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The effect of dendritic cell mobilization on CD8+ T cell responses to influenza A virus

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

THE EFFECT OF DENDRITIC CELL MOBILIZATION ON $CD8^+$ T CELL RESPONSES TO INFLUENZA A VIRUS

(Spine title: Effect of dendritic cell mobilization on flu-specific T cell responses)

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by

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ABSTRACT

Influenza A viruses (IAV) cause respiratory infections with potentially catastrophic consequences. Neutralizing antibodies towards surface proteins of IAV prevent reinfection. However, mutations in these proteins allow the virus to evade these antibodies. Enhancing cytotoxic T cell-mediated immunity has been proposed as an attractive strategy to combat flu. Recombinant human FMS-like tyrosine kinase 3 ligand (FL) is known to mobilize dendritic cells (DCs) in mice. Given that DCs are the most potent antigen-presenting cells, I hypothesized that their mobilization by FL will improve the $CD8^+$ T cell response to IAV. Quantification of $CD8^+$ T cell responses to IAV epitopes by intracellular cytokine staining revealed that FL treatment can indeed increase the frequencies and absolute numbers of mouse T cells specific for select IAV epitopes. Additionally, FL treatment increased the killing function of IAV-specific T cells. My results suggest that FL may exert a therapeutic benefit in the context of flu infection.

KEYWORDS: influenza A virus, dendritic cells, adaptive immunity, antiviral immunity, immunodominance, recombinant human FMS-like tyrosine kinase 3 ligand, $CD8^+$ T cell responses

This is for you, Mom.
Your endless love and support made me the man I am today.

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LIST OF SYMBOLS AND ABREVIATIONS

µg	microgram
µL	microlitre
µm	micromolar
°C	degree Celsius
%	percent
APC	allophycocyanin
pAPC	professional antigen presenting cell
BL/6	C57BL/6
BFA	brefeldin A
BMDC	bone marrow-derived dendritic cell
CO_2	carbon dioxide
CTL	cytotoxic T lymphocyte
iDC	immature dendritic cell
mDC	mature dendritic cell
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorting

FBS	fetal bovine serum
FITC	Fluorescein isothiocyanate
FL	fms-like tyrosine kinase 3 ligand
FSC	forward scatter
GM-CSF	granulocyte monocyte-colony stimulating factor
HA	hemagglutinin
IAV	influenza A virus
ICS	intracellular cytokine staining
ID	immunodominance
IDD	immunodominant determinant
IFN	interferon
IL	interleukin
i.n.	intranasal
i.p.	intraperitoneal
kb	kilobase
LCMV	lymphocytic choriomeningitis virus
M1	matrix protein 1
M2	matrix protein 2
mAb	monoclonal antibody
MHC-I	major histocompatibility complex class I

mg	milligram
mM	millimolar
NA	neuraminidase
NK	natural killer
NP	nucleoprotein
NS1	non-structural protein 1
NS2	non-structural protein 2
p	*p*-value
PA	acid polymerase
PB1	basic polymerase 1
PB2	basic polymerase 2
PBS	phosphate-buffered saline
PEC	peritoneal exudate cell
PFA	paraformaldehyde
RBC	red blood cell
RNA	ribonucleic acid
RPM	rotation per minute
SDD	subdominant determinant
SE	standard error
SSC	side scatter

TAP	transporter associated with antigen processing
TCR	T cell receptor
TNF	tumour necrosis factor

Chapter 1:

Introduction

1.1 Influenza A Virus

1.1.1 Introduction

Responsible for illness, hospitalization and death, the influenza virus is an infectious agent that has threatened the health of humans for centuries (1). A member of the Orthomyxoviridae family, genus Orthomyxovirus, influenza can be categorized into three classes, A, B and C (1, 2). Influenza A viruses (IAV) are single stranded negative-sense ribonucleic acid (RNA) viruses, and are of specific importance as they are the only class fit to readily colonize within humans (3, 4). Given this, IAV imposes tremendous health and economic costs on individuals, groups and countries (5). This can occur locally through epidemics although IAV also has the potential to evoke worldwide pandemic disease (4).

IAV can be further categorized into serotypes designated based on the expression of two proteins known as hemagglutinin (HA) and neuraminidase (NA), which are distributed on the viral surface in a 5 to 1 ratio, respectively (6). HA can bind to glycophorin via sialic acid residues, which is present on the surface of a variety of host cells, including those found in the upper respiratory tract. HA is essential for IAV infection as it allows attachment and eventual entry into host cells (8). On the contrary, NA is an enzyme that cleaves sialic acid groups which allows for detachment from the host cell (9). Currently, there exits sixteen primary subtypes for HA (H1-H16) and nine primary NA subtypes (N1-N9) (5, 10); therefore many arrangements or serotypes of IAV are possible. However, only certain IAV subtypes, mostly H1, H2, H3, N1 and N2 are prevalently in circulation in the human population (10).

1.1.2 IAV Structure and Proteins

The influenza A viral genome consists of eight linear single-stranded RNA spanning nearly 13.5 kilobases (kb) (11, 12). This genome encodes for eleven proteins, with each protein providing an essential feature in facilitating the spread of the virus (13, 14). There are 3 membrane-anchored proteins: HA, NA and matrix protein 2 (M2), which is a transmembrane ion channel protein. There are three subunits of the viral polymerase: acid polymerase (PA), basic polymerase 1 (PB1) and basic polymerase 2 (PB2). In addition, there are two structural proteins: nucleoprotein (NP), which binds viral RNA to form stable ribonucleoproteins and target these for export from the host nucleus to cytosol, and matrix protein 1 (M1). Furthermore there are two non-structural proteins: non-structural protein 1 (NS1) and non-structural protein 2 (NS2). PB1-F2, the latest protein discovered, is responsible for binding to the host mitochondria and sensitizing the cell to apoptosis (13 – 15).

1.1.3 Evasion Strategies

IAV has been a constant burden, both socially and economically (1). This is due to the fact that this dynamic virus is continuously evolving and finding new ways to evade effective treatments (1). IAV undergoes two kinds of antigenic variation; antigenic drift and antigenic shift. Antigenic drift occurs routinely, while antigenic shift occurs infrequently (10, 16).

Antigenic drift refers to small, gradual changes that occur via point mutations caused by selective immune pressure (10). This pressure causes mutations in the genes responsible for the natural assembly of the main surface proteins: HA and NA (10, 17).

These unpredictable mutations readily produce viral strains with the ability to escape recognition by previously produced antibodies (4, 17). This process works as follows: an individual is infected with a specific influenza strain to which an antibody response is mounted. Upon re-infection with the same strain, antibodies circulating within the body will bind specifically to the same HA and NA proteins and eliminate the virus, resulting in no symptoms (16). However, if IAV undergoes antigenic drift, this once effective immune response can no longer recognize the same HA and NA proteins, hence resulting in infection (16, 18, 19). This is the main reason why humans can be seriously infected with IAV more than one time (18, 19). This constant need for protection is the motivation behind formulating new influenza vaccines every year (18). On the positive side, these new subtypes are still related to their predecessors internally and therefore can still be subjected to $CD8^+$ T cell recognition (1, 10, 19).

While antigenic drift is a minor change to the IAV genome, antigenic shift refers to a sudden major change, which has the ability to produce a novel IAV subtype (18, 19). This new subtype contains antigens that are distinct from those of previous strains and is not currently circulating within the population (18). Antigenic shift can occur in two ways. Either through direct animal-to-human transmission or through the mixing of IAV strains from both species (18, 19). Mixing of the genetic materials from different viral strains resulting in the production of a novel strain is termed genetic reassortment (18). This reassortment can cause major changes in the surface proteins, HA and NA, and in the internal proteins such that the virus is no longer recognized by the host immune system at all (18, 20). If history is any indication, humans have had hardly any immunity to these reassorted viruses (18, 20). As such, these viruses have the potential to cause

pandemics (20, 21). A global influenza pandemic can occur if the following three conditions are met: 1) a new subtype of IAV is introduced into the human population; 2) the virus causes serious illness; and 3) the virus has the ability to spread easily from human to human (21). A good example of a recent antigenic shift, which could have resulted in a global influenza pandemic, occurred in Hong Kong in 1997 (22). A few individuals were infected with a novel IAV strain from infected chickens. Mortality in these individuals was significant as 6 out of 18 infected individuals died. Authorities in Hong Kong ordered the mass killing of 1.5 million chickens, after which no others were infected, as the virus (H5N1) did not spread easily from person to person. Thus a possible pandemic was fortunately avoided. Nevertheless, we will not always be this fortunate (22).

1.1.4 Impacts on Public Health

The H3N2, H1N1 and H1N2 subtypes of the virus are currently co-circulating within the human populations (23, 24). IAV causes global health issues and life-threatening infections resulting in roughly half a million deaths per year (18, 21). According to the Center for Disease Control and Prevention (CDC), in the United States alone, roughly 5-20% of the population is annually infected resulting in 200,000 hospitalizations and 36,000 deaths. Not only does IAV cause serious public health issues, but it also can cause serious economic problems. In developed countries, IAV infections can result in many sick days and productivity losses (18). In addition, during the peak infection periods, community clinics and hospitals can be overwhelmed when it comes to resources and ability to treat their patients (18). Taking both of these into account, the

estimated economic burden on the United States every year from IAV is roughly 12-14 billion dollars (18). Health and economic issues caused by IAV will be ruled trivial if a novel IAV strain with the ability to trigger a pandemic is discovered (20, 21, 24).

A pandemic can occur if a novel IAV subtype emerges via antigenic shift and can effectively infect and spread among the human population who lack the appropriate immunity to fight this new subtype (16, 18, 19, 20, 21, 24). Even more concerning has been the reports that interspecies transmission of IAV from avian species to humans has increased significantly in the past 20 years (25). Fortuitously, the World Health Organization (WHO) has determined that no IAV strain that has crossed the interspecies barrier has been able to spread efficiently from human to human. However, if these avian or swine influenza viruses are able to undergo genetic reassortment with the human IAV, they could easily adapt a method allowing for efficient transfer from individual to individual (24, 25). A pandemic of this nature has been estimated to have the capacity to cause greater than 100 million deaths worldwide (24). The IAV pandemic of 1918, known as the “Spanish Flu”, instigated from an adaptation of an avian IAV to human, caused the death of roughly 50 to 100 million people (24).

The best way to prepare for this inevitable event are to maintain general health care practices and perhaps also importantly to boost host immune responses to viral infections. In 2009, the “Swine Flu”, which originated from a novel IAV of swine origin, was the latest IAV pandemic (26). According to WHO statistics, since April 2009, the virus has killed more then 18,000 people. However, there were many deaths in Africa and Southeast Asia that were uncounted for. Experts now agree roughly 295,000 individuals were killed by this novel IAV strain (27). This latest threat is an excellent example of the

problems that surround the emergence of a new strain. Vaccination is the traditional strategy used for many years to prevent influenza infections, but its success is not complete. Predictions of scientists and successful matching of the vaccine antigen with the naturally occurring antigens determine the success of this prevention strategy. After infection, the most effective method to clear the pathogen would be to increase viral clearance though a more efficient antiviral cellular immune response (36). The effective clearance of viral particles would diminish the spread of the virus and significantly reduce the threat of future pandemics.

1.2 $CD8^+$ T cell Activation

$CD8^+$ T cells, otherwise known as cytotoxic T lymphocytes (CTLs) in their activated state, are a crucial subset of T lymphocytes whose principal function is to recognize and eliminate virally infected host cells, tumor cells and other damaged cells (28, 29). Specifically for this project, the interest lays in the ability of $CD8^+$ T cells to participate in an adaptive immune response to intracellular viral pathogens. Naïve $CD8^+$ T cells are only activated by dendritic cells (DCs) (30 – 32). DCs are located in the blood and peripheral tissues and monitor the body for possible antigenic material (33). DCs take up viruses directly into their cytosol either through phagocytic receptors or through direct viral infection (36). Once inside the cytoplasm, the viruses use the host protein machinery to create their own proteins (34, 36). Host cell proteasomes process these viral proteins and break them down into smaller peptides (34). These peptides are then picked up by transporter associated with antigen processing (TAP) proteins, which are embedded in the membrane of the endoplasmic reticulum (ER) (35). TAP proteins effectively transport these antigenic peptides into the ER of the infected cells, where they compete with self

peptides for loading onto major histocompatibility complex (MHC) class I molecules (34, 36). The MHC class I:peptide complex is then transported to the surface of the DC. Once there, the MHC class I:peptide complex will only bind the T cell receptor (TCR) specific for this complex (34, 36). The recognition of the MHC class I:peptide complex by the TCR is known as signal 1 for the activation of antigen-specific $CD8^+$ T cells leading to their differentiation into effector CTLs (34, 36).

The successful activation of naïve T cells requires a second signal, which is a co-stimulatory signal provided by the same antigen-presenting DC (36). Glycoproteins B7.1 (CD80) and B7.2 (CD86) usually found as a homodimeric compound are expressed on the surface of DCs (37). They interact with two receptors on T cells, CD28 and CTLA-4 (34, 36). Binding of B7 to CD28 is crucial for the clonal expansion of these naïve T cells (38), while binding to CTLA-4 negatively regulates the proliferation of already activated T cells upon exposure to their antigen (39).

Of all antigen-presenting cells (APCs), DCs are thought to be the most proficient at priming naïve T cells for a variety of reasons. While circulating within the body and resident in tissues, DCs are present in an immature state (iDCs) where they can effectively trap and process antigens (33). Once DCs have taken up a viral protein or have been infected by a virus itself, they migrate to regional lymph nodes (33). During the migration period, these immature DCs undergo a maturation process, which leads to a decline in their antigenic uptake and processing ability (33, 36). However, there is an enhancement in their ability to present antigens due to increased surface expression of MHC class I and co-stimulatory molecules necessary to activate naïve T cells (33, 36). In addition to the CD80 and CD86 molecules, CD83 is also upregulated on DCs during

maturation (40). CD83 has been shown to influence the antigen-specific activation of T cells by mature dendritic cells (mDCs) (40).

Mature DCs have the ability to bind to naïve T cells in a very efficient manner. (42, 43). This occurs through interactions between lymphocyte function-associated antigen 1 (LFA-1), intercellular adhesion molecule 3 (ICAM-3) and CD2 on the T cell and ICAM-1, ICAM-2 and DC-SIGN on the DCs (43). In particular, the binding of ICAM-3 to DC-SIGN is unique to DCs and T cells (42). This interaction promotes the binding of these two cells creating a perfect "immunological synapse". This has been shown to be crucial to providing enough time for T cells to sample a large number of MHC molecules on the APC for the presence of their specific peptide (42).

Once activated by the appropriate signals, T cells proliferate and differentiate into effector CTLs with the help of the cytokine interleukin-2 (IL-2) (34). CTLs release various cytokines such as IFNγ and tumor necrosis factor (TNF), in addition to various cytotoxic effector molecules such as perforin and granzymes (36, 43, 44). Perforin creates pores in the plasma membrane of target cells to allow granzymes to enter, thus starting a cascade eventually leading to apoptosis (programmed cell death) of virally infected cells (43, 44). Upon activation, CTLs express the surface protein Fas ligand (FasL), which when bound to Fas (CD95) found on target cells induces apoptosis (43 – 45). The upregulation of these effector molecules is required for elimination of infected cells (45).

1.3 Immunodominance

IAV has the ability to provide tens of thousands of potentially immunogenic

peptides, but $CD8^+$ T cell immune responses are always directed towards a few specific peptides (46 - 49). Within this population of peptides, there are those highly immunogenic peptides, termed "immunodominant" determinants (IDDs), and other "subdominant" determinants (49). This concept is known as immunodominance (46 – 49). $CD8^+$ T cell responses are managed by a restricted number of TCRs that are antigen-specific. Those clones with TCRs that are specific for IDDs skew the $CD8^+$ T cell response towards these specific antigens while those $CD8^+$ T cells specific for subdominant epitopes elicit a much more modest immune response. From the variance in response to these immunogenic peptides comes the concept of an immunodominance hierarchy (46, 49).

This immunodominance hierarchy arises from an intricate combination of factors, which encompass several aspects of $CD8^+$ T cell immunology (46, 48, 49). This includes: 1) generation and regulation of the $CD8^+$ T cell repertoire via positive and negative selection (50, 51, 54), 2) interaction of $CD8^+$ T cells with APCs (52), 3) abundance of the antigenic source protein within APCs (53), 4) frequency and specificity of TAP transport of peptides (54), 5) peptide affinity for MHC class I molecules (54), 6) signaling involved in $CD8^+$ T cell activation (53) and 7) immunodomination, which refers to the ability of IDD-specific T cells to suppress subdominant T cell responses (46, 49).

Even though the above factors have been implicated in ID, this phenomenon is not well completely understood. However, its importance in the development of a therapeutic vaccine against viral infections should not be overlooked. $CD8^+$ T cells cannot prevent flu infections, but they can make a major contribution in eradicating viral infections (49, 55). Given this, it is important to understand how immune responses might

be altered by different treatments.

The immunodominance hierarchy of IAV-specific $CD8^+$ T cells in mice has been studied intensely. As mentioned earlier, the IAV genome contains eight linear RNA strands and encodes several proteins (11-14). As such, the immunodominance hierarchy of $CD8^+$ T cells that are specific for well-defined peptides has been established in the IAV system (50, 56, 57). When C57BL/6 ($H\text{-}2^b$) mice are infected with IAV, peptides NP_{366} and PA_{224}, which originate from the viral nucleoprotein and acid polymerase, respectively, elicit the largest $CD8^+$ T cell response (50, 56). Less robust $CD8^+$ T cell responses are also detectable against subdominant epitopes [in order of decreasing $CD8^+$ T cell response: PB1-$F2_{62}$, $PB1_{703}$, $PB2_{198}$, $NS2_{114}$, and $M1_{128}$ (50, 56)]. When Balb/c ($H\text{-}2^d$) mice are infected with IAV, $CD8^+$ T cell responses are against different viral peptides than C57BL/6 ($H\text{-}2^b$) mice due to their differing MHC backgrounds. Given this, in Balb/c mice, the immunodominant peptide NP_{147} elicits the greatest $CD8^+$ T cell response (50, 57). Weaker responses are found towards the other subdominant epitopes (in order of decreasing $CD8^+$ T cell response); $PB2_{289}$, HA_{518}, NP_{39}, NP_{218} and HA_{462} (50, 57). The order of these responses is highly conserved among the $CD8^+$ population; however, changing the immunodominance hierarchy might be useful in the development of an effective therapeutic vaccination (46, 49 – 55).

1.4 Dendritic Cells

1.4.1 Origin and Subsets

To trace the lineage of dendritic cells is not simple. This multifaceted lineage contains several subsets and various maturation stages (67 – 69). DCs develop from one

lymphoid precursor and one myeloid precursor (67 – 69). It has been shown that when lymphoid precursors are transferred into irradiated mice, T cells, natural killer (NK) cells, B cells and DCs can develop (70, 71). The myeloid DCs were first discovered in *in vitro* studies where isolated myeloid precursors gave rise to DCs with the addition of the cytokine granulocyte/macrophage colony-stimulating factor (GM-CSF) (72, 73). Both myeloid and lymphoid DCs originate in the bone marrow, but eventually migrate to non-lymphoid tissues in the body and into peripheral lymphoid organs (67, 74).

For simplicity, two broad classes of DCs will be define in this thesis: conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) (36, 67). cDCs are dendritic cells that are known to participate directly in antigen presentation and subsequent activation of naïve $CD8^+$ T cells (36, 75, 76). Although several subsets of cDCs exist, all subsets have the ability to process and present antigen in an efficient manner (36). cDCs express numerous cell surface markers including high levels of CD11c (77), a type I transmembrane protein and B7 (described previously). Conversely, pDCs secrete significant amounts of type I inteferons (IFN) such as IFNα and IFNβ, but apparently do not have an active role in the activation of naïve $CD8^+$ T cells (67, 75). pDCs express intermediate levels of CD11c on their surface and low levels of B7 – hence their inability to optimally activate naïve $CD8^+$ T cells (36, 78). In contrast to cDCs, pDCs express CD45R (B220) on their surface, which enables us to distinguish between the two subsets (79).

1.4.2 Role of Dendritic Cells in Anti-viral Immunity

DCs, which originate from bone marrow, are present in non-lymphoid tissues as immature dendritic cells (iDCs) (36, 80). This includes areas such as the skin, nose, lungs

and the blood (36). iDCs are unable to activate naïve T cells, but have high endocytic activity (36, 80). iDCs are constantly surveying the environment for antigens with the mission of sampling a pathogenic antigen that may prove detrimental to the body (36). When a pathogen such as IAV is encountered, DCs may release certain cytokines such as IFNα (36, 81, 82). The release of this cytokine causes the activation of other innate effector cells such as eosinophils and macrophages (36). In addition to secreting cytokines, iDCs have the ability to take up and present antigens to adaptive effector cells. DCs are very important APCs as they are the only cell type to induce a primary (initial exposure to pathogen) $CD8^+$ T cell response. Viruses can infect iDCs directly or be taken up by macropinocytosis or phagocytosis (36). After the uptake of an antigen, DCs undergo maturation which results in the upregulation of chemokine receptor 7 (CCR7). This chemokine induces the relocation of DCs from the blood into the lymphatic system/lymph node (36, 83). As DCs mature, they become more potent in activating T cells, but decrease their ability to pick up antigens. As explained above, once in the lymph node, these mature DCs have the ability to activate antigen-specific $CD8^+$ T cells (36). Once activated, $CD8^+$ T cells leave the lymph node and enter circulation, migrating towards sites of infection or injury, where they lyse infected cells (36).

cDCs are clearly important in the activation of naïve $CD8^+$ T cells, but pDCs also have an important role in antiviral responses. pDCs express Toll-like receptor (TLR)-7 and TLR-9, which are important in the recognition of single standard RNA viruses and DNA viruses, respectively (36, 84). Upon recognition of the pathogen, these cells secrete large amounts of type I interferons (IFNα and IFNβ) (36, 84). The release of these

cytokines stimulates an extensive antiviral response in somatic cells, and leads to DC maturation (36).

1.5 FMS-like Tyrosine Kinase 3 Ligand (FL)

1.5.1 FL ligand Background

Flt3 ligand (FL) is a cytokine known to stimulate proliferation and differentiation of stem cells and progenitor cells (58 – 61). Genes encoding FL have been found on a section of mouse chromosome 7 and human chromosome 19q13.3 (62). The ligand, which has been cloned for both mouse and human, is a soluble protein (61, 62). The size and structure of the ligand are strikingly similar to other ligands for tyrosine kinase receptors that are involved in the regulation of proliferation and differentiation of cells (59). These include colony-stimulating factor 1 (CSF-1) and stem cell factor (SCF). All three of these ligands are type I transmembrane proteins that possess a conserved cysteine residue stretch in their extracellular domains (59).

FL is present in both murine and human systems. The cDNA that encodes FL shows a 76% and 72% sequence identity at the nucleotide and amino acid levels in mice and humans, respectively (59, 61, 62). Interestingly, mouse and human FLs can stimulate the proliferation of cells originating from either species at comparable concentrations (59). In fact, multiple studies have used murine FL on human bone marrow cells and vice versa (59 – 62). This is in stark contrast to CSF-1 and SCF. Murine CSF-1 does not show an effect on human cells, and while human SCF does work on murine cells, it is roughly 1000 times less active (59).

1.5.2 Distribution and Function of FL

FL can be found on numerous early progenitor and precursor cells. In addition to being present on stem cells, FL is also expressed on both multipotent myeloid progenitor cells and multipotent lymphoid progenitor cells (64). Additionally, FL is also expressed by various colony-forming unit (CFU) cell types, which are essentially specific precursor cells for particular populations (62, 63). Furthermore, FL is also expressed on progenitors of NK cells.

The receptor for FL is FLT3, which is expressed on stem cells in murine bone marrow (60 – 64). Additionally, in humans, FLT3 is expressed on bone marrow-derived $CD34^+$ cells. CFU-DC is a term that describes $CD34^+$ precursor cells that eventually differentiate into DCs (62, 63). Intriguingly, analysis of FLT3 expression of hematopoietic cell lines indicated the presence of the receptor on pre-DC populations (63).

The primary purpose of FL is to stimulate proliferation and differentiation of hematopoietic and progenitor cells in both murine and human models (58 – 66). Although there are other ligands that stimulate the proliferation of stems cells, FL is unique for multiple reasons. Some important differences include FL's lack of effect on mast cells and its lack of involvement in erythropoiesis (63). However, it does play a role in the proliferative stimulation of B cells and DCs. These results have been obtained in *in vivo* studies using animal models (63). Several studies have shown that delivery of FL in mice for 10 days can induce changes in hematopoietic patterns (63, 65). Furthermore, significant morphological changes in murine spleens have been reported including splenomegaly (enlarged spleen) and a significantly increased number of large cells with

definite DC morphologies (63, 66). Maraskovsky et al. have also shown an increase in the number of functionally active *in vivo* DCs within bone marrow, peripheral blood, spleen and lymph nodes after FL treatment in mice (65).

1.5.3 Impact of FL on DC generation

As previously mentioned, DCs are the most effective APC to stimulate naïve T cells and induce a primary immune response (30 – 33). Prior to the discovery of FL, DC-based immunotherapy was relatively ineffective due to the low yield of functionally active DCs in the blood or lymphoid tissues (63, 66). For example, GM-CSF has been shown to increase the yield of DCs generated *in vitro*, but no growth factor has been identified that increases the quantity of DCs in the lymphoid tissues like FL does (63, 65, 66). In a study by Shurin et al, FL-treated mice had a significant increase in the yield of functionally active DCs in the bone marrow and spleen (63). Functionally active DCs were defined as those with the ability to stimulate proliferation of $CD8^+$ T cells (63). Given the inherent ability of FL to increase the population of functionally active DCs, its assessment as a useful treatment in $CD8^+$ T cell immunotherapy was explored in my project.

1.6 Project Rationale and Aims

Vaccination of the general public against IAV is ineffective in inducing protective immunity o flu. There are still hundreds of thousands of individuals who are infected each year, leading to tremendous health and economic burden. Increasing the antiviral $CD8^+$ T cell immune response may potentially be of preventative/therapeutic value.

The main goal of this project was to determine if FL treatment can boost the magnitude and change the breadth of the anti-IAV $CD8^+$ T cell response as this is

unknown at present. Since CTL responses to viral pathogens play a significant role in antiviral defense, increasing or modulating this response is a logical approach to enhance viral clearance. As mentioned above, DCs play an essential role in the activation of virus-specific CTLs. Additionally, FL has been shown to increase the DC population found throughout the body. Putting these themes together, I hypothesized that with the use of FL to mobilize DC populations, we can increase CTL responses to IAV through increased priming of naïve T cells by DCs.

In order to test this hypothesis, several aims had to be defined. First, FL stock had to be tested to ensure its ability to increase the DC population. This was tested *in vitro* and *in vivo.* If indeed FL did increase the DC population, the next step would be to determine if FL treatment (*in vivo*) can increase the percentage and/or absolute number of flu-specific CTLs. In order to determine if FL treatment increases viral clearance, testing if FL treatment (*in vivo*) could increase the cytotoxic killing function of flu-specific CTLs needed to be conducted. In addition to exploring the primary immune response, when studying IAV, it is important to understand if treatment will help during secondary exposure. As such, investigating the role of FL treatment in recall $CD8^+$ T cell responses was explored. Finally, the effect of FL treatment in a second mouse strain was studied. This study was undertaken to improve our understanding of the overall benefit of FL treatment in the context of a $CD8^+$ T cell-based vaccine or other antiviral treatments.

Chapter 2:

Materials and Methods

2.1 Mice

Adult female C57BL/6 (B6) (H-2^b) and Balb/c (H-2^d) mice were purchased from Charles River Canada Inc. (St. Constant, QC, Canada). Mice were housed in the animal care facility at the University of Western Ontario and cared for according to institutional and national guidelines.

2.2 Recombinant Human FMS-like Tyrosine Kinase 3 Ligand

Original powdered stock of recombinant human **f**ms-**l**ike **t**yrosine **k**inase **3** **l**igand (FL) (5 mg/vial) was diluted with sterile water (Sigma, Oakville, ON) and aliquoted into individual vials at a final concentration of 50 μg/10 μL. These vials were then stored at -30°C until needed.

In preparation for injections, a FL vial was thawed and transferred into 490 μL of PBS giving a final total volume of 500 μL. Mice were subsequently injected via subcutaneous (s.c.) injections with 100 μL effectively delivering 10 μg of FL per mouse. This treatment regimen was adopted from previously published studies (85). Control mice were injected subcutaneously with 100 μL of phosphate-buffered saline (PBS). Injections took place for ten consecutive days.

2.3 Antibodies

All monoclonal antibodies (mAbs) were purchased from eBioscience (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD11c (clone N418, Armenian Hamster IgG) and allophycocyanin (APC)-conjugated anti-mouse CD45R (B220) (clone RA3-6B2, Rat IgG2a) mAbs were used to detect DC populations. In intracellular cytokine staining experiments, APC-conjugated anti-mouse CD8α (clone 53-6.7, rat IgG2a) and FITC-conjugated anti-mouse IFN-γ (clone XMG1.2, rat IgG1) mAb

were used to detect surface CD8 and intracellular IFN-γ, respectively. Anti-CD16/CD32 or Fc Block (clone 2.4G2, rat IgG2b) was used for the prevention of non-specific binding of the above mAbs to Fc receptors.

2.4 Cell Lines

2.4.1 DC2.4

The DC2.4 cell line is an immortalized immature DC line, originally generated from C57BL/6 mice (H-2^b) (86). The cell line was created and provided to our laboratory by Dr. Kenneth Rock (University of Massachusetts Medical School, Worchester, MA). When needed, vials containing DC 2.4 cells that were stored in liquid nitrogen were thawed using a 55°C water bath. After centrifugation (Beckman Coulter, Mississauga, ON) at 1400 RPM, cells were washed three times in complete RPMI 1640 medium (cRPMI): RPMI 1640 containing L-glutamine (Gibco, Grand Island, NY) was supplemented with 1 mM sodium pyruvate, 100 units/mL of each penicillin G and streptomycin (Sigma), 1 mM of non-essential amino acids (Gibco) and 10% fetal bovine serum (FBS) (HyClone, UT, USA). Following washing, DC 2.4 cells were resuspended in 10 mL of cRPMI, seeded into a tissue culture flask (Falcon BD, Mississauga, ON) and incubated at 37°C and 6% CO_2. Cells were sub-cultured into new tissue culture flasks until they reached 80-85% confluency. Given the adherent nature of the DC2.4 cell line, trypsin (Gibco) was used to detach cells from the flask surface. Cells were washed, and re-seeded into a new tissue culture flask with fresh cRPMI.

2.4.2 Balb/c 3T3

The Balb/c 3T3 cell line was generously provided by Dr. Yong Kang (University

of Western Ontario). 3T3 cells are derived from Balb/c (H-2^d) fibroblasts. Thawing and seeding into tissue culture flasks followed the same procedure described above. However, the medium used for the effective growth of these cells was Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% bovine serum (Hyclone). 3T3 cells were incubated at 37°C and 9% CO_2. To effectively maintain a healthy cell culture, cells were sub-cultured every 7-8 days (80-85% confluency) due to their slower growth period. Given the adherent nature of the Balb/c 3T3 cell line, trypsin (Gibco) was used to detach cells from the flask surface. Cells were then washed and re-seeded into a new tissue culture flask with fresh supplemented DMEM medium.

2.4.3 P815 cells

This H-2^d mouse lymphoblast-like mastocytoma cell line was maintained in the similar fashion to the 3T3 cell line above. However, unlike the adherent 3T3 cell line, these cells are suspended in media and therefore do not require trypsin treatment. These cells were used as target cells during the chromium release assay.

2.5 Viral Peptides

The main IAV strain used in this project was the Puerto Rico/8/1934 (PR8) H1N1 strain, which has been heavily studied and is well characterized. As such, the structure and $CD8^+$ T cell immunodominance hierarchies have been well established following the infection of C57BL/6 and Balb/c mice with this IAV strain (50, 56, 57). Peptides are named based on the protein they are derived from in addition to the specific numeric amino acid locations within that protein. Synthetic viral peptides (Tables 1 and 2) used in this project were generously provided to our laboratory by Dr. Jack Bennink and Dr. Jonathan Yewdell (National Institutes of Health, Bethesda, MD). These peptides were

Table 1. ***PR8 viral peptides used in this thesis research (Balb/c)***

Determinant	Abbreviation	Restricting MHC	Sequence
$NP_{147\text{-}155}$	NP_{147}	H-$2K^d$	TYQRTRALV
$NP_{39\text{-}47}$	NP_{39}	H-$2K^d$	FYIQMCTEL
$NP^{218\text{-}226}$	NP^{218}	H-$2K^d$	AYERMCNIL
$HA_{462\text{-}470}$	HA_{462}	H-$2K^d$	LYEKVKSQL
$HA_{518\text{-}526}$	HA_{518}	H-$2K^d$	IYSTVASSL
$PB2_{289\text{-}297}$	$PB2_{289}$	H-$2D^d$	IGGIRMVDI

Table 2. ***PR8 viral peptides used in this thesis research (C57BL/6)***

Determinant	Abbreviation	Restricting MHC	Sequence
$PA_{224-233}$	PA_{224}	$H\text{-}2D^b$	SSLENFRAYV
$NP_{366-374}$	NP_{366}	$H\text{-}2D^b$	ASNENMETM
$PB1\text{-}F2_{62-70}$	$PB1\text{-}F2_{62}$	$H\text{-}2D^b$	LSLRNPILV
$PB1_{703-711}$	$PB1_{703}$	$H\text{-}2K^b$	SSYRRPVGI
$M1_{128-135}$	$M1_{128}$	$H\text{-}2K^b$	MGLIYNRM
$NS2_{114-121}$	$NS2_{114}$	$H\text{-}2K^b$	RTFSFQLI
$PB2_{198-206}$	$PB2_{198}$	$H\text{-}2K^b$	ISPLMVAYM

over 95% pure. All peptides were dissolved in dimethyl sulfoxide (DMSO) and stored at -30°C.

2.6 Viral Inoculation

Influenza A viruses used in this project, PR8 and SEQ12, were propagated within the allantoic cavity of 10-day-old embryonated chicken eggs. Viral particles were isolated from the eggs and aliquoted to a final volume of 1 mL of infectious allantoic fluid per glass vial (VWR, Mississauga, ON). These aliquoted viral stocks were then frozen and stored at -80°C until use.

When determining the effect of FL on primary $CD8^+$ T cell responses to IAV, 1 mL of PR8 was thawed and added to 1 mL of PBS to give a 1:2 dilution. From this mixture, 450 µL was injected into each mouse intraperitoneally (i.p.), which was estimated to deliver approximately 600 hemagglutinating units. This dosage has been shown to efficiently induce IAV-specific $CD8^+$ T cell responses in mice as previously reported (87). Injection of PR8 occurred on day 0 in all experiments.

When determining the effect of FL on $CD8^+$ T cell recall responses, mice were primed via an i.p. injection of PR8 on day 0 followed by injection of SEQ12 via the i.p. route on day 30.

2.7 *In vitro* infection of DC2.4 or 3T3 cells for viral antigen presentation

To gauge for a representative "overall" antiviral response of all flu-specific $CD8^+$ T cells, DC2.4 cells or 3T3 cells were infected with IAV. The following procedure outlines the infection methodology. Cells were collected from their respective tissue culture flasks and placed in a 15 mL conical tube (Falcon BD). The tube was then filled with sterile PBS (Sigma) and centrifuged at 1400 RPM for 4 minutes. After

centrifugation, the supernatant was discarded and cells were resuspended in 1 mL of PBS. From here cells were counted with the aim to collect 8-10 million cells. After another PBS wash, cells were resuspended in 1 mL of PBS. Similar to the preparation for inoculation in mice, a two-fold dilution was completed by adding 1 mL of IAV infectious allantoic fluid to 1 mL of PBS containing 8-10 million cells. Cells, now in the presence of IAV, were incubated for 1 hour at 6% CO_2 and 37°C while being constantly mixed via a cell rotator. After 1 hour, 6 mL of complete medium was added and cells were left inside the CO_2 incubator for another 5 hours. After this incubation period, cells were taken out and washed three times in complete medium to remove any excess viral particles. Cells were now considered infected with IAV and ready to be used in my ICS experiments.

2.8 Intracellular Cytokine Staining

Intracellular cytokine staining (ICS) for interferon IFNγ is a highly sensitive and quantitative technique used to determine the frequencies and absolute numbers of antigen-specific $CD8^+$ T cells (Figure 1), as it is the primary cytokine produced by stimulated $CD8^+$ T cells. The ICS assay was conducted seven days after PR8 inoculation, a time point at which antigen-specific $CD8^+$ T cells response are known to be at their peak (87). This method allowed us to precisely examine $CD8^+$ T cell responses to virally infected cells, in addition to synthetic peptides corresponding to specific epitopes of IAV.

On day seven post-inoculation, spleens and peritoneal exudate cells (PECs) were isolated from mice in treatment and control groups (FL and PBS, respectively). The spleen was isolated as it is a major peripheral lymphoid organ housing a significant pool of $CD8^+$ T cells and represents the systemic immune response to IAV (87). PECs were isolated from the peritoneal cavity as they are indicative of a local immune response to

i.p. injection.

Isolated spleens were individually homogenized in 5 mL of PBS and then transferred into a 15 mL Falcon tube. Next, both splenocyte and PEC suspensions were centrifuged at 1400 RPM for 4 minutes at 4 °C. The cell pellets were then racked and 4 ml of ACK lysis buffer (0.15M ammonium chloride, 1.0 mM potassium bicarbonate and 0.1mM ethylenediaminetetraacetic acid) was added for precisely 4 minutes to eliminate erythrocytes. To stop cell lysis, 10 mL of PBS was added to each tube and cells were spun once more (same setting as above). The cell pellet was then racked and cells were resuspended in 10 mL of complete medium. Large cellular debris, such as fat, was removed from the splenocyte suspension by rotating Falcon tubes while transferring the cellular suspension into new 15 mL Falcon tubes. After re-centrifugation, both splenocytes and pooled PEC suspensions were counted and seeded into a U-bottom, 96-well polystyrene plate (Falcon BD). Splenocytes were seeded at 2×10^6 cells/well, while PECs were seeded at $5 \times 10^5 - 1 \times 10^6$ cells/well.

Once in their respective wells, medium alone (nil), irrelevant peptide (control) or specific synthetic IAV-derived peptides were added. Peptides were added at a final concentration of 500 nM. The irrelevant peptide used was nucleoprotein (NP) 118, which is a immunogenic peptide of the lymphocytic choriomeningitis virus (LCMV). This peptide is restricted by H-2^d. NP_{118}-specific $CD8^+$ T cell responses are not detectable in IAV-inoculated mice because the mice were not infected with LCMV. In parallel with control and cognate peptides, the *in vitro* flu-infected cells were added into the wells containing bulk splenocytes or PECs to account for an overall anti-IAV $CD8^+$ T cell response. Roughly 200,000 flu-infected cells were added per well. Once the peptides and

flu-infected cells were added, the plate was placed in an incubator (37 °C; 6% CO_2). After a two-hour incubation period, brefeldin A (BFA) (Sigma-Aldrich) was added to the wells at a final dose of 10 μg/mL. BFA inhibits the transport of proteins from the endoplasmic reticulum (ER) to the Golgi and thus leads to the accumulation of IFNγ, among other soluble mediators, inside activated *in-vivo* primed T cells. Cells were then placed into the incubator for another 3 to 4 hours after which time they were stained.

Following this incubation, cells in the U-bottom plate were spun at 2100 RPM for 5 minutes. Once confirming the presence of cellular pellets, the supernatant was discarded and the plate was gently vortexed to loosen the pellet. 20 μL/well of Fc Block was added to prevent non-specific binding of fluorochrome-labeled antibodies to Fc receptors. After 20 minutes, APC-conjugated anti-mouse CD8α mAb was diluted in PBS/bovine serum albumin (BSA) in a 1:200 ratio. Fifty μL of the diluted antibody was added per well via a multi-channel pipette and left on ice for 30 minutes. Cells were then washed with PBS and fixed using 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 20 minutes at room temperature in the dark. Cells were washed three times, and then left to incubate overnight with 50 μL of diluted FITC-conjugated anti-mouse IFN-γ mAb. The antibody was diluted with cold PBS, which contained 0.1% saponin (Calbiochem), whose purpose is to permeabilize the cellular membrane allowing the IFN-γ mAb to gain access to the interior of the cell, for instance to the ER. After the overnight incubation, cells were washed thrice, resuspended in 200 μL of PBS/BSA and transferred to individual fluorescence activated cell sorting (FACS) tubes (Falcon BD) to be read by flow cytometry.

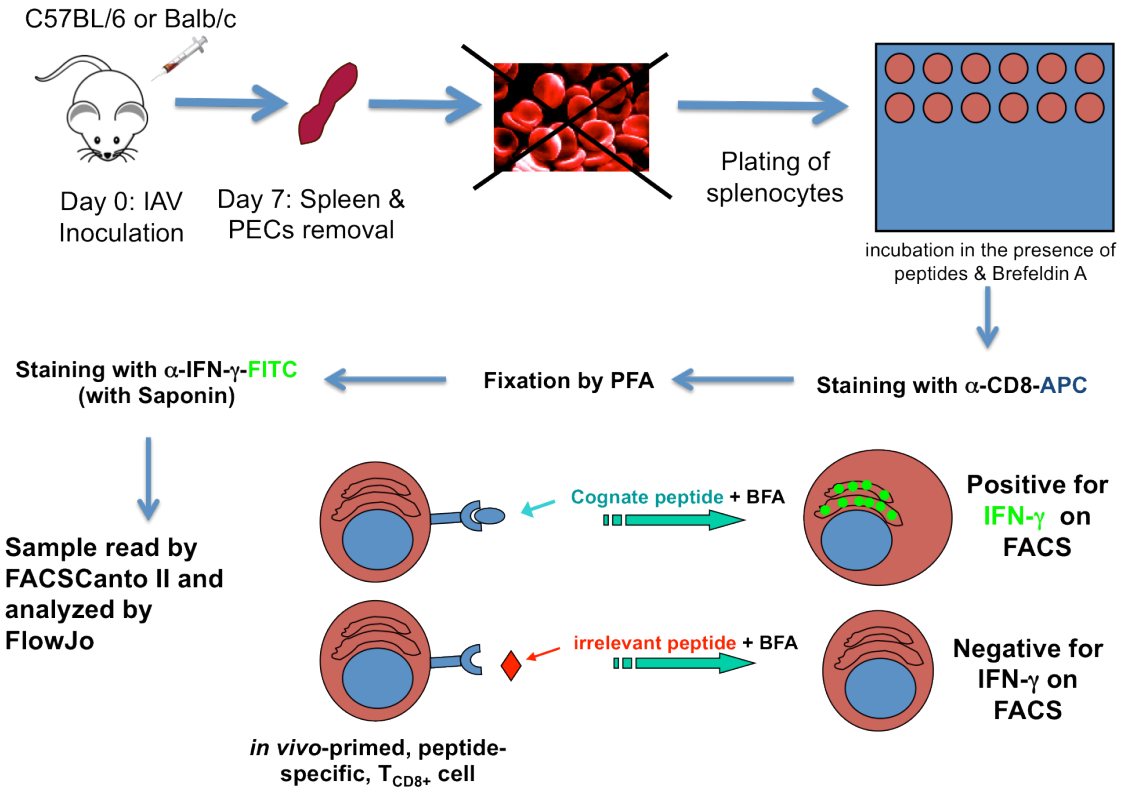

Figure 1: Intracellular cytokine staining overview. In brief, spleens and PECs were harvested from Balb/c mice seven days after PR8 inoculation. Splenocytes and PECs were purified and plated in the presence of synthetic IAV-specific peptides in order to re-simulate *in-vivo* primed $CD8^+$ T cells. Cells were stained for CD8 and intracellular IFNγ, after which samples were read by a FACSCanto II flow cytometer.

2.9 Bone Marrow Isolation

Balb/c mice were euthanized via cervical dislocation. Bone marrow-derived DCs (BMDCs) were generated as previously described (63). In brief, femurs and tibias were dissected and placed into a tissue culture dish (Falcon BD) containing PBS. Using scissors and forceps the muscle was stripped away eventually isolating the bones. Holding the bare bone with forceps, the top and bottom of the bone were cut, exposing a hole on each end. A 25G needle (Becton Dickinson) attached to a 10 cc syringe (Becton Dickinson) filled with PBS was inserted into one side of the bone and used to flush out all marrow cells and particles, directly into a 50 mL Falcon tube until the bone turned white and was completely washed out. Once collected, the marrow cells were centrifuged for 4 minutes at 1400 RPM. The cells were subsequently racked and treated with ACK lysis buffer to remove all erythrocytes. PBS was added to neutralize the buffer, and once again the cells were centrifuged. After racking and resuspending the cells in PBS, the suspension was filtered through a 40 μm cell strainer (Falcon BD) into a new 50 mL Falcon tube to remove non-cellular particles. Cells were centrifuged once again and finally resuspended in 2 mL of cRPMI. Cells were counted and adjusted to 1 million cells/mL (in complete medium). Four million BM cells (4 mLs) were then transferred into each well of a 6-well plate (BD Falcon) and incubated at 37°C and 6% CO_2.

2.10 Flow Cytometry Analysis

Cytofluorimetric analyses were performed using a BD FACSCanto II flow cytometer (BD Biosciences). A minimum of 1×10^5 events were acquired for splenocytes and bone marrow samples, while 5×10^4 events were gathered for each PECs sample. After collection, the data was analyzed using FlowJo software (Tree Star, Ashland, OR).

Once events were displayed in FlowJo, using forward and side scatter, all live cells were gated on, excluding dead cells. Forward scatter is indicative of cell size, while side scatter specifies cellular granularity and/or content complexity. Dead cells have low forward and side scatter profiles and are therefore represented in the bottom left corner (Figure 2A). Analyzing from the live cell population only, the x-axis was changed to the APC-anti-$CD8^+$ stain. The population of live cells expressing CD8 on their surface was then specifically gated (Figure 2B). From here, any possible differences in the percentage of $CD8^+$ cells due to FL treatment can be noted and analyzed. Expression of intracellular IFNγ was then analyzed on the gated live $CD8^+$ cell population. The percentage of $CD8^+$ T cells that expressed IFNγ was then calculated (Figure 2C and 2D). $IFN\gamma^+$ $CD8^+$ live cells represent the IAV-peptide-specific $CD8^+$ T cell population in the spleen (or among PECs) of treated animals for each individual peptide tested. In order to provide an accurate calculation of the response to each peptide, the percentage of nil-treated $CD8^+$ $IFN\gamma^+$ of each spleen (or PECs) was subtracted from the percentage of peptide treated $CD8^+$ $IFN\gamma^+$ for each cognate peptide. Another method used to ensured accuracy and fair analyses involved placing the $CD8^+$ IFN-γ^+ rectangular gate on only the nil samples first (for each individual spleen). The exact locations of the nil gates were then applied to all peptides and virus-infected cell samples within their respective spleen or PEC sample.

2.11 Assessment and Staining of FL-Treated Bone Marrow Cells

After bone marrow cells were plated and incubated in a 6-well plate, 200 ng of FL was added to each well. Pictures of FL-treated wells and non-treated cultures were taken from day 0 to day 6. On day 6, 1 million cells from each well (3 treated and 3 non-treated) were transferred into six separate 5 mL BD FACS tubes. Two mL of staining

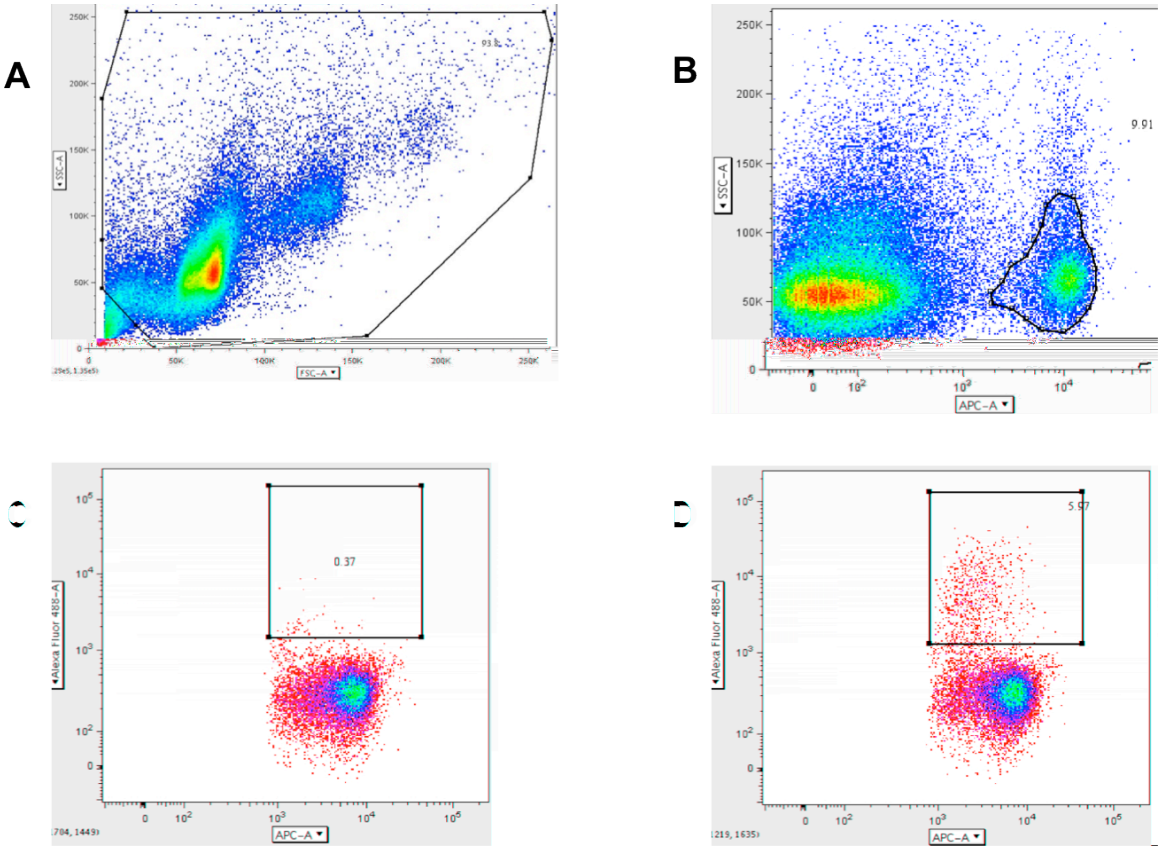

Figure 2: Gating strategy employed to identify IAV-specific CD8+ T cells. Each dot represents one event. This figure is a representative example of one analyzed sample. *A*, Initially the total live cell population is gated upon. Then, live cells positive CD8 were gated on (*B*). Then, $CD8^+$ gated live cells that co-expressed intracellular IFN were gated. *C,* represents the $CD8^+$ $IFN\gamma^+$ control population, when cells were incubated in medium alone (0.37%). *D*, represents the $CD8^+$ $IFN\gamma^+$ population, cells were re-stimulation with the NP147 peptide, the most immunodominant peptide epitope of IAV in the Balb/c strain.

buffer (PBS +1% FCS) was then added into all tubes and centrifuged at 1400 RPM for 4 minutes. After the spin, each tube was incubated with 20 μL of Fc block for 20 minutes on ice to prevent non-specific binding of mAbs. Without washing off the Fc block, 50 μL of a master mix containing FITC-anti-CD11c and APC-anti-B220 mAbs diluted in the staining buffer was added to each tube. After 30 minutes of incubation in the dark, the stained cells were washed twice, once with staining buffer and once with PBS. Cells were then fixed, resuspended in 500 μL of 1% paraformaldehyde and read by a FACSCanto II flow cytometer.

2.12 Chromium Release Assay (^{51}Cr release assay)

To determine the effect of FL on CTL-mediated killing, a standard ^{51}Cr release assay was used. Briefly, splenocytes were isolated using the same methodology explained above for my ICS experiments. However, rather than being treated with peptides and virally infected cells, these splenocytes were used as effector cells against chromium-labeled P815 (H-2^{d} mouse lymphoblast-like mastocytoma cells) target cells. These target cells, prior to cultivation with effector cells, were labeled with ^{51}Cr and pulsed with each specific IAV peptide. After this incubation period, target cells incorporate ^{51}Cr within their cellular membrane and present the respective peptide they are pulsed with via MHC Class I molecules. An "overall" killing percentage to IAV was determined by using IAV-infected P815 cells (similar to the *in vitro* infection done on DC2.4 and 3T3 cells). This allows a natural display of peptide antigens on MHC Class I by P815 cells. In the presence of the splenocyte pool, effector CD8^{+} T cells that have seen the IAV peptide before will lyse the target cell effectively releasing ^{51}Cr into the supernatant.

Effector splenocytes were seeded into a 96-well plate and 10^{4} target cells were

added per well. Cells were plated to provide an effector to target cell ratio of 30:1, 100:1 and 300:1. The cells were left to incubate together for 9 hours at 37°C and 6% CO_2. After the incubation period, the supernatant was harvested from each sample and the ^{51}Cr release was calculated on a gamma counter. Specific killing by $CD8^+$ T cells was calculated using the following formula: $\frac{ER-SR}{TR-SR}$ x 100. ER is defined as experimental release, which is the authentic ^{51}Cr release from wells containing effector and target cells. SR, or spontaneous release, is determined from wells containing target cells with the addition of media only. This provides the amount of ^{51}Cr that is naturally released in this assay, a simple negative control. On the contrary TR or total release, is determined from wells receiving target cells in addition to Triton X-100 (Sigma-Aldrich). Triton X-100 is a biochemical reagent that causes the lysis of all cells and serves the purpose of being a positive control, indicating the total amount of ^{51}Cr that can be released.

2.1.3 Statistical Analysis

For ICS results, graphed values were adjusted by subtracting background levels of IFN-γ. Background levels were obtained from wells receiving only medium. Statistical comparisons were performed using Student's t-test. Error bars represent the standard error of the mean. Graphs generated using GraphPad Prism software (GraphPad Prism Software, Inc. CA, USA). Significant values $p < 0.05$ and $p < 0.01$ are denoted by * and **, respectively.

Chapter 3:

Results

3.1 The effect of FL on DC generation *in vitro*

Before testing the ability of FL in DC mobilization leading to augmentation of antiviral $CD8^+$ T cell responses, as hypothesized, it was necessary to determine the effectiveness of the FL stock in expanding DC populations. According to the literature, the dosage of FL given should be sufficient to promote the transition of bone marrow cells into a DC phenotype (88). Originally, this was tested *in vitro*, by incubating bone marrow cells isolated from Balb/c mice with 200 ng/ml of FL or nil (control), for 6 days. The cultured cells were then examined both morphologically and phenotypically for their differentiation into DC. Microscopic pictures of both treated and non-treated cells were taken daily (Figure 3). From these pictures, it is evident that bone marrow cells treated with FL showed an increase in number compared to the control. In addition, on day 6, dendrite projections were evident on FL-treated bone marrow samples. While an apparent overall increase in the number of cells and dendritic projections in the FL treatment group, this was not sufficient in proving that FL was expanding DC populations. This question was answered through staining for CD11c and B220 on day 6 (Figure 4). From this in vitro stain, it is clear that FL-treated bone marrow sample contains more $CD11c^+$ $B220^+$ cells (2.6% control vs. 12.5% FL treatment), which is indicative of plasmacytoid DCs (78, 79). Furthermore, there was an increase in the $CD11c^+$ $B220^-$ cell population, which represents cDCs (0.36% control vs. 11.6% FL treatment) (77).

3.2 The effect of FL on DC populations *in vivo*

Although it was reassuring that our FL stock had the ability to expand DC populations *in vitro*, it was essential to test the ability of FL to expand these populations *in vivo*. Therefore, to determine the effectiveness of FL treatment *in vivo,* Balb/c mice

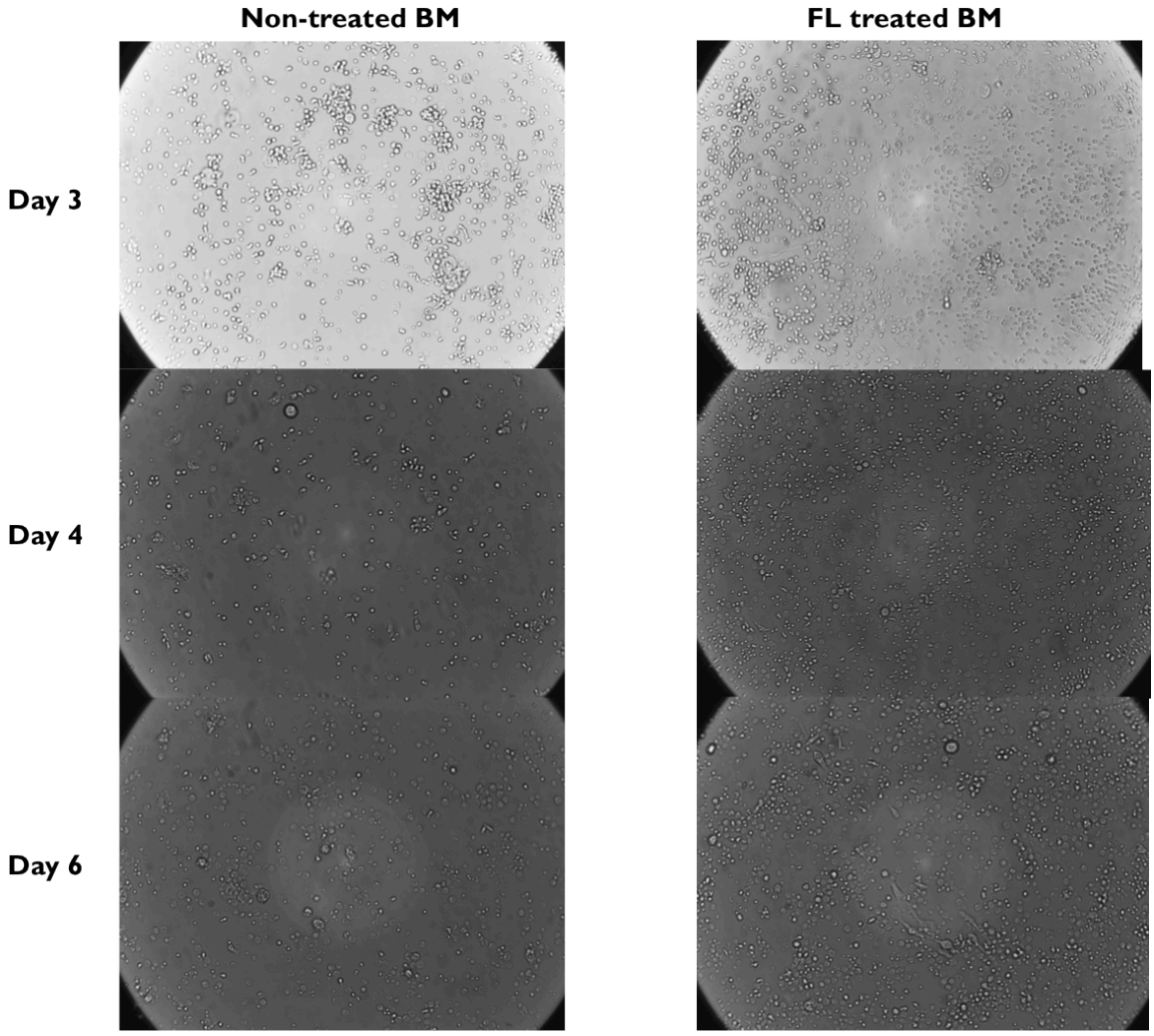

Figure 3: Microscopic pictures of bone marrow cells treated with FL. Isolated bone marrow cells (4 x10^6 cells) were incubated for 6 days either in the presence or absence of FL (200 ng/ml). From day 3 the effect of FL treatment is apparent and leads to formation of dendrites that are visible around day 6. Pictures were taken from VWR VistamodelVison microscope (model #: 82026-630) at 25x magnification.

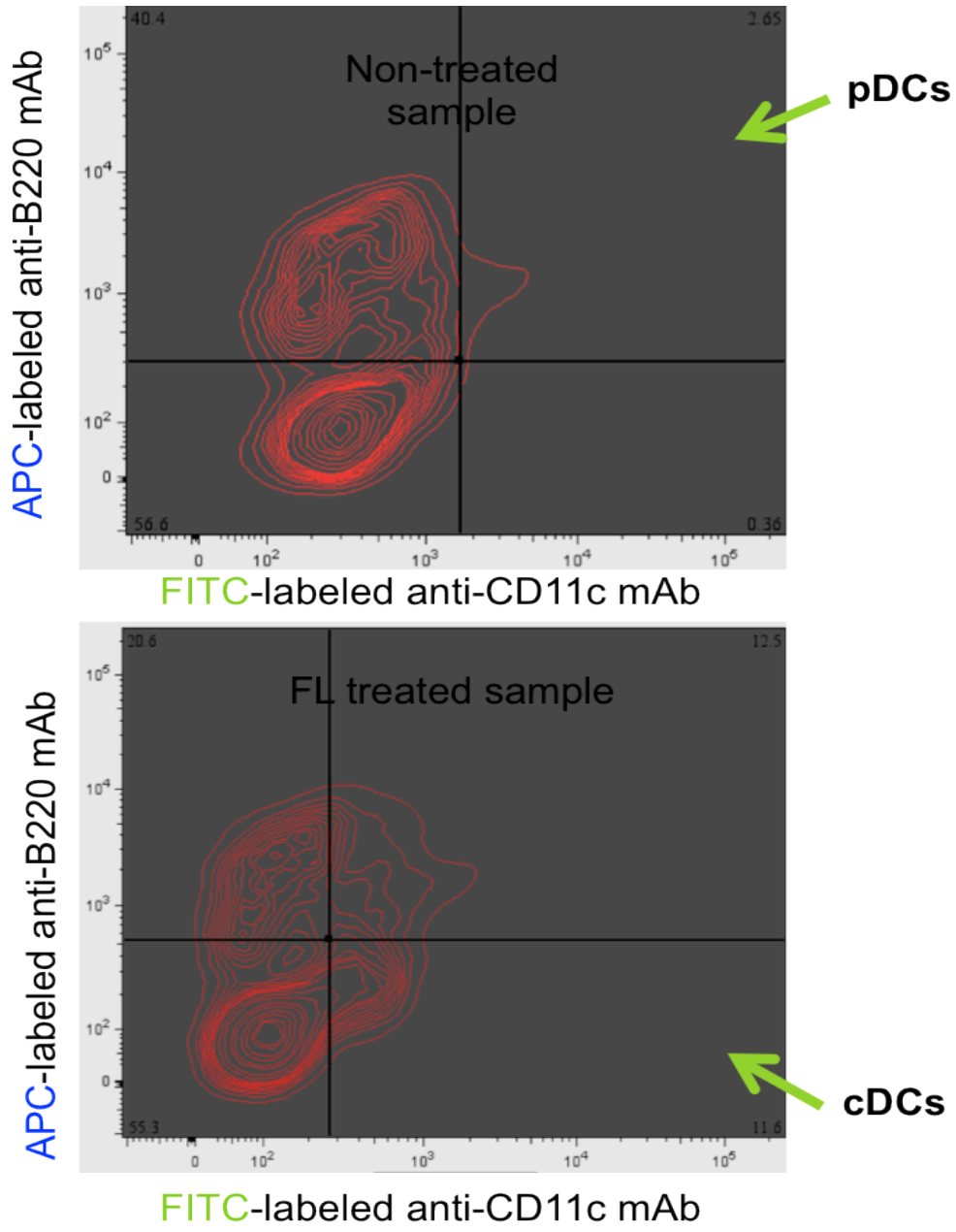

Figure 4: Contour plot of *in vitro* cultured Balb/c bone marrow cells. FL-treated and untreated bone marrow cells were washed and stained for CD11c and B220 in order to test the effectiveness of FL treatment in expanding DC popultions. Gates for each sample were individually placed due to a shift in population during reading. Contour plots indicate the presence of multiple cells populations rather than individual cells. FL treatment leads to an increase in both DC populations *in vitro* (plasmacytoid and conventional).

were subcutaneously injected with either FL (10μg in 100μl of PBS) or sterile PBS (control) for 10 days as previously described (85). On day 10, three measures were used to determine the ability of FL to expand the DC populations. The first measure was the ability of FL to increase the spleen size (splenomegaly), which is customary during FL treatment (63). Spleens of both FL-treated mice and PBS-treated mice were isolated and compared for their size (Figure 5). This picture illustrates that FL treatment did in fact lead to splenomegaly. The next step was to determine that this increase in size was due to an increase in splenocytes and not due to other possible effects (e.g. increase in the amount of fat, fluid, red blood cells, etc.). Once isolated, spleens were homogenized and splenic cells were counted. Figure 6 illustrates an increase in the number of cells found in FL-treated spleens. Finally, the last step was to determine if the increase in cells was due to an expansion of DC populations. Splenocytes were stained using the same mAbs (CD11c and B220) (Figure 7). Splenocyte staining clearly showed an increase in both $CD11c^+$ $B220^+$ and $CD11c^+$ $B220^-$ populations, indicative of an increase in the DC compartments.

3.3 Confirmation of the ID Hierarchy

Prior to determining the ability of FL to increase the $CD8^+$ T cell responses to various viral epitopes, as hypothesized, it was important to confirm that the IAV ID hierarchy does in fact stay consistent in my hands. To determine this, mice were injected with PBS subcutaneously for 10 days, mimicking the upcoming FL treatment regimen. On day 0, mice were injected with IAV (PR8 strain). Seven days later, a time point at which PR8-specific $CD8^+$ T cell responses reach their peak (87) mice were euthanized and an ICS assay for IFNγ was conducted on the spleens of these mice. In the literature,

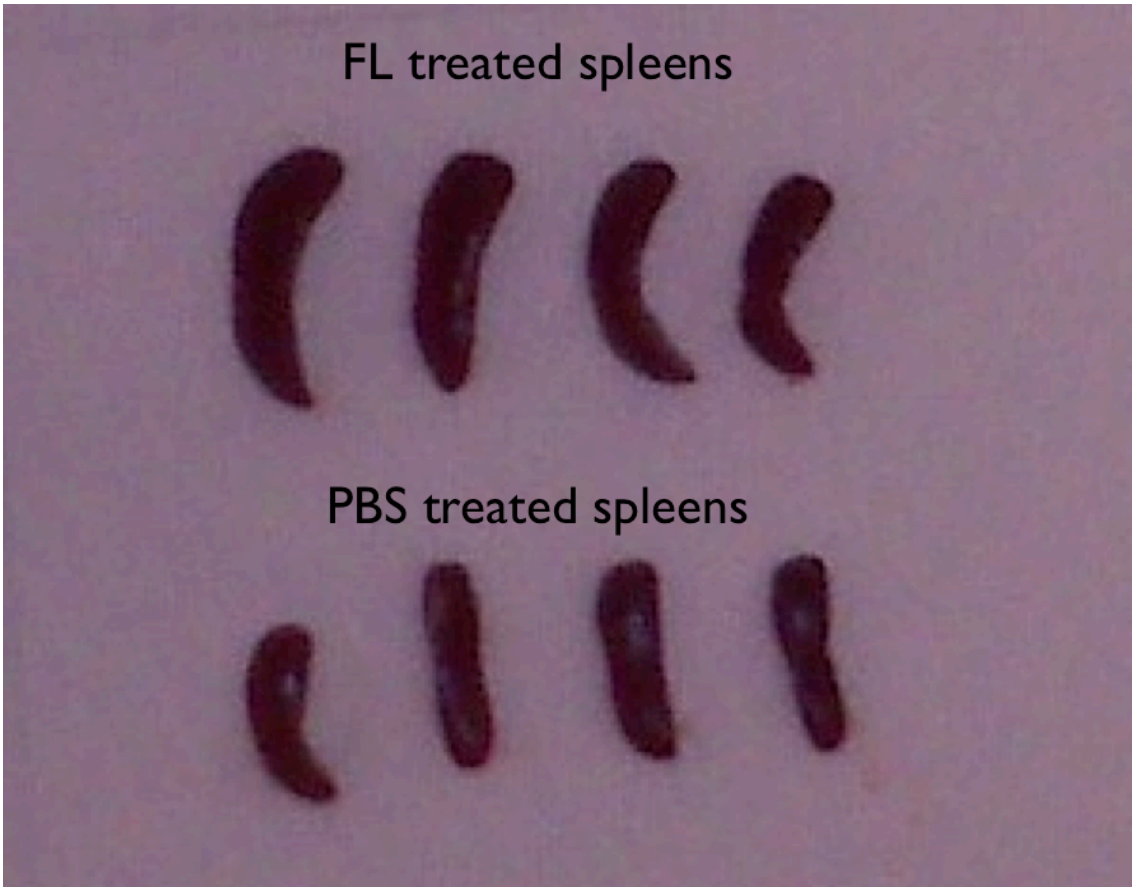

Figure 5: Pictures of isolated spleens from FL treated and control Balb/c mice. Mice were treated with either FL (10 μg) or PBS for 10 days. On day 10, spleens from all mice were isolated and photographed.

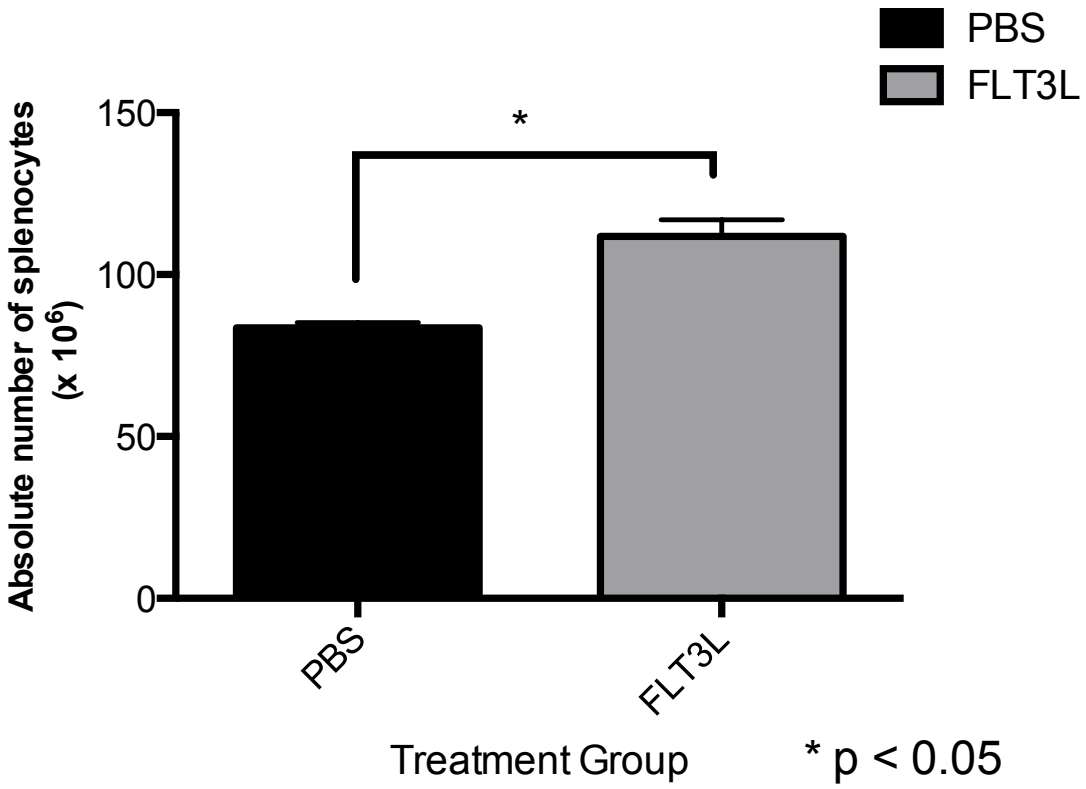

Figure 6: Absolute numbers of splenocytes from FL-treated and control Balb/c mice. Mice were treated with either FL (10μg) or PBS for 10 days. On day 10, spleens from all mice were homogenized after isolation. The data represents one experiment of three independent experiments. * denotes a significant difference, $p < 0.05$ (PBS n=4, FL n=4).

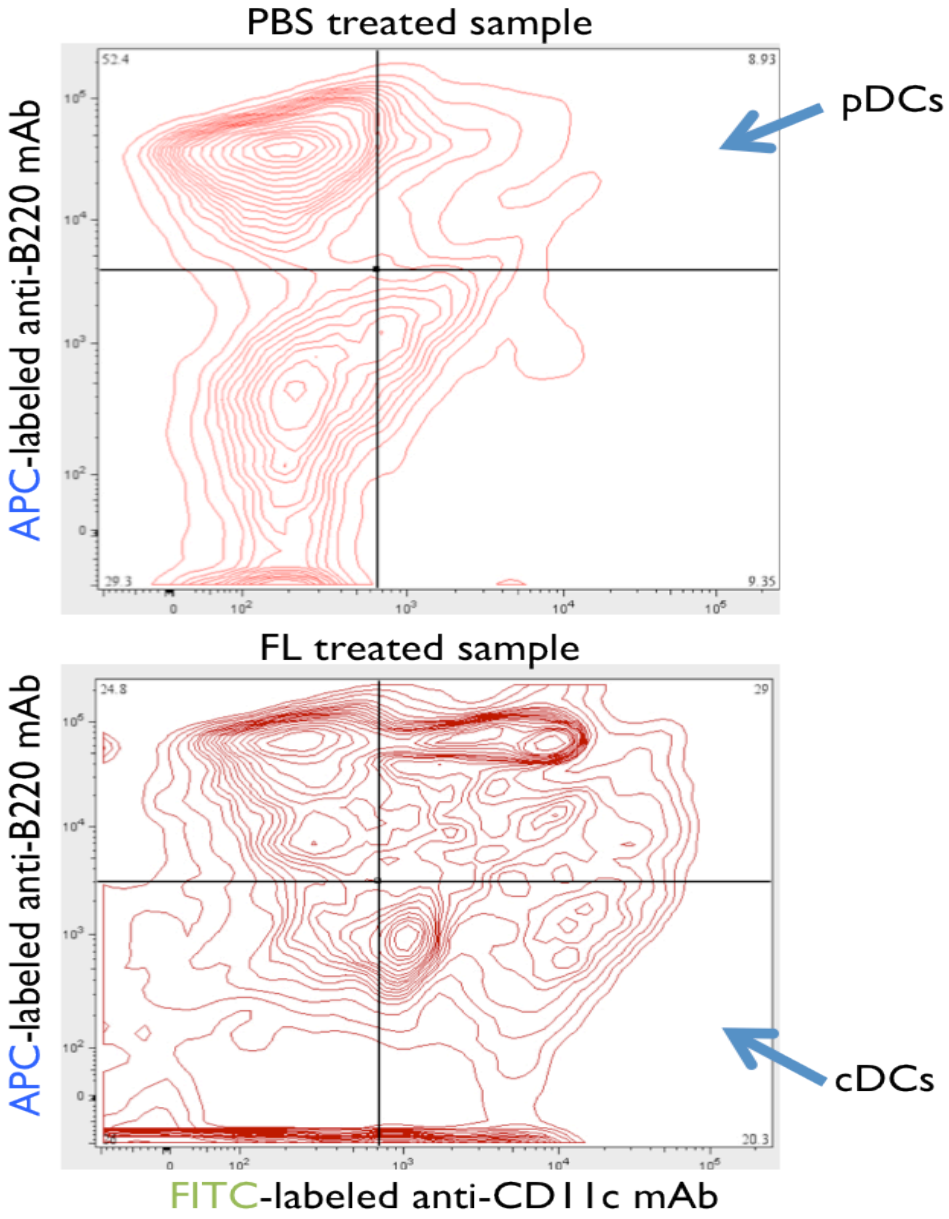

Figure 7: The frequencies of conventional and plasmacytoid DCs in the spleen of FL-treated and control Balb/c mice. Mice were treated with FL (10µg) or PBS for 10 days. On day 10, spleens from all mice were isolated and homogenized followed by staining for CD11c and B220.

previous studies have reported that $CD8^+$ T cell CTL responses to IAV in Balb/c mice are favoured towards the NP_{147} immunodominant peptide (50, 57). Weaker responses are also detectable against so-called subdominant epitopes. These are $PB2_{289}$, HA_{518}, NP_{39}, NP_{218} and HA_{462} in order of decreasing $CD8^+$ T cell response they induce. These experiments showed that $CD8^+$ T cell response follow this well-established immunodominance hierarchy (Figure 8).

3.4 Frequency of $CD8^+$ cells

Prior to determining the absolute number of epitope specific $CD8^+$ T cell responses, I had to determine the frequency of $CD8^+$ cells among live-gated splenocytes. Figure 9 illustrates that both FL- and PBS-treated mice have the same frequency of total $CD8^+$ cells found in their spleen, typically ranging between 12-15%. Therefore, the noted increase in the absolute number of epitope-specific $CD8^+$ T cells likely reflects increases in splenic cellularity and also importantly to heightened frequencies of epitope-specific $CD8^+$ T cells.

3.5 The effect of FL on frequencies of epitope-specific $CD8^+$ T cells

In order to determine if FL has an effect on the percentage of flu-specific $CD8^+$ T cells, mice were injected with FL or PBS for 10 days (day -3 to day 7). On day 0, mice were injected with IAV (PR8 strain). Mice were then euthanized seven days later, which correlates with the peak CTL response to IAV in the literature (89). Immediately after sacrifice the ICS procedure for IFNγ was performed on the splenocytes and PECs of all mice. Subsequently, samples were acquired by a flow cytometer and $CD8^+$ T cell responses were analyzed by FlowJo using the aforementioned gating strategies. After gating on $CD8^+$ $IFN\gamma^+$ populations, the percentage of $IFN\gamma^+$ cells from the $CD8^+$

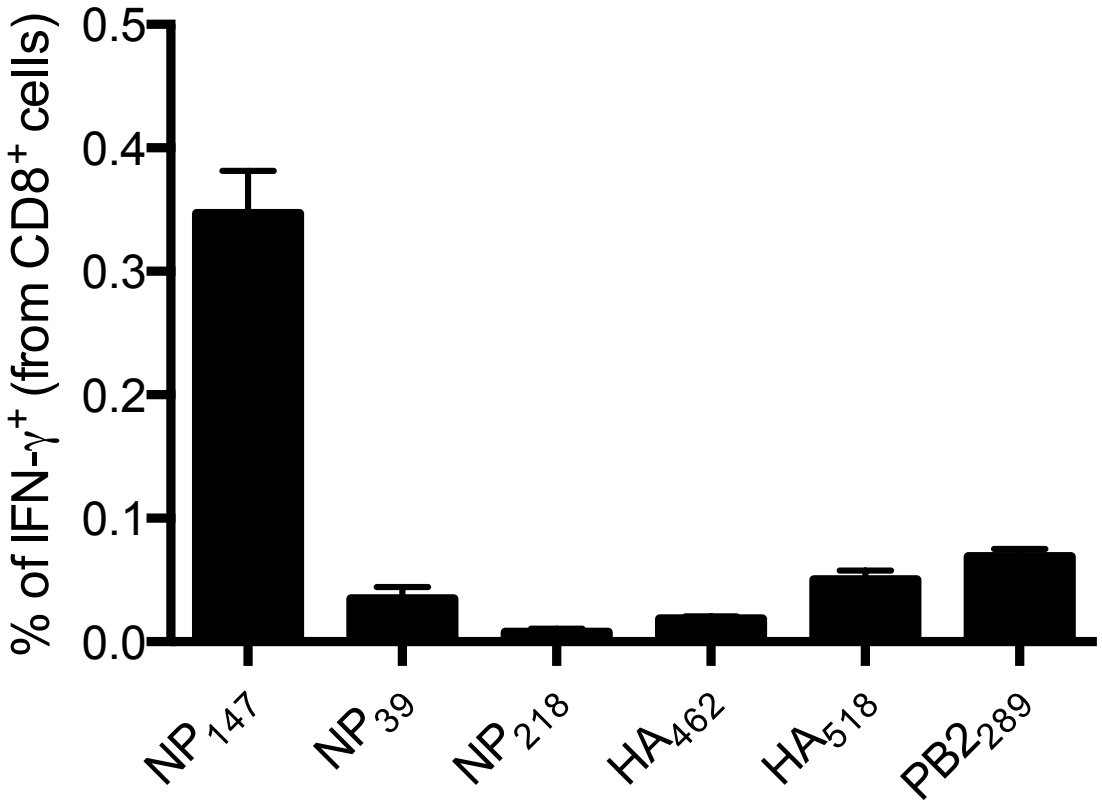

Figure 8: ID hierarchy in $CD8^+$ T cell responses to IAV (PR8 strain) in Balb/c mice. Mice were inoculated with IAV and seven days later an ICS for IFNγ was conducted. Specific peptides corresponding to IAV epitopes were added to splenocytes. $CD8^+$ cells were gated on, followed by determination of $IFN\gamma^+$ producing cell frequencies among $CD8^+$ cells. Error bars represent standard errors of the mean calculated for 4 mice. The observed hierarchical pattern was reproducible in all ICS experiments in this study.

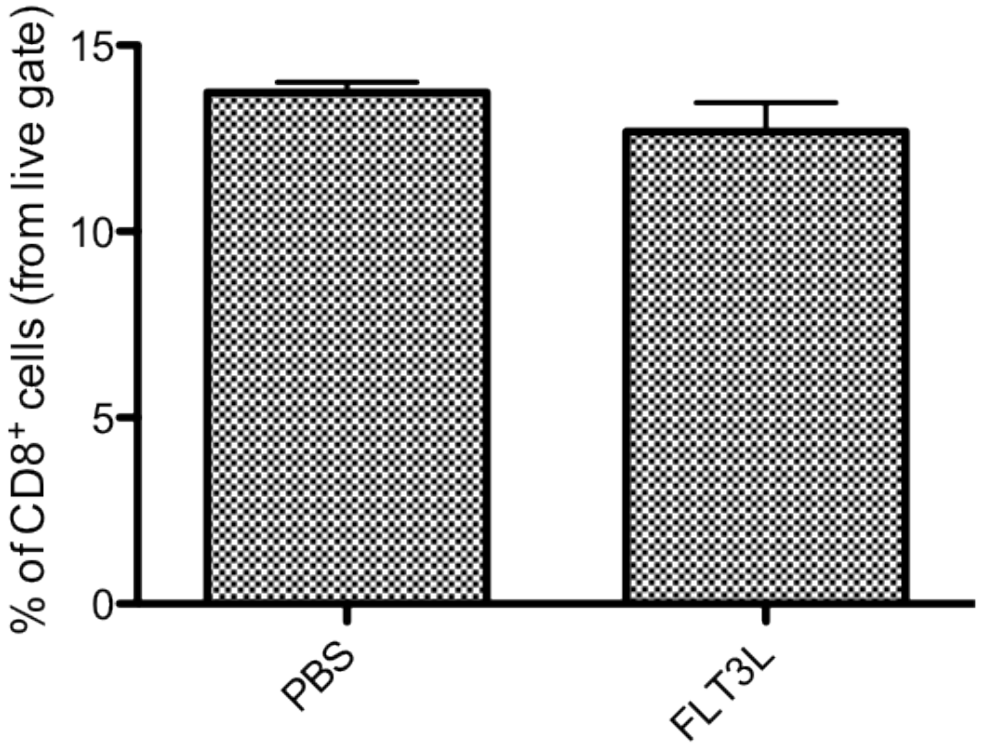

Figure 9: Frequency of $CD8^+$ cells from live splenocytes of Balb/c mice. Mice were treated with FL (10 μg) or PBS for 10 days (day -3 to 6). On day 0, all mice were injected with IAV (PR8 strain). On day 10, mice were euthanized and an ICS was conducted. FL treatment did not alter the frequency of $CD8^+$ cells. This data represents the reproducible trend from one of multiple experiments typically using 4 mice per group.

population was graphically represented (Figures 10 and 11). Figure 10, which reflects the percentage of epitope-specific $CD8^+$ T cells in the spleen, illustrates a clear increase in splenic response for several epitopes in FL-treated mice. Additionally, FL treatment resulted in an increase in the percentage of $CD8^+$ T cells responding to flu-infected cells. In fact, the increase in response for FL-treated mice in three of the six peptides reached statistical significance ($p < 0.05$). Furthermore, it is important to note the consistency of the immunodominance hierarchical pattern. There was no change in the IDD, as NP_{147} remained the most immunogenic peptide. In addition, the other subdominant peptides followed the established hierarchy. The data presented in figure 10 is a compilation of three separate ICS experiments. In total, 9 PBS-treated mice and 12 FL-treated mice were analyzed. Figure 11, which illustrates the $CD8^+$ T cell response in the peritoneal cavity, as a picture of the local response, shows a surprising decrease in percentage of $CD8^+$ T cell response across all epitopes in the FL treatment group. Once again, the ID pattern did stay constant for both treatment groups. The data represented in this figure is the average of three separate ICS experiments, where PECs were pooled from all mice from the same treatment group. Note that it was difficult to conduct ICS assays on PECs obtained from each individual mice because many thousands of cells cannot be recovered from the peritoneal cavity – hence my experiments on pooled PECs for each treatment group.

3.6 The effect of FL on absolute number of flu-specific $CD8^+$ T cells

The absolute number of epitope-specific $CD8^+$ T cells was determined by taking into consideration the number of cells per each spleen, the percentage of $CD8^+$ cells among live splenocytes and the frequencies of epitope-specific $CD8^+$ T cells (i.e. $CD8^+$

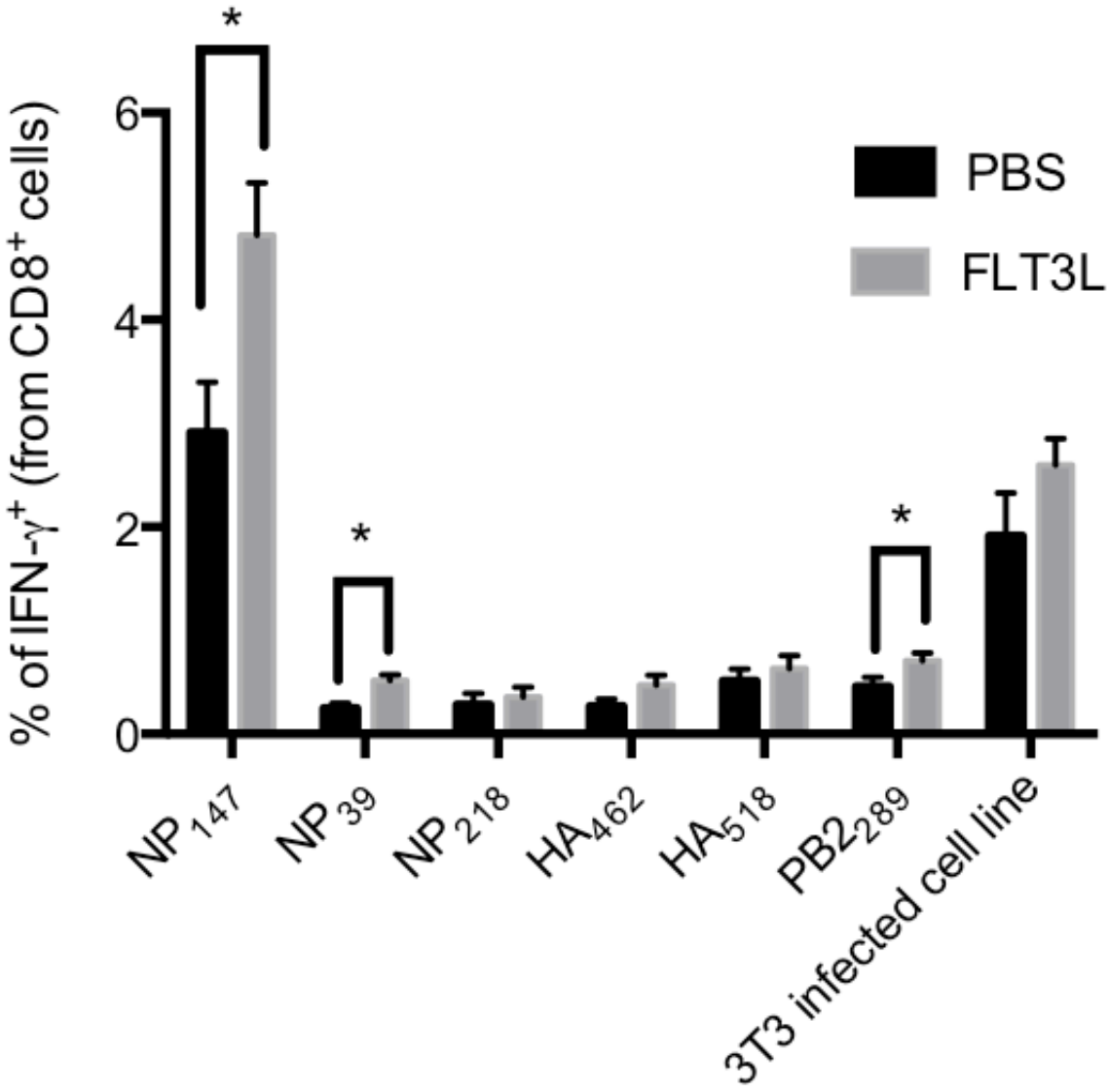

Figure 10: Frequencies of $CD8^+$ $IFN\gamma^+$ cells in the spleens of FL-treated and control Balb/c mice. Mice were treated with FL (10 μg) or PBS for 10 days (day -3 to 6). On day 0, all mice were injected with IAV (PR8 strain). On day 10, mice were euthanized and an ICS was conducted on splenocytes to determine the percentage of the double positive ($CD8^+$ $IFN\gamma^+$) cells, which represents epitope-specific $CD8^+$ T cells. * denotes a significance difference, $p < 0.05$ (PBS n=9, FL n=12).

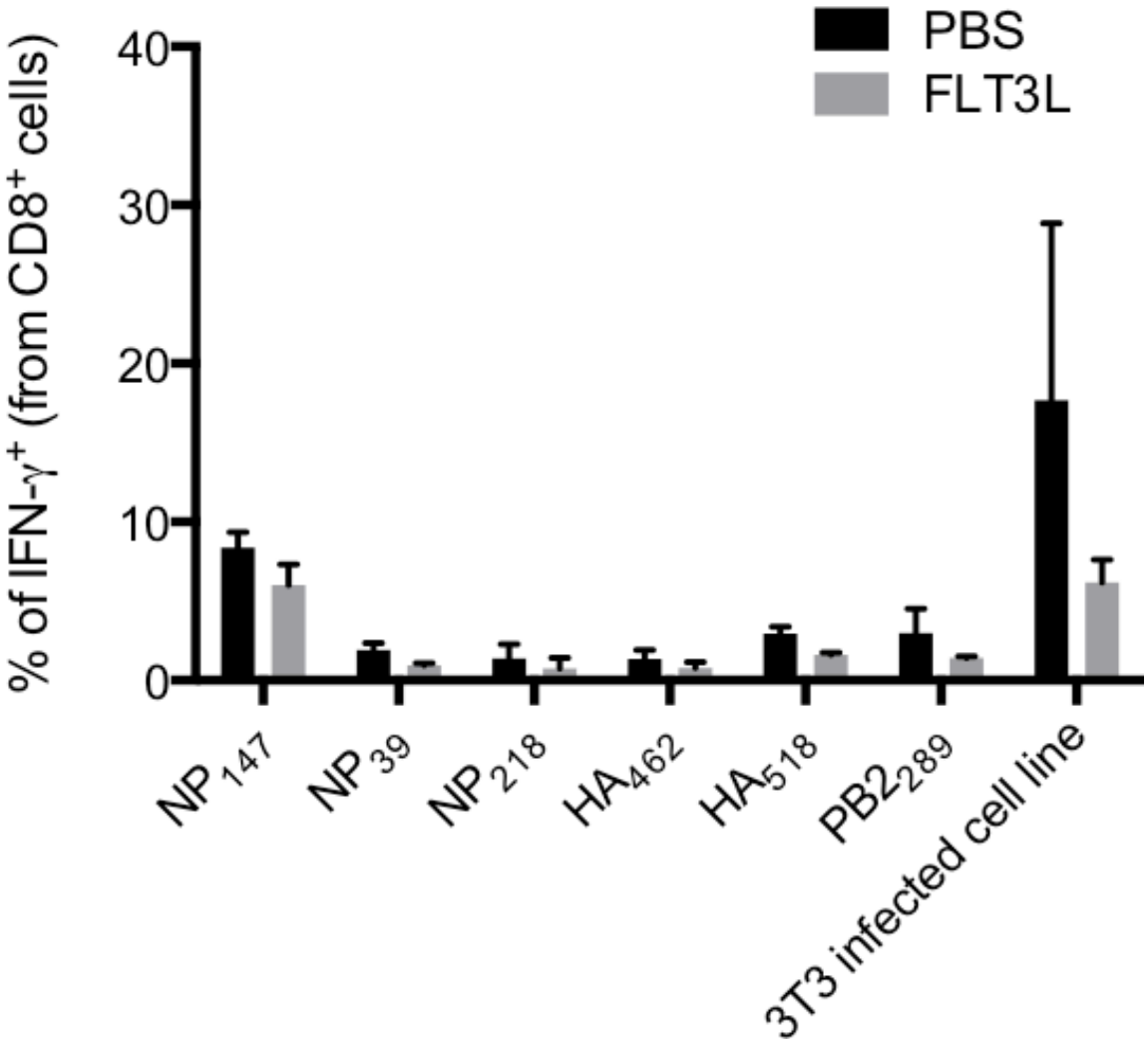

Figure 11: Frequencies of flu-specific $CD8^+$ T cells among PECs of FL-treated and control Balb/c mice. Mice were treated with FL (10 μg) or PBS for 10 days (day -3 to 6). On day 0, all mice were injected with IAV (PR8 strain). On day 10, mice were euthanized and an ICS was conducted to determine the percentages of peritoneal epitope-specific ($CD8^+$ $IFN\gamma^+$) T cells. N = 3 (three separate experiments, pooled PECs).

IFNγ^+ cells). These double positive populations, which represent epitope-specific CD8$^+$ T cells, are illustrated in a graphical representation (Figure 12 and 13). Figure 12 shows an increase in the absolute number of splenic CD8$^+$ IFNγ^+ cells across all epitopes and flu-infected cells as a result of FL treatment. In fact, two epitopes and the overall response, as determined by using flu-infected cells in lieu of individual peptides, reached statistical significance ($p < 0.05$). This is consistent with the data shown in Figure 13, which displays an increase in the absolute number of peritoneal CD8$^+$ IFNγ^+ cells in FL-treated mice. It is of interest to note that although the percentage of CD8$^+$ T cells decrease in PECs after FL treatment, the absolute numbers of effector cells actually increase due to FL treatment.

3.7 The effect of FL on CTL effector function

The primary function of CTLs is to kill virally infected cells. In order to test if FL treatment enhances CTL mediated killing, the gold standard test of chromium release (as mentioned in the Material and Methods) was used. Target cells were incubated with individual peptides in parallel and labeled with 51-chromium simultaneously. During this incubation period viral peptides are presented on the surface of these cells via MHC class I molecules. Figures 14 and 15 depict the killing ability of CTLs isolated from PBS and FL-treated mice. The y-axis, "% killing", was calculated using the formula found in the Methods section, which takes into consideration total and spontaneous chromium release. Figure 14 specifically looks at the killing ability towards cells that present the most immunodominant peptide (i.e. NP_{147}). As expected, the killing percentage towards cells that presented NP_{147} was higher than those presenting subdominant peptides (Figure 15), regardless of treatment. FL treatment significantly increased the killing ability of CTLs

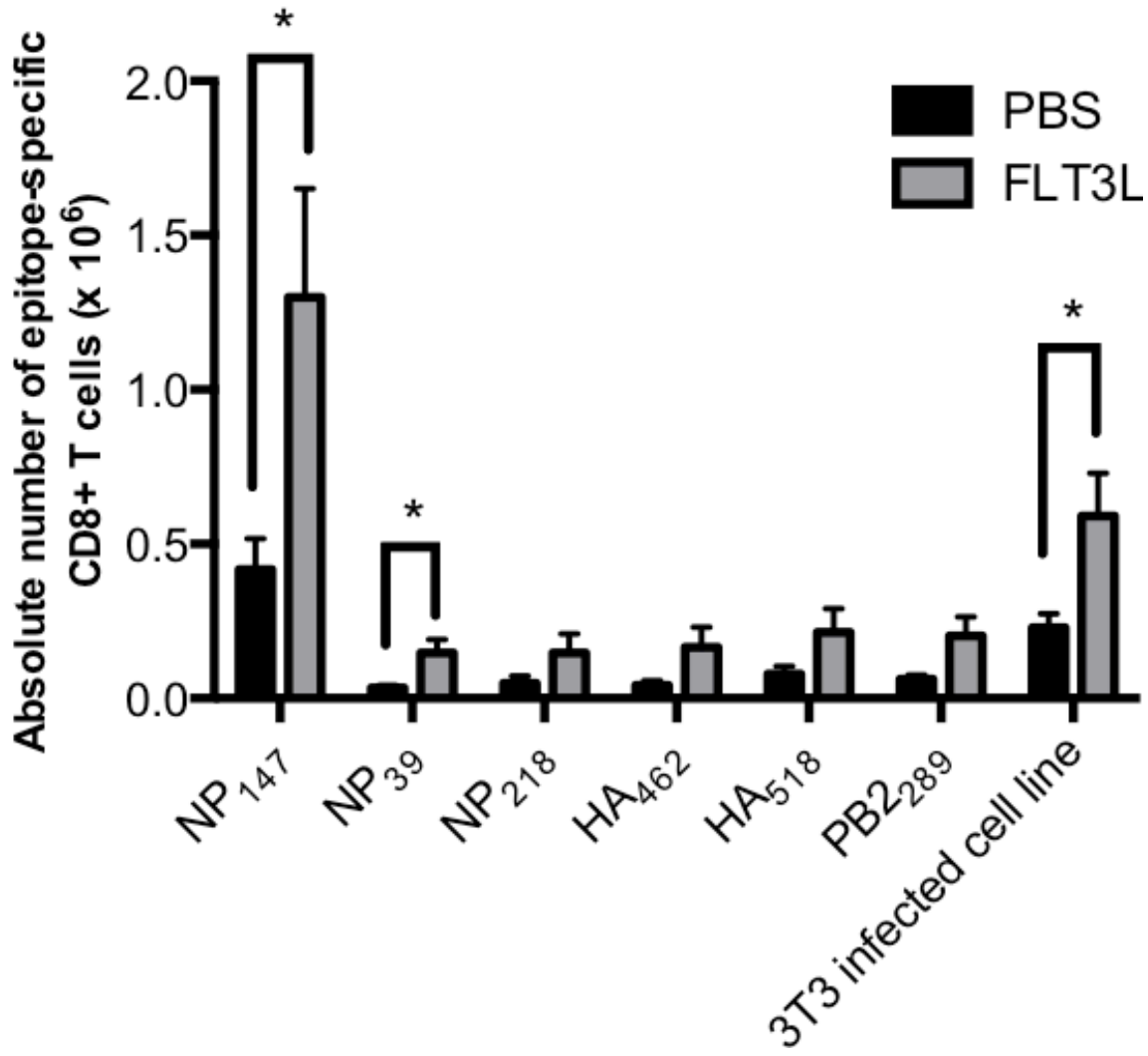

Figure 12: Absolute number of $CD8^+$ $IFN\gamma^+$ cells in the spleen of FL-treated and control Balb/c mice. Mice were treated with FL (10 μg) or PBS for 10 days (day -3 to 6). On day 0, all mice were injected with IAV (PR8 strain). On day 10, mice were euthanized and an ICS was conducted on splenocytes. Absolute number of $CD8^+$ T cells was then calculated as described in the text. * denotes a significance difference, $p < 0.05$ (PBS n=9, FL n=12).

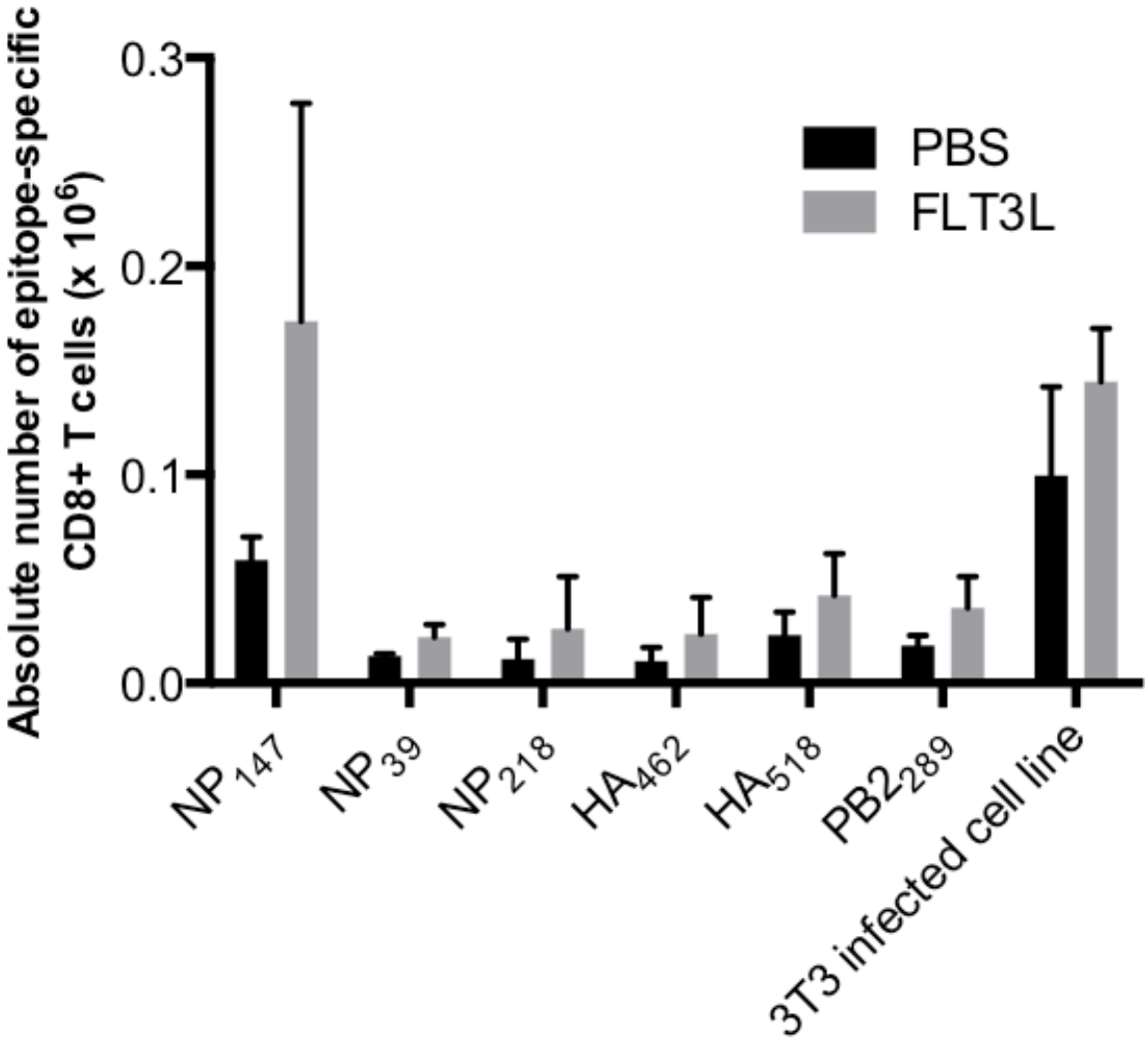

Figure 13: Absolute number of peritoneal $CD8^+$ $IFN\gamma^+$ cells in FL-treated and control Balb/c mice. Mice were treated with FL (10 μg) or PBS for 10 days (day -3 to 6). On day 0, all mice were injected with IAV (PR8 strain). On day 10, mice were euthanized and an ICS assay was conducted on PECs and absolute number of flu-specific $CD8^+$ T cells was calculated. N = 3 (three separate experiments, pooled PECs)

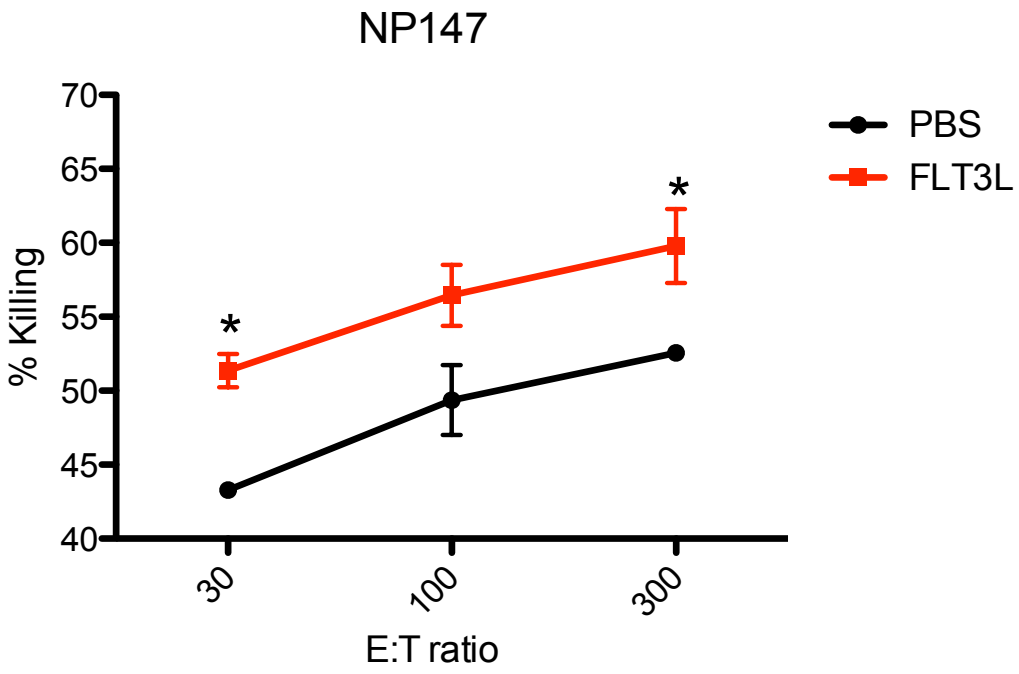

Figure 14: CTL-mediated killing of NP_{147}-pulsed target cells following FL treatment. P815 target cells were pulsed with 51-chromium and NP_{147}. Target cells were then incubated with splenocytes isolated from mice that had received PBS or FL treatment. * denotes a significance difference, $p < 0.05$ (PBS n= 4, FL n= 4)

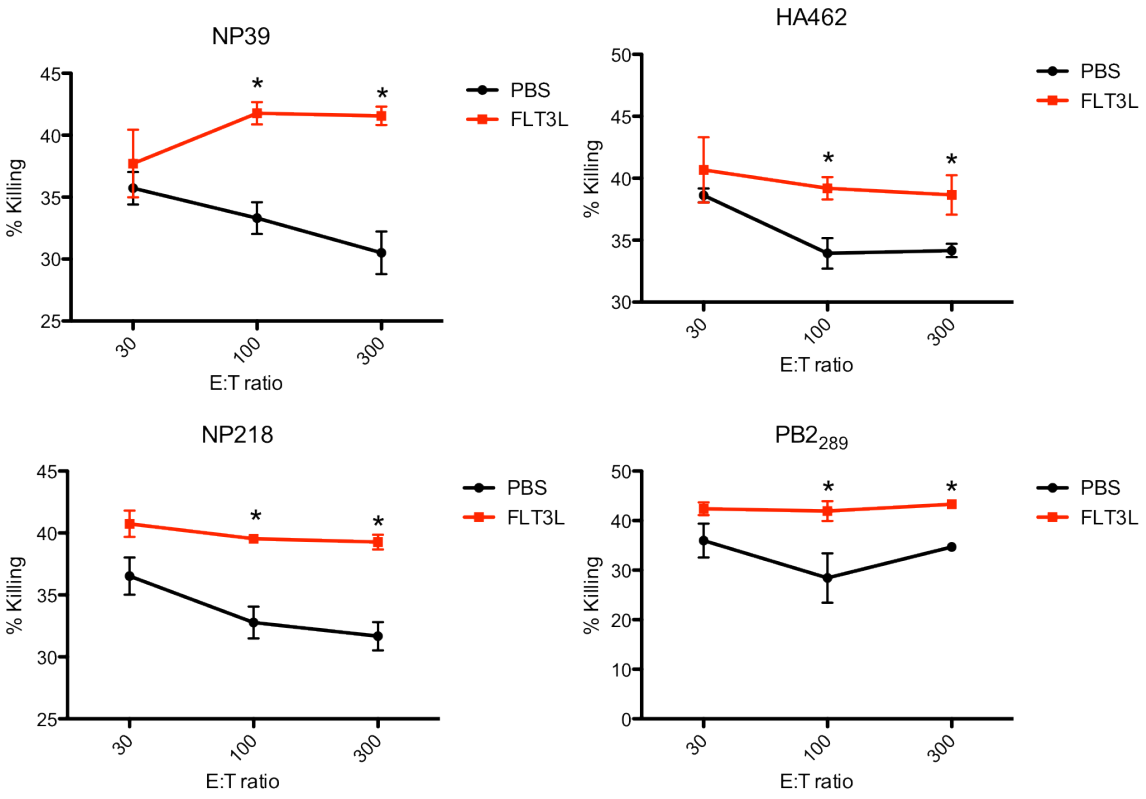

Figure 15: CTL-mediated cytotoxicity exerted by splenocytes obtained from FL- and PBS-treated mice. Target cells were pulsed with 51-chromium and subdominant peptides. Target cells were then incubated with splenocytes (effector cells) isolated from mice that had received PBS or FL treatment. * denotes a significance difference, $p < 0.05$ (PBS n= 4, FL n= 4)

against NP_{147}. Figure 15 demonstrates that FL treatment increased the killing ability of CTLs against some subdominant peptides as well.

3.8 The effect of FL on recall flu-specific $CD8^+$ T cell responses

In order to determine if FL treatment increases the ability of $CD8^+$ T cells to respond to secondary exposure of IAV, a different time course experiment was designed. For ten days, mice were given either FL or PBS treatment subcutaneously (day -3 to day 6). Similarly to previous experiments, on day 0, IAV (PR8 strain) was administered to all mice. Instead of performing an ICS seven days later, mice were allowed to recover from this viral inoculation (day 7 to day 29). However, on day 30, mice were once again injected with IAV (SEQ12 strain). Seven days later, an ICS assay was conducted on the spleens of these animals. It should be noted that SEQ12 was used for the secondary inoculation to avoid an established antibody response. SEQ12 maintains the same epitopes as PR8, but avoids antibody recognition due to its altered external protein coat (87). The ICS methodology and data analysis for this experiment and previously explained experiments remain identical. Figures 16 and 17 demonstrate the effect of FL treatment on CTL recall responses. Figure 16, which takes into consideration the percentage of $CD8^+$ T cell response, shows a decrease in recall response across all epitopes. This is in agreement with Figure 17, which shows a decrease in the absolute number of epitope-specific $CD8^+$ T cells across all epitopes. However, it is important to note that the ID hierarchy remains unchanged in recall experiments.

3.9 The effect of FL on CTL responses to IAV in BL/6 mice

In order to determine whether or not the selectively enhancing effect of FL on IAV-specific CD8+ T cell responses is limited to one mouse strain ($H\text{-}2^d$ Balb/c) only, I

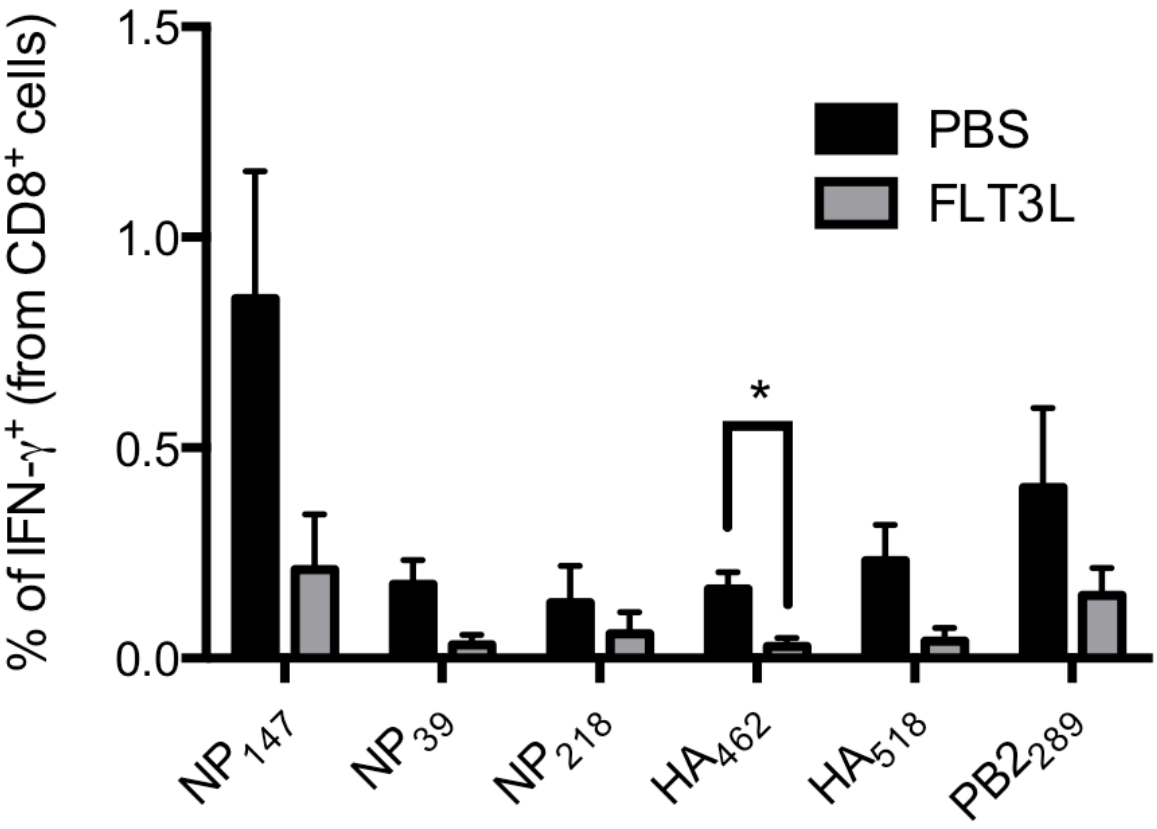

Figure 16: Frequencies of flu-specific CD8$^+$ T cells following secondary exposure of FL- and PBS-treated mice. Mice were treated with FL (10 μg) or PBS for 10 days (day -3 to 6). On day 0, all mice were injected with IAV (PR8 strain). Mice were allowed to overcome this initial viral inoculum (day 7 to 29). On day 30, mice were re-exposed to IAV (SEQ12 strain). Seven days later, mice were euthanized and an ICS was conducted on splenocytes. * denotes a significance difference, $p < 0.05$ (PBS n=4 FL n=4)

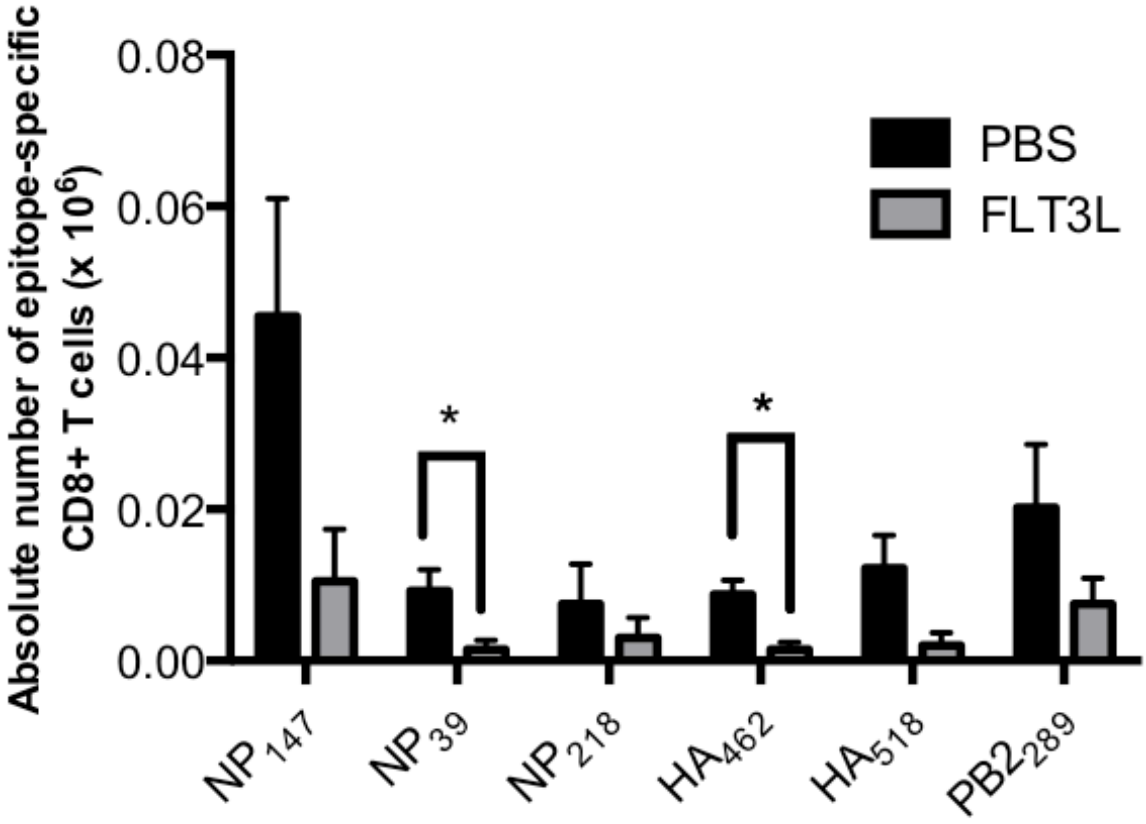

Figure 17: Absolute number of flu-specific $CD8^+$ T cells following secondary exposure in FL- and PBS-treated mice. Mice were treated with FL (10 μg) or PBS for 10 days (day -3 to 6). On day 0, all mice were injected with IAV (PR8 strain). Mice were allowed to overcome this initial viral inoculum (day 7 to 29). On day 30, mice were re-exposed to IAV (SEQ12 strain). Seven days later, mice were euthanized and an ICS was conducted on splenocytes and the absolute number of flu-specific $CD8^+$ T cells was calculated as described in the text. * denotes a significance difference, $p < 0.05$ (PBS n=4 FL n=4)

examined anti-IAV CD8+ T cell responses of H-2^b C57BL/6 mice following their treatment with FL using a similar treatment regimen. Mice were injected with FL or PBS for 10 days (day -3 to day 6). On day 0 of this experiment, BL/6 mice were injected with IAV (PR8 strain). Mice were then euthanized seven days later and an ICS assay for IFNγ was conducted using splenocytes obtained from the spleens of these animals. Previous studies have reported that IAV-specific $CD8^+$ T cell responses in BL/6 mice are favoured towards two immunodominant peptides, namely NP_{366} and PA_{224} (50, 56). Weaker responses are found towards several subdominant epitopes. These are PB1-$F2_{62}$, $PB1_{703}$, $PB2_{198}$, $NS2_{114}$, and $M1_{128}$, in order of decreasing $CD8^+$ T cell response they induce. A difference in the methodology is that since BL/6 mice possess an H-2^b MHC background, their $CD8^+$ T cells can only respond to cells presenting antigen with the same MHC (rule of MHC class restriction) (36). Therefore, DC2.4 cells, which share this H-2^b MHC background, were used (86). As in Balb/c mice, the percentage and absolute number of $CD8^+$ $IFN\gamma^+$ are presented graphically (Figures 18 and 19). Figure 18, which illustrates the percentage of cognate $CD8^+$ T cells within the spleen, shows no difference between PBS and FL treatment. This is contrary to the results I obtained in Balb/c mice (Figure 10), which did show a selective increase in the percentage of $CD8^+$ T cell responding to several epitopes. On the other hand, Figure 19 indicates an increase in the absolute number of some cognate $CD8^+$ T cells, which is likely a reflection of increased overall splenic cellularity, due to FL treatment. Therefore, I found a large increase in the absolute number of $CD8^+$ T cells responding towards both immunodominant epitopes in BL/6 mice. It should be noted that the ID hierarchy did not change as a result of FL treatment.

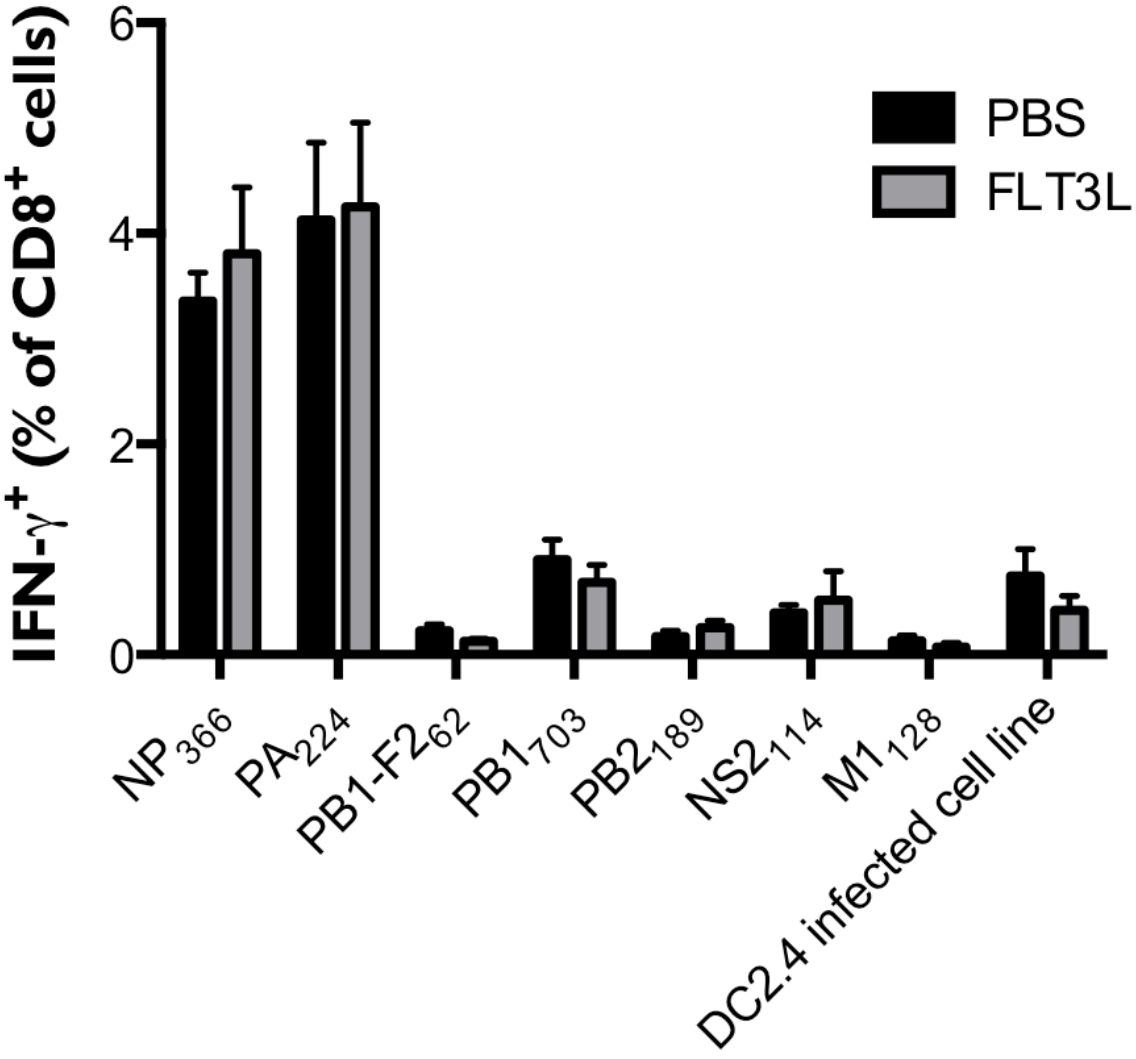

Figure 18: Frequencies of $CD8^+$ $IFN\gamma^+$ cells in the spleens of FL-treated and control BL/6 mice. Mice were treated with FL (10 μg) or PBS for 10 days (day -3 to 6). On day 0, all mice were injected with IAV (PR8 strain). On day 10, mice were euthanized and an ICS was conducted using splenocytes to determine the percentage of the double positive ($CD8^+$ $IFN\gamma^+$) cells, which represent epitope-specific $CD8^+$ T cells. * denotes a significance difference, $p < 0.05$ (PBS n=8, FL n=8).

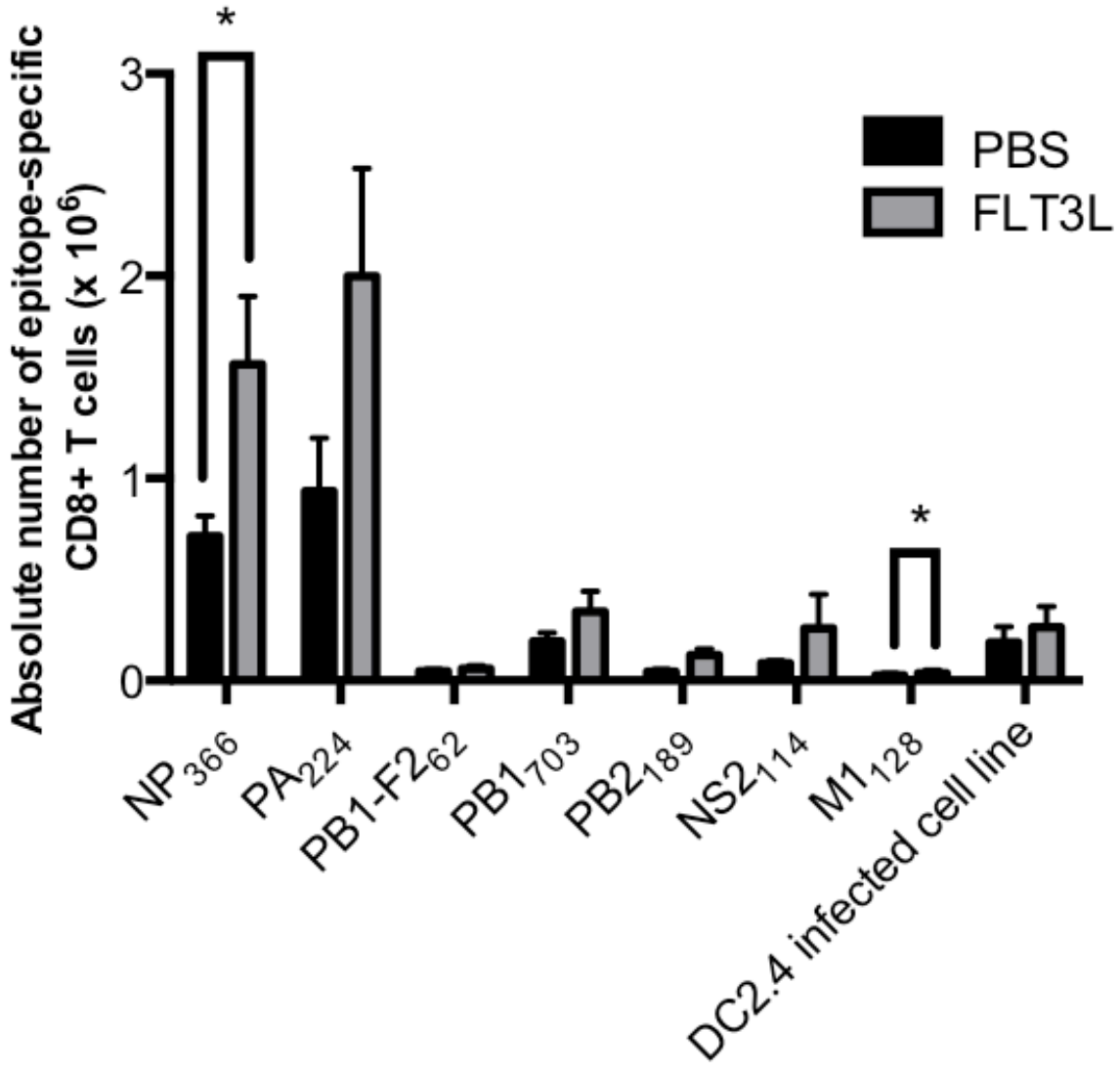

Figure 19: Absolute number of splenic $CD8^+$ $IFN\gamma^+$ cells in FL-treated and control BL/6 mice. Mice were treated with FL (10 μg) or PBS for 10 days (day -3 to 6). On day 0, all mice were injected with IAV (PR8 strain). On day 10, mice were euthanized and an ICS was conducted on splenocytes. Absolute number of $CD8^+$ T cells was then calculated as described in the text. * denotes a significance difference, $p < 0.05$ (PBS n=8, FL n=8).

Chapter 4:

Discussion

In this study, the beneficial effects of FL treatment on $CD8^+$ T cell responses to IAV were examined. Previous studies have illustrated the ability of FL treatment to enhance immune responses to other viral infections (79, 90). However, the effect of FL on cognate $CD8^+$ T cell response to IAV has not been explored. In addition, it was not known whether treatment with FL affects the breadth of IAV-specific $CD8^+$ T cell responses. In my initial experiments, the ability of FL to expand the two major DC populations, namely pDCs and cDCs was explored. As reported in the literature, a 10-day treatment schedule (10 μg daily) usually leads to splenomegaly as a result of expanding splenic populations (63, 85). This observation was confirmed during the treatment period with FL. I also demonstrated that both *in vitro* and *in vivo*, FL treatment effectively expands the population of both plasmacytoid and conventional dendritic cells.

Given important roles these DC subsets play in the context of viral immunity, future studies need to uncover which population contributes to the augmentation of IAV-specific CD8+ T cell responses (if not both). Several studies have reported the presence of at least three subtypes of cDCs in the spleens of mice (76, 91). cDCs are of prime importance to anti-pathogen immunity due to their innate ability to uptake and present antigens to naïve T cells (36, 75, 76). All cDC subsets are $CD11c^{high}$ (91) and as such, are not distinguishable based on the cell staining shown. The cDC subsets are $CD8^+$ DCs, $CD4^+$ DCs and $CD8^-CD4^-$ (double negative) DCs (91). It has been shown that $CD8^+$ DCs, in particular, play a large role in the cross presentation of antigen (76, 91). Cross presentation is simply defined as the capture and presentation of antigen from exogenous sources that is mediated by professional APCs such as DCs. During cross presentation leading to cross priming, host APCs process and present antigens taken up from other

cells (92). For example, if a cell is infected with a virus but lacks the appropriate co-stimulatory molecules, viral antigens or other materials from that infected cell can be taken up by DCs and presented via MHC class I to naïve T cells (36, 92). DCs have high levels of co-stimulatory molecules such as B7-1 and B7-2 and can form stable and productive immunological synapses with naïve T cells, which is a prerequisite for their activation to activate naïve T cells (36, 37). With cross presentation having the ability to play a significant role in viral responses it would be beneficial to monitor if this specific $CD8^+$ DC population is expanding. In the future, staining the splenocytes of FL-treated mice with a combination of CD11c, B220, CD4 and CD8 mAbs may provide a more accurate indication of which DC subset(s) is/are having the biggest contribution to $CD8^+$ T cell responses to IAV.

In order to test the function of these $CD8^+$ DCs during FL treatment a simple experiment can be suggested. The exact same experimental outline and timeline used in mice can be done in $Batf3^{-/-}$ mice. These mice are deficient in $CD8^+$ DCs only (94) and, as such, comparing the responses between PBS and FL treatment groups between $Batf3^{-/-}$ and BL/6 mice will provide an accurate assessment of the effect of $CD8^+$ DCs in promoting an increase in percentage and absolute number of epitope-specific $CD8^+$ T cells. Although cDCs do possess an increased ability to activate naïve T cells, pDCs do have an important role in antiviral responses. As mentioned, pDCs possess TLRs 7 and 9, which are essential in the recognition of single standard RNA viruses and DNA viruses respectively (36, 84). Once these TLRs are activated by the presence of viral particles, pDCs secrete multiple cytokines, including large amounts of IFNα and IFNβ (36). In addition, the cytokines secreted by pDCs enhance the cross presentation ability of cDCs

(92). Given this information, it may be important to determine if the pDC population is having an effect directly or indirectly on $CD8^+$ T cell responses to IAV. It is quite possible that the increased secretion of cytokines by pDC may create a microenvironment that promotes and enhances $CD8^+$ cDC function. Nevertheless, for the purpose of this thesis, there is a clear expansion in both the DC populations.

My findings indicate that FL treatment increases both the percentage and the absolute numbers of $CD8^+IFN\gamma^+$ cells, which represent $CD8^+$ T cells specific for multiple epitopes. This is a novel finding as far as the magnitude and breadth of IAV-specific $CD8^+$ T cell response are concerned. By graphically representing the percentage of $CD8^+IFN\gamma^+$ cells, any changes in overall frequency of responding epitope-specific $CD8^+$ T cells can be accurately recorded. Additionally, displaying the percentage of responding cells allows for an accurate comparison of responsiveness towards different IAV-derived peptides and the detection of any potential changes in the ID hierarchy of IAV. ID dictates that certain peptides, due to previously described factors; elicit a more robust $CD8^+$ T cell response (IDD) in comparison with other subdominant determinants (SDD) (46 – 49). ID is a hurdle in the creation of effective vaccines that are designed to increase the $CD8^+$ T cell response (46). Each virus has the ability to create tens of thousands of possible immunogenic peptides, however, significant $CD8^+$ T cell responses are usually limited to few, sometimes one or two, IDDs. Having a strong immune response to only one or two possible peptides out of tens of thousands may not be very efficient, especially due to the potential ability of viral pathogens to easily mutate. Therefore a treatment option that increases the $CD8^+$ T cell response to various peptides of a pathogen is thought to be significantly beneficial.

In my study, FL treatment did increase the splenic frequency of responding cells to each epitope, in addition to the overall detection of flu-infected cells. In fact, the frequency of $CD8^+$ T cells responding to immunodominant peptide NP_{147} and subdominant peptides NP_{39} and $PB2_{289}$ increased significantly. However, FL treatment failed to increase peritoneal $CD8^+$ T cell responses IAV-derived peptides. As mentioned, the responsiveness of peritoneal exudate cells (PECs) provides a picture of local CD8+ T cell responses to the viral inoculum that has been injected via the i.p. route. This is contrary to splenic CD8+ T cells that represent systemic responses. I found that CD8+ T cells detected in the peritoneal cavity and in the spleens of IAV-inoculated mice show different susceptibilities to FL treatment.

This can be due to a multitude of reasons, such as the differing antigen presenting cells present in each environment (95, 96). In addition, there are many other cell types among PECs, such as macrophages (95, 96), which may have an effect on the microenvironment through the release of various cytokines, which may alter the immune response as well. For instance, my experiments revealed a lower frequency of $CD8^+$ T cells among PECs of FL-treated mice. This decrease in frequency may be due to an increase in other effector cells recruited to the peritoneal cavity as a result of FL treatment. Moreover, the peritoneal cavity contains a unique B cell subset, called B1 B cells (97). These innate-like B-lymphocytes may exhibit regulatory properties due to their ability to secrete significant amounts of interleukin (IL) 10 (97). As reported in the literature, IL-10 may have the ability to dampen the $CD8^+$ T cell response (98). This may explain, at least partially, why FL treatment is not as effective in the peritoneal cavity. It should be noted that although FL treatment did increase the frequency of $CD8^+$ T cells in

the spleen, the ID hierarchy did not change in either the splenic or peritoneal responses. Interestingly, the frequency of overall splenic $CD8^+$ cell population (out of live cells) remained similar for both PBS and FL treatment groups. Therefore, an increase in the absolute number of $CD8^+$ T cells is likely a reflection of an increase, at least in part, in the percentage of epitope-specific $CD8^+$ T cells. In order to determine the absolute number of $CD8^+$ T cells in the spleen and PECs, the absolute number of $CD8^+$ cells was first calculated. This was accomplished by multiplying the percentage of $CD8^+$ gated cells by the overall splenocyte count. From here, the absolute number of $CD8^+$ cells was multiplied by the percentage of double positive cells ($CD8^+$ $IFN\gamma^+$) to effectively give the absolute number of IAV-specific $CD8^+$ T cells. It is clear that FL-treated mice have increased numbers of epitope-specific $CD8^+$ T cells in both the spleen and PECs. In fact, there was a significant increase in the absolute number of splenic $CD8^+$ T cells responding to the immunodominant peptide NP_{147}, NP_{39} and the flu-infected cells. When dealing with a viral infection, the absolute numbers of virus-specific $CD8^+$ T cells obviously carry more weight than their frequencies do. The goal of a $CD8^+$ vaccine is to increase the rate of viral clearance. This is not achieved through an increase in the percentage of cells, but rather by increasing the absolute number of effector cells. In this case, effector cells are epitope-specific $CD8^+$ T cells, which lyse virally infected cells. In a patient, a high number of responding cells is crucial as it provides an improved ability to remove virally infected cells from the body and prevents morbidity and mortality. In fact, the results found in this portion of my thesis, which indicate FLs ability to increase the percentage and absolute number of $CD8^+$ T cell response to IAV correlates well with the literature. Previous studies show that FL treatment has the ability to increase the

$CD8^+$ T cell response to pathogens such as malaria parasite and listeria (99, 100). As such, the ability of FL treatment to increase the anti-IAV $CD8^+$ T cell response coincides with these findings.

In my experimental system, IAV is injected directly into the peritoneal cavity. Although this system does allow the accurate assessment of the percentage and absolute number of epitope-specific $CD8^+$ T cells, it does not completely represent productive infection with this virus. T cells can be activated through direct priming, usually when a DC is infected with the virus itself, or through cross priming (explained above). In the future, it may of interest to determine the effect of FL treatment on the activation of $CD8^+$ T cells via direct or cross priming pathways. In order to test the effects of FL treatment on cross priming, the following experiment may be used.

One can infect a cell line with a $H\text{-}2^b$ background with IAV. Once the cell line is appropriately infected, the cells can be irradiated and washed to remove any excess viral particles on the surface of these cells. Also, incubation with an anti-hemagglutinin (anti-HA) antibody will block any remaining viral particles on the surface of these cells. After anti-HA treatment, these flu infected cells can be washed, irradiated, and injected into Balb/c mice ($H\text{-}2^d$) who have/will receive PBS or FL treatment 3 days prior to injection and 6 days post-injection. In this experiment $CD8^+$ T cell activation is thought to be induced via cross priming (92, 103). This is due to three main reasons: i) the infected and injected cells are allogeneic to the host and therefore can not directly activate host T cells according to the rule of MHC class restriction; ii) gamma irradiation kills the virus and prevents its spread to other cells that could potentially induce direct priming; iii) the anti-HA antibodies prevent any viral particles on the surface of the injected cells to be directly

recognized by host cells (92, 103). Following the same ICS procedure as above, the effect of FL treatment on cross priming and its ability to increase the frequency and absolute number of epitope-specific $CD8^+$ T cells can be accurately measured.

As mentioned, when dealing with a viral infection, the absolute numbers of effector cells play a significant role. However, just as important is the efficiency of these effector cells at performing their designated tasks. The main effector function of CTLs lies in their ability to effectively kill virally infected cells thus preventing the further spread of the virus (36). When activated, CTLs release cytotoxic effector molecules such as perforin and granzymes eventually leading to apoptosis of the virally infected cell (36, 43, 44). In addition, activated CTLs express surface protein FasL, which plays a role in inducing apoptosis of virally infected cells (43 – 45). In order to assess the cytotoxic effector function of CTLs the ^{51}Cr release assay was used. As mentioned before, the goal of a $CD8^+$ T cell vaccination is the promotion of viral clearance (cell death), as such; the ^{51}Cr release assay allows for accurate measurement of this function. While other CTL killing assays, such as Granzyme B ELISpot, have the advantage of directly measuring the number of cytokine-releasing CTLs, they fail to assess authentic killing function of CTLs. In addition, they may miss killing pathways not contributed by granzymes (e.g., the Fas-FasL pathway). My findings indicate that FL treatment enhances the killing function of these epitope-specific CTLs across all effector-to-target ratios. Not only did FL enhance the killing of target cells pulsed with the IDD, but it also enhanced the killing of target cells pulsed with subdominant epitopes. It should be noted that P815 cells were used as target cells during the chromium release assay for two main reasons. The first reason is the $H\text{-}2^d$ background of this mouse lymphoblast-like mastocytoma cell line

allows for syngeneic recognition of peptide:MHC I complexes according to the rule of MHC restriction (101). Therefore, these effector cells can lyse the virally infected target cells once recognized via MHC class I:peptide presentation. Secondly, the P815 cell line is resistant to NK cell-mediated killing (101). NK cells, unlike CTLs, fail to express a TCR, but rather respond to cells that are stressed and are missing "self" markers (36). By using the P815 cell line during the ^{51}Cr assay, one can conclude that the killing to be reported is done by epitope-specific CTLs, not by NK cells. The ability of FL to increase the percentage, absolute number and effector killing function of these CTLs is consistent with the hypothesis that DC mobilization by FL enhances various aspects of anti-IAV CD8+ T cell responses.

In order to determine the sustainability of FL treatment, I examined the effect of FL on IAV-specific $CD8^+$ T memory responses. This experiment allowed for the comparison of primary and secondary $CD8^+$ T cell responses in FL- or PBS-treated mice. To quickly recap, mice were administered PBS or FL during the prime period (day -3 to day 6) and received a PR8 inoculum on day 0. The resolution period was next (day 7 to day 29) as mice were allowed to recover from initial inoculations. On the first day of the boost period (Day 30 to Day 37) mice were injected with SEQ12. As mentioned in the results portion, SEQ12 maintains the same epitopes as PR8, but avoids antibody recognition due to its altered external protein coat (87). This enables the examination of recall CD8+ T cell responses to IAV without worrying about the ability of neutralizing Abs to remove viral particles before CD8+ T cells find an opportunity to mount a secondary response. Seven days after the secondary exposure, I examined CD8+ T cell responses to IAV in PBS- and FL-treated animals, similar to those in the primary

response experiments. Although further experiments are required to validate these results, FL treatment administered during the prime phase decreased the percentage and absolute number of epitope-specific $CD8^+$ T cells. This result holds true for all epitopes tested, including the immunodominant peptide NP_{147}. Interestingly, the ID hierarchy for IAV remains constant and similar to the pattern established during the primary response. Although these results were not quite what I expected, they may be explained, at least theoretically, by what we know about the function of FL. Contrasting to the primary ICS, the absolute number count of splenocytes between the two treatment groups showed no change (data not shown). As such, it is possible that the effect of FL treatment lasts for a certain duration, as seen in the primary response, but given an extended period the effect fades.

This however, does not seem to be the case as indicated by Mosley *et al*, who have reported the ability of FL treatment in the expansion of effector-memory T cells (102). Using the same FL dosage scheme as in my experiments, Mosley *et al*, illustrated that FL treatment increases the percentage and absolute number of effector memory cells (102). The staining for CD44, which is a standard effector-memory cell marker, indicated effector memory cells (102). For the future of this project, a repeat of this experiment must be conducted, as there is evidence for the role of FL in the development of effector-memory cells.

With the possibility of FL treatment enhancing the development of effector-memory $CD8^+$ T cells, it would be of interest to treat mice with FL or PBS as in the primary response, and then during the resolution period, stain for CD127 (IL-7 receptor α chain) and KLRG1 (103). It would be important to determine if FL treatment enhances

the frequency of memory $CD8^+$ T cell precursors ($CD127^{high}KLRG1^{low}$) or short-lived effectors ($CD127^{low}KLRG1^{high}$) (103). Additionally, the administration of FL throughout the entire 37-day process or just during the final boost period can be conducted to determine if there is any change in the percentage or absolute number of epitope-specific $CD8^+$ T cells following these treatment regimens.

In the final portion of this project, the exploration on the effect of FL treatment in BL/6 mice was conducted in order to examine whether the increase in percentage and absolute number of $CD8^+$ T cells is mouse-strain-specific. Although, FL treatment did not increase the percentage of epitope-specific $CD8^+$ T cells, it did increase the absolute numbers of these cells across all epitopes. Even though this experiment was conducted once with 4 mice per group, the absolute number of $CD8^+$ T cells responding to the IDD (NP_{366}) for IAV in the BL/6 reached statistical significance. Although the percentage of IAV-specific $CD8^+$ T cells in BL/6 mice did not change significantly due to FL treatment, the absolute number, which is important in any viral infection, did dramatically increase.

The difference in the responsiveness between the two strains, BL/6 and Balb/c, may be due to their natural immune responses. BL/6 mice are traditionally known to favour a TH1 skewed immune response, which is usually characterized by the functions of several proinflammatory cytokines such as IFNγ (102). On the contrary, Balb/c mice are TH2-dominant, which is more anti-inflammatory through the release of cytokines such as interleukin (IL) 4 and 5 (102). Given this, the effect of FL treatment, which has been shown to increase the percentage and absolute number of $CD8^+$ IFNγ releasing T cells, may be more pronounced in a Balb/c strain which posses an inherent deficiency in

this type of response.

Although the exact mechanism underlying the beneficial effect of FL treatment effects on $CD8^+$ T cell antiviral responses remains to be uncovered it is clear that this treatment option is a promising therapeutic candidate in the fight against IAV. The results illustrated in this thesis have implications with regard to the FL's potential adjuvanticity in effective antiviral treatment and vaccination. ID is an important factor to consider in rational vaccine design. IAV mutations may cause their antigenic epitopes to be no longer detectable by $CD8^+$ T cells and thus inhibiting the ability to eliminate the virus from the infected host (18, 19). FL treatment has shown to broaden and increase the magnitude of $CD8^+$ T cell responses to multiple epitopes of IAV, which allow for a more efficient $CD8^+$ T cell response. As previously described, current vaccination attempts have focused on the neutralization of surface proteins HA and NA (16, 18, 19). Nonetheless, these proteins are under constant immune pressures and are constantly changing (18, 19, 103). However, FL has shown to augment the $CD8^+$ T cell response to relatively conserved internal viral antigens. For example, the nucleoprotein gene in IAV has a conservation rate of 90% in varying IAV subtypes (H1N1, H2N2 and H3N2) (103). Promoting an increased response to these segments may protect individuals from emerging reassorted IAV viruses.

From hospitalizations and death to economic burden, IAV has been and will continue to be a constant hindrance on our society. With the continuously expanding reservoir of possible IAV mixing and high mutation rates, attempts to develop an effective and efficient treatment option have not been successful. As such, the idea of therapeutic agents, which have the ability to alter immune responses to viral antigens, is

being explored. My experimental findings reveal the prospective therapeutic benefits of FL treatment in the framework of a $CD8^+$ T cell vaccine or other antiviral treatments.

Chapter 5:

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Appendix

Western

September 17, 2010

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2014

Dear Dr. Haeryfar:

Your Animal Use Protocol form entitled:
Cell-mediated Immune Responses to Influenza A Virus
Funding Agency CIHR

has been approved by the University Council on Animal Care. This approval is valid from **September 17, 2010 to September 30, 2011.** The protocol number for this project is **#2010-241 which replaces 2006-065 which has expired..**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 4 Years

Species	4 Year Total Numbers Estimated as Required	List All Strain(s)	Age / Weight
Mouse	8584	wild-type B6, wild-type Balb/c, B6.CD1d-/-, Balb/c.CD1d-/-, B6.Ja18-/-, B6.Rag1Va14-TG, B6.IL4-/-, B6.IL13-/-, B6.IFNg-/, B6.IDO-/-, B6.HLA-DR4-TG	up to 6 months of age

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. Approval- M. Haeryfar, M. Harding, W. Lagerwerf

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
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Curriculum Vitae

Name: Adil Shivji

Post-secondary Education and Degrees:

McMaster University
Hamilton, Ontario, Canada
September 2006 - April 2010
Graduated with Bachelor of Science, Honours Life Science

The University of Western Ontario
London, Ontario, Canada
January 2011 – January 2013
Master of Science Candidate, Microbiology and Immunology

Honours and Awards:

Recipient of the Western Graduate Research Scholarship
2011

Graduated on the Deans Honours List
2010

Graduated with Distinction from McMaster University
2010

Research Experience

$CD8^+$ T cell response to IAV
Effect of Rapamycin on graft acceptance/rejection
The University of Western Ontario
2010 – 2013

Publications:

Maleki Vareki S, Harding MJ, Waithman J, Zanker D, **Shivji AN**, Rytelewski M, Mazzuca DM, Yekta MA, Chen W, Schell TD, Haeryfar SM: Differential regulation of simultaneous anti-tumor and alloreactive CD8+ T cell responses in the same host by rapamycin. Am J Transplant 2012; 12:233-239