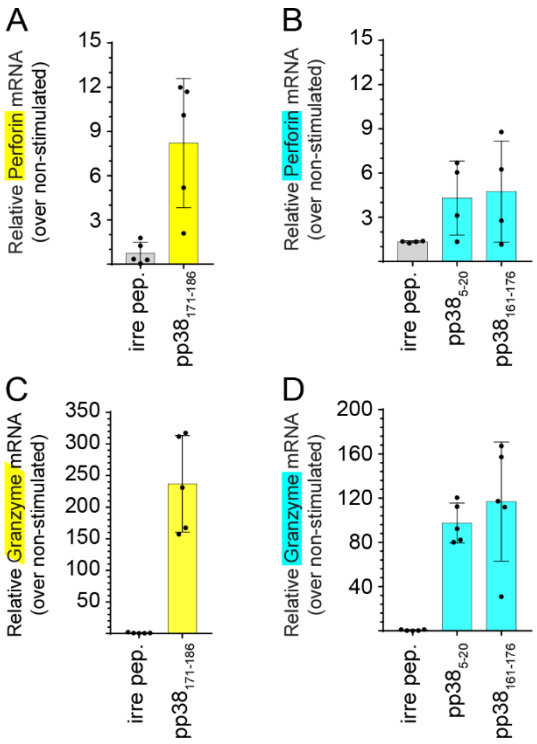

Figure 32: Upregulation of Perforin and Granzyme B in an antigen specific manner

Real-Time PCR was used to analyzed fold change gene expression of Perforin and Granzyme B in mononuclear cells stimulated with $pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ or $pp38_{171\text{-}186}$ peptides. Fold change expression of **(A)** perforin and **(B)** Granzyme B in splenocytes isolated from the MD-resistant (Yellow) and susceptible (Blue) lines of chicken are shown. Data is representative the mean of experiment triplicates performed from 5 individual chickens on different days. * and ** Indicates a statistically significant difference compared to control ($P < 0.05$, 0.001). Irre pep. indicates irrelevant peptide (influenza HA peptide: $H5_{246–260}$) and NS indicates no significant difference.

4.4.13 Induction of memory pp38 specific T cells in MDV infected chickens

Short-term T cell lines were expanded *in vitro* from spleen mononuclear cells isolated from the MD-susceptible line recognizing the immunodominant $pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ and from the MD-resistant line recognizing $pp38_{171\text{-}186}$ peptides (Figure 33A). After 8 days, the frequencies of peptide specific chIFN-γ producing effector T cell were analysed in both the MD-resistant and susceptible lines of chicken (Figure 33B and C). Here we outline for the first time that MDV-specific effector T cells are detected using *ex vivo* ELISPOT assay, While chicken antigen-specific memory T cells can be detected using cultured ELISpot assay for chicken IFN-γ (Figure 21 and 23). Short term T cell lines stimulated with the MDV-derived peptide epitope elicited antigen specific T cell recall response in all 6 chickens tested from both the MD-resistant and susceptible lines

of chicken. Antigen specific memory T cell responses were only detectable at week 4 post either challenge, vaccine-challenge or vaccine-boost in both the MD-resistant (Figure 33B) and susceptible (Figure 33C) lines of chicken. Furthermore, no detectable antigen specific memory T cell responses were observed within 1, 2 or 3 weeks post infection *in* both the MD-resistant (Figure 33B) and susceptible lines of chicken (Figure 33C), suggesting that memory T cells are generated 4 weeks post vaccination or infection. No response was detected in the cells, isolated from vaccinated or infected chickens, cultured with an irrelevant peptide. No antigen specific T cell response was detected in the mock infected control chickens. Antigen specific T cell responses were detected in both the MD-resistant and susceptible lines of chicken that were either vaccine-challenged or vaccine boosted. Interestingly, a lower frequency of antigen specific memory T cell responses was detected in the MDV-infected MD-susceptible lines of chicken ($pp38_{5\text{-}20}$; mean = 56.3 SFU/10^6 and $pp38_{161\text{-}176}$; mean = 50.5 SFU/10^6 cells) compared to that in the MD resistant lines of chicken ($pp38_{5\text{-}20}$; mean = 202.9 SFU/10^6).

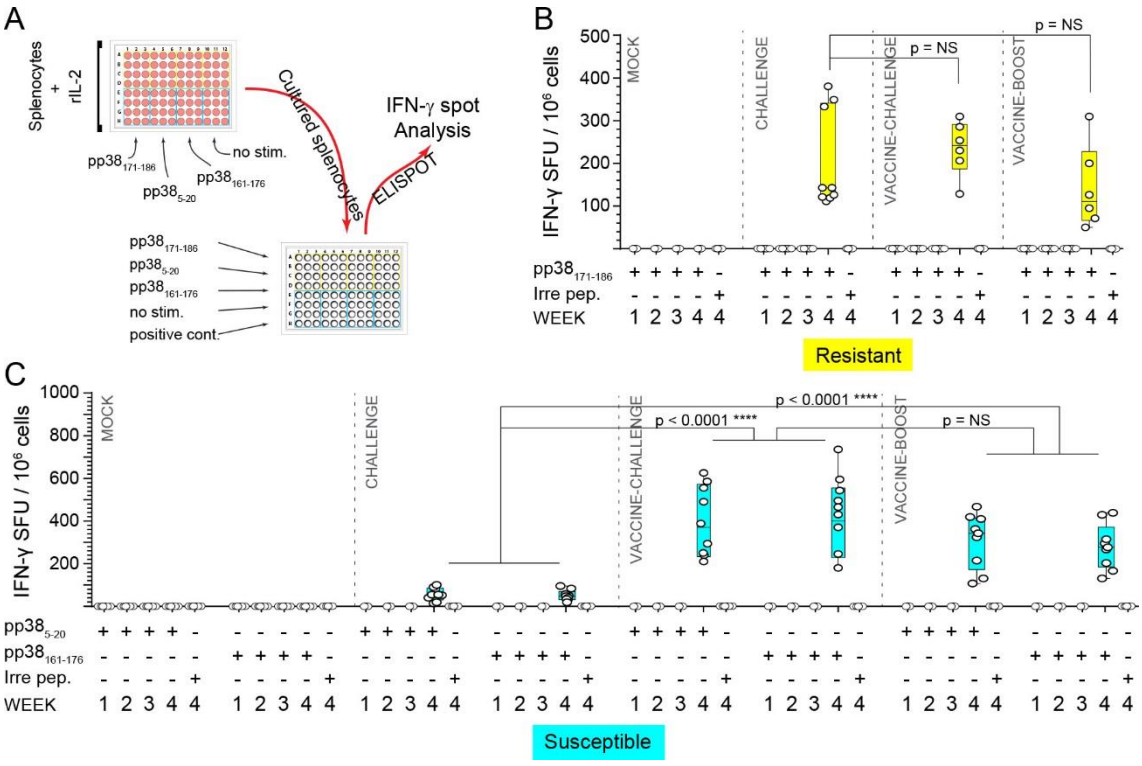

Figure 33: MDV infection or vaccination induces memory MDV-specific T cell responses

Short term peptide specific T cell lines were generated *in vitro* from MDV infected or vaccinated MD-resistant (B21) and susceptible (B19) lines of chickens to detect antigen specific memory T cell responses. The cells were expanded from the birds isolated from mock infected, challenged, vaccine-challenged or vaccine boosted groups, and the presence of antigen-specific IFN-γ producing T cells were analysed using a cultured ELISPOT assay as **(A)** outlined in the schematic diagram. The frequency of antigen specific IFN-γ producing T cells (spot forming unit; SFU / 10^6 cells) are presented for 1-4 weeks post infection/vaccination for both **(B)** the MD resistant (Yellow) and **(C)** susceptible (Blue) lines of chicken. All experiments were performed in triplicates from 6 different chickens. ******** Indicates a statistically significant difference compared to control ($P < 0.001$). Irre pep. indicates irrelevant peptide (influenza HA peptide: $H5_{246–260}$) and NS indicates no significant difference.

4.5 Discussion

Marek's disease virus (MDV), an *alphaherpesvirus*, is the etiologic agent for Marek's disease (MD) and causes a lymphoproliferative disease in chickens. Immunity elicited by vaccination inhibits formation of deadly lymphoma and thus prolongs host survival without inhibiting persistent viral infection, viral replication or transmission. The question of how vaccines provide protection against tumour formation while failing to prevent infection has not been fully addressed, and there are contrasting reports on the role of T cells in the control of tumour growth and infection. One of the first steps of analysing the role of T cells in vaccine-induced immunity is to identify and characterize MDV-specific T cell responses in infected and vaccinated chickens. The present study is the first report which present data on identification and characterisation of MDV-derived T cell epitopes which are restricted to the MD-susceptible (B19) and resistant (B21). In this study, we utilized an epitope mapping approach and synthesized a library of overlapping peptides spanning the length of the MDV MEQ and pp38, and examined anti-MDV T cell responses using various immunological techniques including *ex vivo* and cultured ELISpot assays for detection of effector and memory T cell responses. For this study, we selected two immunogenic MDV antigens that are expressed at different phases of virus replication, the early protein pp38 and the major oncogene MEQ expressed during lytic replication and transformation. Selection of MEQ and pp38 was based on previous work demonstrating that antigen specific responses could be detected in vaccinated chickens against pp38 and MEQ (25) in an *in vitro* chromium release assay. However, no T cell epitope was identified and the quality and quantity of T cell responses to these MDV antigens were not studied. In this study, we identified

immunodominant T cell epitopes within pp38 antigen restricted to B19 ($pp38_{5\text{-}20}$ and $pp38_{161\text{-}176}$) or B21 ($pp38_{171\text{-}186}$) haplotype in chickens that were either challenged, vaccine-challenged or vaccine-boosted. The $pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ and $pp38_{171\text{-}186}$ peptides were found to be fully conserved in GaHV-2 strains of virus regardless of their origin. These immunodominant peptides were not found to be cross-reactive between B19 and B21. Moreover, we identified $CD4^+$ $TCR\nu\beta_1^+$ T cells as the reacting T cells to these immunodominant epitopes, in both the MD-susceptible (B19 haplotype) and resistant (B21 haplotype) chickens. Further characterising T lymphocytes confirm that the identified immunodominant peptides elicit IL-2, IL-4, IL-10, gene expression in an antigen specific manner. For the first time, in this study we report a method for identification and characterization of memory T cell responses in chickens, and demonstrate that memory T cell responses are detectable only after 4 weeks post infection or vaccination against MDV.

More importantly, our results demonstrate that MDV infection impaired T cell degranulation in both antigen specific and antigen non-specific manner. The induction of multi-functional virus-specific effector T cells, which have cytotoxic ability and produce cytokines, are believed to be critical for immune defence against viral infections and current opinion is that this should be considered for vaccine development. T lymphocyte cytotoxicity can occur through direct secretion of lytic granules containing perforin and granzymes into immunological synapses and this process is called "degranulation. Virus-specific $CD4^+$ and $CD8^+$ cytotoxicity T cells plays an important role in deciding the outcome of viral infections. Our results demonstrate a cytotoxic dysfunction resulting in a decrease frequency of both $CD4^+$ and $CD8^+$ T lymphocytes that degranulate ($CD107a^+$) either in response to pp38 antigen or PMA/ION stimulation. Antigen-non-specific stimulation of T cells from both naïve MD-resistant and susceptible chickens demonstrated that there is no significant intrinsic ability of T cell degranulation between chicken lines. However, T cell degranulation in MD-susceptible line of chicken was significantly lower than MD-resistant chickens after MDV infection. This may explain why higher virus titres are detected in MD-susceptible chickens than that in the resistant chickens. Taken together the evidence presented here indicate that a major determinant of pathogenicity in B19 birds could be a lack of T cell degranulation within cytotoxic $CD4^+$ and $CD8^+$ T cells. The differential

T cell responsiveness to pp38 peptide epitope in B19 and B21 could provide an indication for MDV pathogenesis.

Beyond just cytokine secretion and proliferation, a new expanding role of cytotoxic activity (CD107a$^+$, perforin$^+$ and granzyme A$^+$) have been identified for antigen experienced effector CD4$^+$ T cell (266). The ability to kill virus infected cells is a key feature to prevent dissemination and provide sterile immunity. However chronic infection is usually associated with a failure to either recognize, control or kill infected cells. Cytotoxic CD4$^+$ T cells responding to WNV infection are essential for resolving infection and increased survival rate in mice (279). The antiviral response is mediated by secretion of granzyme B and IFN-γ production. Furthermore, CD4$^+$ T cells secreting perforin protect against lethal influenza infection in an antibody independent manner (280). In macaques, antiretroviral therapy have been shown to elicit cytotoxic CD8$^+$ T cells (281) which when depleted resulted in decrease prognostic, and increased viral load. Our results demonstrate that splenocytes isolated from MD-susceptible and resistant lines of chicken can recognize MDV antigen, and unlike T cell degranulation, are able to express both perforin and granzyme B. Based on our results, we concluded that dysfunction of T cell degranulation is not associated with their failure to express perforin and granzyme B. Our group has previously shown in a peptide specific manner induction of perforin and granzyme B transcripts in CD8$^+$ T cells post vaccination with TROVAC^{TM}-AIV H5 vaccine in B19 haplotype birds (277). In MDV infection of B19 haplotype, infiltrating CD8$^+$ T cells have been identified in tumor environment (37). However the progression and eventual deadly lymphoma in B19 haplotype birds suggest a failure of cytotoxic activity. Here the results demonstrate that MDV impairs both CD4$^+$ and CD8$^+$ T cell cytotoxic function as indicated by a reduction in the frequency of CD107a$^+$ T cells post PMA/ION stimulation. Peptide specific degranulation was further impaired in CD4$^+$ and CD8$^+$ T lymphocytes in birds either challenged, vaccine-challenged or vaccine-boosted. Taken together the evidence presented here indicate that vaccination is not sufficient to restore either CD4$^+$ or CD8$^+$ T cell cytotoxic activity in the face of challenge.

Following a rapid proliferative antiviral response, the majority of effector CD4$^+$ T cells contract due to exhaustion (282, 283), leaving a much smaller population of antigen specific memory CD4$^+$ T cells that persists. Memory T lymphocytes have unique

functional attributes which allow for a rapid and effective anti-viral response which is an important part of a long-lived protective immune response following both vaccination and infection (284). The mechanism of virus mediated clearance hasn't been filly elucidate but rapid expansion of effector $CD4^+$ T cell mediated antiviral activity against corona virus induced encephalomyelitis is critical for mice survival (285). In addition, lung resident memory $CD4^+$ T cell against the Influenza HA in a mice infection model has been shown to elicit protective and antiviral responses independent of $CD8^+$ T cell. Therefore, directed protective immunity through elimination of bystander contributing factors can be generated depending on the balance between T_{H1}/T_{H2} responses even in the absence of B cells (286). However in EBV infection, dendritic cells pulsed with viral antigen were shown to elicit a $CD8^+$ T lymphocytes responsiveness (271). Antigen specific long lived memory $CD8^+$ T cells have been identified against CCHFV (272). Regardless of the antigen, the mechanism of presentation will dictate activation of specific immune cell subsets. In HSV-2 infection, re-activation of antigen specific memory $CD4^+$ T cells mediate NK cell activation for enhance antiviral function (287). Our results demonstrate that *in vivo* generated memory T cells can be cultured *in vitro* and differentiated into effector T cell in both MD-susceptible (B19) and resistant lines of chickens (B21) in a peptide specific manner based on recall response in an IFN-γ ELISPOT assay. Moreover, we could identify a higher frequency of peptide specific effector T cell derived from memory T cells in vaccinated but not MDV infected MD-susceptible chickens. Higher frequencies of MDV-specific memory T cells were generated in MD-resistant chickens that were infected with MDV or vaccinated against MDV. A better understanding of memory T cells functions will allow us to evaluate their potential contribution to immunity when they are induced by either infection or vaccination and also following re-infection or viral re-activation.

To develop into effector populations that combat viral infections, naive $CD4^+$ T cells need to recognize peptide antigens presented by MHC class II molecules on activated APCs. In humans, Dengue virus infection leads to expansion of antigen specific effector $CD4^+$ T cells which is associate with protective immunity (288). It has been previously shown that effector $CD4^+$ T cells play a critical role in the clearance of HSV-1 infection of sensory ganglia and spinal cords (289). Furthermore this helper response can play a major role in long lived immunity against subsequent viral reactivation. Similarly,

varicella zoster (269) and West Nile virus infection (279) elicits a broad yet effective primary $CD4^+$ T cell response sufficient to elicit antiviral protection. Activation of effector $CD4^+$ T cells results in IL-2, IL-4 and IFN-γ production which support lymphocyte activation. The frequency of antigen experience effector $CD4^+$ T cells is maximum at 2 weeks post infection and secret IL-2 and IFN-γ in an antigen dependent manner. During Respiratory syncytial virus infection, tissue resident effector $CD4^+$ T cell in the lung play a critical role in protection against infection (290). Furthermore, enrichment of primed effector $CD4^+$ T cells during HSV-1 infection leads to improved prognostic in mice as well as effective viral clearance from sensory ganglia and spinal cords supported by IL-2 production (289). Expression of IFN-γ is not always required for $CD4^+$ T cell IL-4 and IL-10 cytokine production (280). Our group has shown that peptide specific induction of cellular proliferation, and IFN-γ production can be detect in chickens vaccinated against influenza (277). This is due to the promiscuity of antigen presentation and/or T cell recognition of varying length peptides. In accordance, the *ex vivo* depletion studies confirmed that $pp38_{5\text{-}20}$, $pp38_{161\text{-}176}$ and $pp38_{171\text{-}186}$ peptide specific responses observed following MDV infection were a results of eliciting responses from $CD4^+$ and $TCRv\beta_1^+$ but not $CD8^+$ and $TCRv\beta_2^+$ cells. Furthermore, expansions of peptide specific $CD4^+$ and not $CD8^+$ T cells during natural infections following *ex vivo* flow-cytometric analysis reveals a highly polarized phenotype. *In vitro* peptide pulse splenocytes isolated from both MD susceptible and resistant chickens are able to express IL-2, IL-4 and IL-10 transcripts. The induction of $CD4^+$ T cell subset activation demonstrate that peptide presentation is highly haplotype restricted and does not exhibit promiscuity as we would expect p-MHC-II/B-F presentation to $CD4^+$ T cells.

Taken together, we have identified, for the first time, a $CD4^+$ T cell restricted immunodominant epitope against pp38 in both MD-susceptible and resistant chickens. In addition to cytokine production, our data show that both $CD4^+$ and $CD8^+$ T cells lack cytotoxic function post infection. Memory T cell response can be generated against MDV however the abundance and maintenance of the cellular compartment is unknown. The model presented here should be of value in determining the correlates of protection against MDV infection as well as a platform for screening further MDV proteins which could be used in combination for subunit vaccines.

Chapter 5: General discussion

5.1 Overall objective and perspective

The last 30 years in MDV research has been marked by implementation of a vaccine (CVI988/RISPENS), the first of its kind against an oncogenic virus; which provided protection against severe neurological symptoms and subsequent transformation and proliferation of $CD4^+$ T cell lymphoma in susceptible chickens (4). Migratory and backyard birds act as a reservoir for MDV in the wild and may contribute to virus spread into poultry production facilities. Surveillance data have demonstrated an increase in virulence of field MDV strains marked by severe acute onset of MD (20). Vaccinated day-old chicks still become infected, at the expense of already pre-established biosecurity measure, and shed virus from the feather follicles as part of dust and dander into the environment (3). As a result, renewed emphasis is being placed for research into new therapeutic interventions so as to prevent further losses associated with culling of infected birds. MD is a very attractive proposition as a model to study the mechanism of herpesvirus induced atherosclerosis (246), lymphomagenesis (37) and mode of virus infection and replication (11) in its target host. Therefore, translating already established tools (206, 278) for studying immune responses (16, 35, 277) in the avian host and elaborating on host-pathogen interaction will help us elucidate new research opportunities (217).

A medley of growth factors and cytokines such as myelomonocytic growth factor (MGF) (291) and IFN-γ (292) have been considered as immuno-modulators and vaccine adjuvants against MDV. The treatment of MDV-susceptible chickens with MGF, a growth and activation factor for monocytes/ macrophages, reduced viral load, increased survival rate and reduced tumour incidence after challenge (291). It is postulated that MGF increases the number of macrophages and their response to stimuli as shown by an increase in NO production by activated macrophages. However the role of macrophages and other innate immune system cells during MD is still unclear. As a cell associated *alphaherpesvirus*, induction of cell mediated immunity is regarded as the primary defence against virus infected cells and clearance (217). Our group have recently shown that administering HVT vaccine with a plasmid expressing recombinant

chicken IFN-γ enhanced the protective efficacy of the vaccine against MDV and reduced viral load and tumour incidence (292). IL-18 has been shown to stimulate IFN-γ production from $CD4^+$ T cells and can also indirectly stimulate $CD8^+$ T cell proliferation (293). In addition, IFN-γ can enhance the development of T_{h1} type responses which is known to be important in the control of viral infections as well as activating innate immune system cells such as macrophages and NK cells.

In the present work, an *in vitro* and an *in vivo* approach was utilized to investigate two main objectives. The primary objective was to establish an *in vitro* understanding of essential macronutrients for virus infection and replication. This could then be utilized to interrogate whether they are antiviral and immune modulating factors. The secondary objective was to understand in an *in vivo* model whether either pathogenic (RB1B) or vaccinal (CVI988/RISPENS) strains of MDV can elicit a cell mediated immune response. Furthermore, the specific cell mediated immune responses could be characterized in the MD-resistant (B21) or MD-susceptible (B19) lines of chickens.

In the first objective of this study, we initially confirmed that MDV infected CEFs have an accumulation of lipid droplets. Therefore, MS-GC was utilized to interrogate induction of lipogenesis during MDV infection. The results demonstrated firstly that the small pharmacological inhibitors are effective antiviral molecules and can also be utilized to validate the MS-GC data. Essential pathways and nutrients dispensable to the host cell but not MDV were mapped thoroughly. β-oxidation is dispensable as other intracellular sources of energy support MDV infection and replication. Secondly, a link was established between induction of lipogenesis and eicosanoid synthesis, specifically PGE_2, here demonstrated as essential for viral replication. Our groups have previously reported that MDV transformed cell lines produce soluble factors such as PGE_2 which inhibited T cell proliferation and migration (240). Therefore we concluded that PGE_2 is an essential factor for viral replication and immune evasion tactic. Furthermore, this can then be translated to an *in vivo* model as a multi subunit antivirals to assess the quality of cell mediated immune response in conjuncture with supressing viral replication and dissemination.

In the second objective, T cell specific responses were evaluated in both the MD-resistant (B21) and MD-susceptible (B19) lines of chickens. Although significant effort

have been invested to characterise MDV gene function, limited information is available on MDV antigens or cell mediated immunity. Previous work have demonstrated that MDV MEQ and pp38 are able to elicit a cytolytic T cell response and potentially are highly antigenic (25, 26). Even though MD is controlled effectively by vaccination, the antigens associated with protective immunity are poorly defined. Some degree of protection is afforded to all chickens regardless of their MHC haplotype (294). However, using MHC specific defined line of chickens is an attractive proposition to elucidate clonal similarities or difference in T cell responses. Therefore, both MDV MEQ and pp38 were selected to characterise the quality and quantity of antigen specific T cell responses in both the MD-resistant (B21) and MD-susceptible (B19) lines of chickens. Both the vaccine (CVI988/RISPENS) and pathogenic strain (RB1B) of MDV elicited $CD4^+$ T cell responses directed against the pp38 peptides; $pp38_{5\text{-}20}$ and $pp38_{161\text{-}176}$ in the MD-susceptible line of chicken and $pp38_{171\text{-}186}$ in the MD-resistant line of chicken. The fact that these peptides are conserved among vaccinal strains (SB-1, HPRS24 and CVI988/RISPENS) of MDV confirms that protective and long lasting immunity could be afforded through pp38 and not MEQ. An alternative, but not mutually exclusive scenario is that the immunoreactivity pattern observed may be a line-specific response. It is important to note that the chickens currently utilized in poultry production facilities, but not backyard operations, are derived from the MD-resistant line. As such, the findings reported here are highly significant in the broader scope for next generation subunit vaccines.

5.2 Concluding Remarks

Our understanding of innate and adaptive immunity to MDV infection is continuously improving. This growing knowledge can be further enhanced by our understanding of immunity to human *alphaherpesviruses* and host-pathogen interaction. However, there are many differences between the human and avian immune system as well as different characteristics between MDV and human *alphaherpesviruses* (VZV, HSV-1 and 2). MDV is a highly cell associated virus which imposes certain limitations. In terms of vaccine research and development, the developments of potent cell-free vaccines that can inhibit infection as well as the disease are paramount to research in this area. Going forward, the research presented here have outlined alternative avenues for antiviral research and established a baseline for vaccine design.

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