THE IMPACT OF L-SELECTIN/CD62L ON THE CO-STIMULATION AND MIGRATION OF $CD8^+$ T CELLS DURING VIRUS INFECTION

Rebar N. Mohammed

A thesis submitted to Cardiff University in candidate for the degree of Doctor of Philosophy

Institute of infection and immunity
School of Medicine
Cardiff University
Cardiff
United Kingdom

February 2016

Declaration

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidate for any degree or other award.

Signed …… (Candidate) Date ……

STATEMENT 1

This thesis is being submitted in partial fulfillment of the requirements for the degree of …… (insert MCh, MD, MPhil, PhD etc, as appropriate)

Signed …… (Candidate) Date ……

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated.

Other sources are acknowledged by explicit references. The view expressed are my own

Signed …… (Candidate) Date ……

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organizations.

Signed …… (Candidate) Date ……

STATEMENT 4: PREVIOUSLY APPROVED BAR ON ACCESS

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access previously approved by the Academic Standards and Quality Committee.**

Signed …… (Candidate) Date ……

Acknowledgments

First of all, I would like to specially thank Dr. Ann Ager for her greatest support and patience and continuous believes in me during the last years of my PhD study. I am very proud to have such a fantastic supervision. I would like to thank members of Ager group (present and in the past) for providing me with a great support and a team-working environment. Also, I would like to thank a number of people in the institute of infection and immunity, Cardiff University including Dr. Ian Humphreys, Prof. Awen Gallimore, Prof. David Price, Dr. Edward Wand, Dr. Bernhard Moser, Dr. Matthias Ebrel for providing me with support and knowledge.

I would like to extend a massive thanks to my family and friends for their encouragement and support during my PhD study.

Finally, I would like to thank my lovely wife for providing me with love, passion, support and encouragement at all the time, also to be a good mother of our lovely twin boys (Landy and Lawy).

Abstract

The strategy of the adaptive immune system in eliminating viruses from infected tissues is the activation of $CD8^+$ T cells with specific T cell receptor in the LN draining the site of virus entry and subsequent migration of these cells to the sites of the viral infection. L-selectin, a well characterized LN homing receptor, is variably expressed on virus peptide activated $CD8^+$ T cells, regulated through two separate mechanisms of early ectodomain shedding and late gene silencing. The role of L-selectin in homing of activated CD8+ T cells to sites of virus infection is not studies in detail. Here we show that despite being primed normally in the draining LN, there were a hierarchy in homing ability of adoptively transferred $CD8^+$ T cells expressing mutant L-selectin (which resist shedding and gene silencing upon T cell activation), wildtype L-selectin and deficient in L-selectin (Ko) to the site of virus infection. The L-selectin specific recruitment was confirmed by using antibody blockade strategy and short-term competitive homing experiments. Furthermore, L-selectin dependent homing of virus specific $CD8^+$ T cells rather than hyper-functional or hyperproliferative T cells conferred anti-viral immunity against two evolutionarily distinct viruses, vaccinia and influenza viruses which infect mucosal and visceral organs, respectively.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ADAM: A Disintigrin and Metalloproteinase
AEC: 3-Amino-9-ethylcarbazole
APC: Allophycocyanin
APC-Cy7: Allophycocyanin-Hilite 7
APP: Amyloid precursor protein
B6: C57BL/6 mouse
Blimp-1: B-lymphocyte induced maturation protein-1
CAMs: Cell adhesion molecules
CD: Cluster of Differentiation
$Cacl_2$: Calcium chloride
$CuSO_4$: Copper sulphate
CFSE: CarboxyFlourescein Succinimidyl ester
CLA: Cutaneous lymphocyte antigen
CXCR3: C-X-C motif chemokine receptor 3
CCL5: CC chemokine ligand 5
CXCL9: C-X-C chemokine ligand 9
CXCL10: C-X-C chemokine ligand 10
DUSP-1: Dual-specificity phosphatase 1
DMF: Dimethyl Formamide
DMEM: Dulbecco's Modified Eagle Medium
EDTA: Ethylenediaminetetracetic acid
EdU: 5-ethynyl-2'-deoxyuridine;
EAE: Experimental autoimmune encephalomyelitis
F5 TCR: Transgenic F5 T cell receptor
F5/B6: mice or T cells expressing F5 TCR
F5/LΔP: mice or T cells expressing F5 TCR and mutant L-selectin that resists shedding and gene silencing
FFU: focus-forming units
FMO: fluorescence minus one
FCS: Foetal calf serum
FA: Formaldehyde
FOXO1: Forkhead box O 1
GVHD: Graft Versus Host Disease
GlyCAM-1: Glycosylation Cell Adhesion Molecule-1
GM-CSF: Granulocyte-macrophage colony-stimulating factor
H_2O_2: Hydrogen peroxide
HEV: High endothelial venules
HP: Homeostatic proliferation
HBSS: Hank's balanced salt solution
IgSF: Immunoglobulin superfamily
ICAM-1: Intercellular adhesion molecule-1
Ig: Immunoglobulin
IgG2a: Immunoglobulin G2a
i.n.: intranasal
i.p.: intraperitoneal
i.v.: intravenous;
JAMs: Junction adhesion molecules
KLF2: Kruppel like factor
L-$sel^{-/-}$: L-selectin deficient mice or T cells;
LN: lymph nodes;
LFA-1: leukocyte function antigen-1
LCMV: lymphocytic choriomeningitis virus

MFI: median fluorescence intensity;
MCDK cels: Madin Darby Canine Kidney cells
MACS: Magnetic activating cell sorting
Mac-1: Macrophage antigen-1
MAdCAM-1: Mucosal Addressin cell adhesion molecule-1
mTOR: Mammalian target of rapamycin
MCA tumor: Methylcholanthrene tumor
MCP-1: Monocyte chemoattractant protein-1
MAPK: Mitogen-activated protein kinase
NK cells: Natural Killer cells
ovdLNs: ovary draining LNs
PE: Phycoerythine
PE-Cy5.5: Phycoerythin-Cyanin 5.5
PE-Cy7: Phycoerythin-Cyanin 7
PerCP/Cy5.5: Peridinin chlorophyll- Cyanine 5.5
PFA: Paraformaldehyde
PFU: Plaque forming units
PBS: Phosphate buffered Saline
p.i.: Post infection
Pmel-1 $CD8^+$ T: CD8+ T cell expressing Pmel-1 transgenic TCR
PCLP: podocalyxin-like protein
PSGL-1: P-selectin glycoprotein-1
PNAd: Peripheral node addressin
PMA: Phorbol myristate acetate
PI3K: phosphoinositide 3 kinase
$RAG^{-/-}$: Mice knockout for Recombination Activating Gene
RBC: Red Blood cell
RPMI: Roswell Park Memorial Institute medium
TCR: T cell Receptor
TK- cell: Thymidine Kinase deficient cell line
tet: tetramer
VaccNP: recombinant vaccinia virus expressing nucleoprotein peptide NP366-374
V(D)J: Variable Diversity and joining
VCAM-1: Vascular cell adhesion molecule-1

Chapter one

1. Introduction

1.1. Immune system

The immune system is a vital system of the body, where its main role is to protect against invading pathogens and cancer. The immune system is widely distributed in the body through its various cells and organs. The biological processes that are generated during an immune response are an interaction between the cells of the immune system, the organs of the immune system and the invading pathogens or cancer. The key importance in the immune system is its' ability to specifically identify a wide range of invading pathogens; this makes the immune system unique and a vital system in the body (Janeway et al. 2001).

Patients suffering from X-linked severe combined immunodeficiency disorder, which is a genetic disorder characterized by inability of the patient to produce T lymphocyte and natural killer cells (NK cells), clearly show the importance of the immune system, since these patients suffer from recurrent bacterial infection early during first three months of life and followed by viral infections (Allenspach et al. 2003).

The organs of the immune system, which include bone marrow, thymus, spleen and lymph nodes, play important roles ranging from development of the various immune cells and providing an environment for the immune response reaction to occur between the immune cells and the pathogen. Bone marrow and thymus are sites of lymphocyte generation and maturation (Fig. 1.1). Spleen and lymph nodes are sites of lymphocyte activation and differentiation (Fig. 1.1).

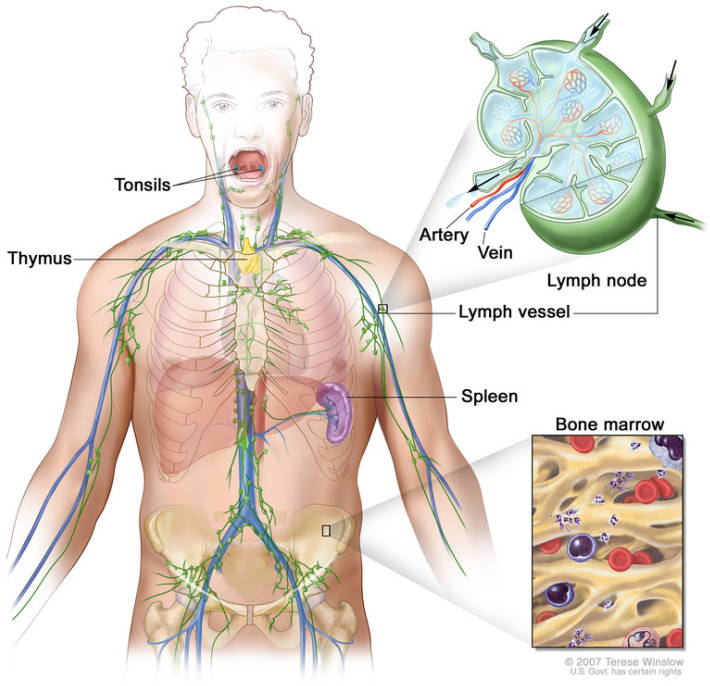


Figure 1.1: Organs of the immune system. Immune system consists of a number of organs and cells. Organs include lymph nodes, spleen, tonsils, thymus and bone marrow.
https://www.meb.uni-bonn.de/Cancernet/CDR0000258030.html

The immune system can be classically categorized into innate and adaptive immune system. The innate immune system, which is also called first line of defence, consists of neutrophils, NK cells, macrophages and dendritic cells. Physical barriers such as the skin and other epithelial surfaces, mucous and hair, also constitute a major part of the innate immune system and participate in the very early protection of the body from entry of pathogens. Skin can also protect the body from the lethal factors in the surrounding environment such as irradiation, heat and poisonous substances (Alberts et al. 2002).

Innate immune system do not require highly specific activation and differentiation that is why their action is much immediate with regards to responding to invading pathogens since they are either in the tissues such as dendritic cells and macrophages or they can be recruited directly from the blood stream such as monocyte, eosinophil, neutrophil and NK cell.

1.2. Adaptive immune system

Any invading pathogens that are not eliminated by the innate immune system will be dealt with through the second line of defence, the adaptive immune system. This system is more specifically deal with the invading pathogens such as bacteria, viruses, fungi and parasites. It can distinguish between self and non-self-antigens through specific receptors, which evolve during their maturation process. Also, the adaptive immune system has the long life protection scheme, which does not exist in the first line of defence through the generation of memory cells (Janeway et al. 2001).

The cells of the adaptive immune system are T and B lymphocytes (which I refer here and after as T and B cell, respectively). The adaptive immune system cells have their own duty. B cell is one of the arms of the adaptive immune system; its main role, antibody production in the body, is highly important in the elimination of invading pathogens (Vazquez et al. 2015).

T cell, similar to the other leukocytes, originate from pluripotent hematopoietic stem cells in the yolk sac, early during the embryo development, and later in foetal liver and bone marrow, and in adult, the bone marrow is the main first site of T cell generation unless there is a defect in the bone marrow which in turn makes the liver become haemopoietically active (Alberts et al. 2002). After their first generation step in the bone marrow as pro T cells, they migrate to the thymus for their maturation. A multistep processes in the thymus controls T cell development through production of the T cell receptor (TCR)(von Boehmer 1994). The TCR molecules consist of either an alpha (α) and beta (β) chain or gamma (γ) and delta (δ) chain. The αβ or γδ TCR are originated through genes in

the variable region of the DNA called V(D)J rearrangement. The genes in these regions are encoding for the TCR. The VDJ encoding has to undergo rearrangement in order to generate a TCR that is specific for a conformational shape of certain peptides. The VDJ rearrangement occurs through the action of lymphoid-specific recombination activity enzyme, which is also called VDJ recombinase. The enzyme VDJ recombinase is the product of the genes called RAG1 or RAG2 (Schatz et al. 1992; Alt et al. 1992). T cells can recognize thousands of various infectious and non-infectious pathogens through their diverse TCRs.

1.2.1. T cell immune surveillance and subsequent activation

$CD8^+$ T cells will not participate in an immune response unless they are specifically activated through their TCR. Taking the specific activation into consideration, $CD8^+$ T cells with a given peptide specificity has to circulate continuously searching for its cognate antigen in order to be activated. The $CD8^+$ T cells generated and matured in the thymus leave the site of their production and enter circulation. They migrate through blood and lymphatic entering the secondary lymphoid organs, such as spleen, lymph nodes and Peyer's patches, searching for their cognate antigen in a process called surveillance. At this moment, since they have not seen any antigen, a part from self-antigens in the thymus, these $CD8^+$ T cells are called naïve $CD8^+$ T cells (Masopust & Schenkel 2013).

As part of its immune surveillance, naïve $CD8^+$ T cell enters the lymph node either via the afferent lymphatic or through the specialized blood vessels called high endothelial venules (HEV). Naïve $CD8^+$ T cell has its characteristics and phenotype. They are identified through a number of activation molecules and cell adhesion surface receptors. Some of the well-characterized receptors are the late activation antigen (CD44) and the surface adhesion molecule also called lymph node homing receptor, L-selectin (CD62L). $CD8^+$ T cells, which express low level of CD44 and high level of CD62L is phenotypically characterized as naïve $CD8^+$ T cells (Sallusto et al. 1999).

In order to enter the lymph node via HEV, naïve $CD8^+$ T cells use the peripheral lymph node homing receptor CD62L binding to peripheral node addressin (PNAd). The CD62L-PNAd engagement allows the first capture of the $CD8^+$ T cells and allows the cell to roll on the surface of the HEV (Gallatin et al. 1987). Furthermore, the cell will reach the stage of activation-induced arrest mediated by CC-chemokine receptor (CCR7) engagement with the CC-chemokine ligand (CCL21) and lymphocyte function-associated antigen-1 (LFA-1) engagement with intracellular adhesion molecule (ICAM-1)(Masopust & Schenkel 2013).

Whereas, when naïve $CD8^+$ T cells enter the lymph node via the afferent lymphatic, it first arrives to the lymph node sinus. Since T cells have its zone in the lymph nodes, then they enter the paracortex T cell zone in the lymph node in a chemokine dependent manner (CCR7)(Figure 1.2)(Masopust & Schenkel 2013).

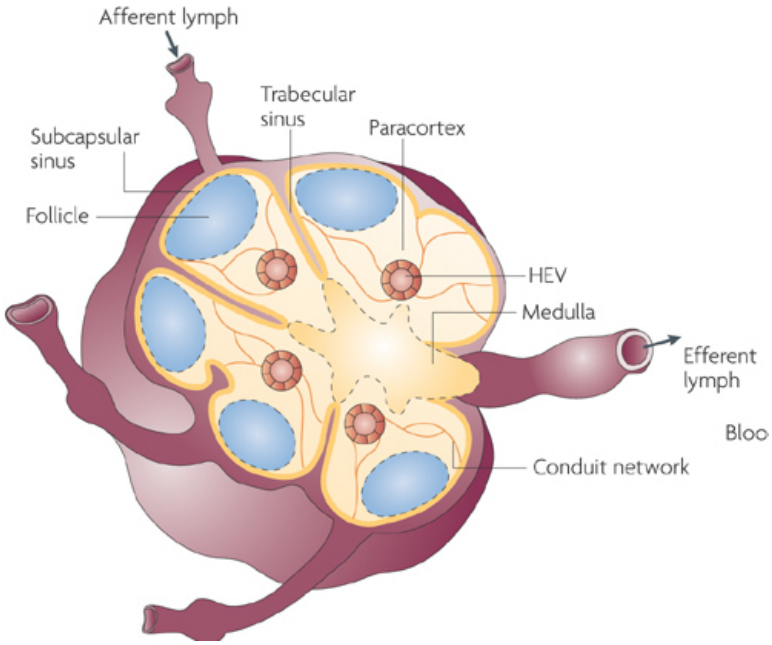


Figure 1.2: lymph node structure. Lymph node is one of the most important organs of the immune system. Structurally, It consists of the capsule, which also contains subscapular sinuses, cortex and medulla. The cortex is further divided into B and T cell zone and medulla contains mainly blood vessels.

The fundamental achievement of T cell immune surveillance is the finding of antigen bearing cells by the naïve $CD8^+$ T cells in the secondary lymphoid tissues such as in the paracortical T cell zone of lymph nodes. Naïve $CD8^+$ T cells recognize peptide, which is derived from the pathogens, in the context of major histocompatibility complex (MHC) class I on antigen presenting cells (APCs) such as cells of the innate immune system, dendritic cells (Medzhitov & Janeway 2000). In order for the naïve $CD8^+$ T cells to perform its function, they must migrate to the site where they can find the peptide presented APCs (Germain & Stefanova 1999). $CD8^+$ T cells have its unique TCR that recognizes specific peptide, except for a few $CD8^+$ T cells that can recognize peptide from any pathogen.

The diversity of the TCR makes the adaptive immune system specific and potent with regards to long life immunity and specific protection of the body. The T cell repertoire has about 25-100 million unique TCR for a diverse range of antigens (Arstila et al. 1999). The APCs that gather information and peptide through out the body provide useful information for the naïve $CD8^+$ T cells. Once naïve $CD8^+$ T cells have been presented with their cognate antigen in the context of MHC class I in the presence of essential co-stimulation, the cell undergoes proliferation to produce thousand of its clone to fight the infection specifically. Naïve $CD8^+$ T cells can be activated at any secondary lymphoid organs such as spleen, lymph node, tonsil and Peyer's patches near to the site of the pathogen entry.

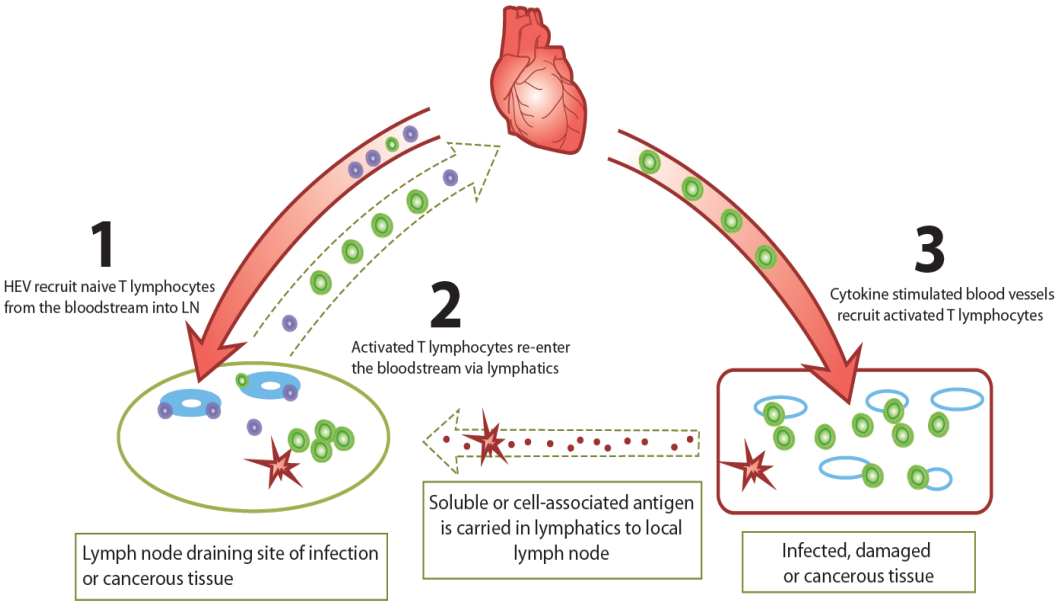


Figure 1.3: T lymphocyte re-circulation and activation. Naïve $CD8^+$ T cells re-circulate in and out of secondary lymphoid organs via lymphatic or blood vessels searching for its cognate peptide. Once they have been presented with the antigen in the context of APCs, they will be activated, exit the lymph node, re-enter the circulation and migrate to the site of infection. HEV play an important role in the recruitment of naïve $CD8^+$ T cells into the lymph nodes.
Ager and May, 2013

1.2.2. T lymphocyte differentiation

Upon activation through cognate peptide in the context of MHC class I with appropriate co-stimulation, the $CD8^+$ T cells differentiate into effector $CD8^+$ T cells and later into memory $CD8^+$ T cells. Cytokines play an important role in the process of $CD8^+$ T cell differentiation. For example, IL-7 cytokine is required for $CD8^+$ T cell differentiation and expansion during homeostatic proliferation, which is a process of maintaining the number of various immune cell in a balanced condition (Schluns et al. 2000), whereas the key cytokine that is controlling antigen-specific $CD8^+$ T cells differentiation and clonal expansion is interleukin-2 (IL-2) (Kalia et al. 2010).

IL-2 engaging with its receptor, which is composed of 3 subunits α, β and γ, which are also called CD25, CD122 and CD132, respectively, stimulates $CD8^+$ T cell proliferation. IL-2 can also cease clonal expansion by activation-induced cell death (Lenardo 1991). Upon an increase in the TCR binding to its cognate peptide MHC I on APCs, the level of high-affinity IL-2R (CD25) is increase and the amount of IL-2 production by the $CD8^+$ T cells itself is also increase (Wong & Pamer 2014).

Since IL-2 is an important factor in the process of $CD8^+$ T cells clonal expansion, IL-2 has been approved for a numbers of metastatic cancers treatments such as melanoma and renal cancer. IL-2 has a short half-life in the body; combining IL-2 with its monoclonal antibody provided promising results with regards to activated $CD8^+$ T cells expansion (Tomala et al. 2009). IL-2 is not only having a short half-life, IL-2 leads to adverse side effects including pulmonary damages which are caused by integration of IL-2 with its IL-2R on the lung endothelial cells (Krieg et

al. 2010). Using IL-2 in combination with its monoclonal antibody as a complex was increased its short half-life and also reduced the severe adverse effects (Tomala et al. 2009; Krieg et al. 2010).

During the activation process of $CD8^+$ T cells, which up-regulates low level of IL-2 receptor (IL-2R), have the fate to turn into memory cells very early during the activation stage without fighting the infection and reside in locations through out the body (Laouar et al. 2008), and also cells which up-regulate high level of IL2R have the fate of terminal effector $CD8^+$ T cells. One of the keys essential in the differentiation of effector to memory $CD8^+$ T cells is the B-lymphocyte induced maturation protein-1 (Blimp-1). Cytokine such as IL-2 is inducing the Blimp-1 expression that in turn induces the conversion of effector to memory cells (Boulet et al. 2014).

IL-15 is a growth factor maintaining the memory $CD8^+$ T cell's pool (Ku et al. 2000). Another factor controlling the naïve $CD8^+$ T cells differentiation to effector cells is the physiological exposure to a various range of hyperthermia. A research study by (Mace et al. 2011) suggesting that exposing to a mild hyperthermia 39.5°C prior to antigen specific activation of Pmel-1 $CD8^+$ T cells was resulted in an increase in the numbers of the cells which eventually differentiated to effector $CD8^+$ T cells.

1.2.3. $CD8^+$ T cells trafficking to peripheral tissues

The leukocyte migration from blood and lymphatic to peripheral tissues and back to blood is controlled through a multi-cascade process involving a numbers of adhesion molecules and their counter ligands. Leukocyte extravasation from

blood to tissues and back to blood occurs continuously in order for the leukocytes to undergo their circulation and searching for antigen. Different vascular phenotype at different location and also the phenotype of the $CD8^+$ T cells determine their fate and the end point of the cells during normal circumstances against situations where there is an infection at some part of the body (Brinkman et al. 2013).

Various subsets of $CD8^+$ T cells, such as naïve, effector and memory cells, have different migration or homing capacity. Naïve and memory $CD8^+$ T cells, because of their functions of first to find their cognate antigen, are mostly found in the secondary lymphoid organs. They usually circulate and they are found either in circulation or in secondary lymphoid organs. Whereas the effector $CD8^+$ T cells, in which its function is to fight the infection, are mostly found in the peripheral non-lymphoid tissue (Sallusto et al. 1999). Although Naïve $CD8^+$ T cells, due to the nature of their function, are either usually found in the secondary lymphoid organs or their site of development such as thymus, these cells were found in the wall of aorta and liver (Golden-Mason et al. 2004; Galkina et al. 2006).

The endothelium plays a major role in the attraction and recruitment of T cells into the site of infection. During immune surveillance, naïve $CD8^+$ T cells use specialized blood vessels called HEV in the secondary lymphoid organs and, in particular lymph node, to enter and seek their cognate antigen. Whereas effector $CD8^+$ T cells preferentially attracted to the site of the inflammation and use the conformation changes in the endothelium and post-capillary venules as a result of an inflammation, to enter (Streeter et al. 1988).

Effector $CD8^+$ T cells, critical for protection against viral infection, enter the lung interstitium from the pulmonary vasculature and retain for a long period of time

via other adhesion molecules such as leukocyte function antigen-1 (LFA-1). The function of these effector $CD8^+$ T cells from the pulmonary vasculature into the lung interstitium in part is driven by the CC chemokine ligand 5 (CCL5) expressed by the lung (Galkina et al. 2005).

Furthermore, T cells can use other mechanism in order to enter peripheral tissues. For example, a population of T cells called T regulatory cells (T reg) can enter the tissues via recognition of self-antigen, which is expressed in the context of MHC class II molecules on the surface of endothelium. This Treg recruitment phenomena relies on the IFN-γ mediated up-regulation of MHC Class II by the endothelium and the PI3K P110δ pathway activation via the TCR (Fu et al. 2014).

Activated T cells use another chemokine interaction mechanism to enter the site of Graft Versus Host Disease (GVHD) in the skin. This mechanism relies on the interaction of C-X-C motif chemokine receptor 3 (CXCR3) expressed by the T cells binding to C-X-C chemokine ligand 9 and 10 (CXCL9) and (CXCL10) expressed by the epidermal cells of the skin (Villarroel et al. 2014).

Whereas, at the peripheral non-lymphoid sites of tumour, or at the periphery of the tumour mass, HEV can develop and facilitate the recruitment effector $CD8^+$ T cells to the site of infection (Bento et al. 2015). Because of the ligand and receptors expressed by the HEV, it is an ideal surface for the T cells to use in order to home to the site of interest. There was a strong correlation between the higher numbers of the infiltrating T cells with the higher numbers of the HEV in a mouse model of chemical carcinogenesis called methylcholanthrene (MCA) tumor (Gallimore et al. 2013). HEV not only allow the recruitment of either naïve T cells to the lymph node or effector T cells into the site of inflammation, if HEV exist at the site, but also, it allows the monocyte-derived macrophages to enter

the lymph node through monocyte chemoattractant protein-1 (MCP-1), which will act as APCs for the T cell activation and priming (Palframan et al. 2001).

Moreover, cytokines such as IL-6 have been implicated in enabling effector $CD8^+$ T cells infiltration into tumor microenvironments in mice and human. A research study by has shown that a systemic thermal therapy (39.5°C $\pm$ 0.5°C) leads to an increase in the level of IL-6 secretion by the stromal cells which binds to the soluble IL-6 receptor-α that further leads to E/P-selectin- and ICAM-1-dependent recruitment of effector $CD8^+$ T cells (Fisher et al. 2011).

1.3. Adhesion molecules

Adhesion molecules, such as members of selectin family, immunoglobulin superfamily (IgSF) and integrin family, play an important role in the process of T cell migration. In order for a T cell to enter the secondary lymphoid organs or peripheral sites of infection, it must undergo a multi-step process orchestrated by the adhesion molecules and their counterpart ligands. T cell itself expresses surface adhesion receptors, and endothelium of blood vessels also expresses a number of adhesion receptors. Although these cell adhesion molecules (CAMs) are mainly involved in the capturing and adhesion processes, they provide extracellular signalling communications.

One the most common families of adhesion molecules is the integrin family. It is composed of a heterodimer of α and β chains. Both chains of the integrins are required in order to exert it's binding and signalling function. The most common integrin's involved in leukocyte-endothelial interaction cascade are a member of the β_7 subfamily, three from the β_2 and one from β_1 subfamily (Hynes 1992).

Another family of cell adhesion molecules is the Immunoglobulin superfamily (IGSF). Intercellular adhesion molecule-1, 2 and 3 (ICAM-1, 2 and -3) are members of IGSF. They are receptors expressed by endothelial cells and leukocytes that mediate and participate in the leukocyte-endothelial cells interaction. Vascular cell adhesion molecule-1 (VCAM-1) is also a member of the IG superfamily (Pepinsky et al. 1992). Under normal circumstances, these adhesion receptors are very minimally expressed by endothelial cell lining blood vessels, in order to allow the normal flow of the immune cells to take place. In inflammation situation and in the presence of cytokine such as TNF-α and IL-1b, their expression is up-regulated near to the site of the inflammation and allows the enhanced recruitment of the necessary cohort of the cells to the site of the inflammation (Gearing & Newman 1993).

The last family in the cell adhesion molecules is the selectin family. Members of the selectin family are L-, P- and E-selectin, which is expressed by leukocytes, platelet and endothelium, respectively. They are glycoprotein in nature and their participation in leukocyte-endothelial cell interaction is pivotal (Bevilacqua 1993). The cyclical expression status of members of selectin family varies according to the different members, for example, L-selectin is constitutively expressed by the leukocyte under normal circumstances, but it is expression is altered during the leukocyte activation. E- selectin is expressed in similar manner to the integrins family with a response to inflammatory stimuli, whereas the P-selectin is stored in the intracellular cytoplasmic compartments and up-regulated to the surface according to inflammatory stimuli. Selectins are first involved in capturing and rolling of leukocyte on the surface of the blood vessels. This sequential event of leukocyte-endothelium interactions, such as neutrophil recruitment during inflammation, is initiated through selectins expressed by the leukocyte called L-

selectin binds to cutaneous lymphocyte antigen (CLA) and, selectins expressed by endothelial cells P- and E-selectin binds to P-selectin glycoprotein-1 (PSGL-1) on the leucocyte (Hill et al. 2003). This first process is called primary capturing of leukocytes on the surface of the endothelium. Where as in lymph nodes, L-selectin expressed by T cells use PNAd binding to interact with the endothelial cells lining HEV within the lymph node.

The process of capturing and rolling is followed by the next step, which is mediated by integrins and will lead to the integrin-mediated leucocyte arrest through integrin α4β7, leukocyte function-associated antigen-1 (LFA-1), very late antigen-4 (VLA-4) and macrophage antigen-1 (Mac-1). After that, leukocytes crawl along the wall of the blood vessels and later they have to transmigrate into the tissues using either of the two mechanisms which are mediated by integrins and IgSF. First mechanism is paracellular, in which the T cell transmigrates between two adjacent endothelial cells by dissociating the junction between the cells engaging with the junction adhesion molecules (JAMs, such as JAM-A, JAM-B), PECAM/CD31, ICAM-1, ICAM-2 and CD99. In the second mechanism, T cells transmigrate in a process called transcellular migration, in which the T cells transmigrate through a single endothelial cell by engaging with F-actin containing VCAM-1 and ICAM-1 rich podosomes (Figure 1.4)(Vestweber 2007; Ager 2012).

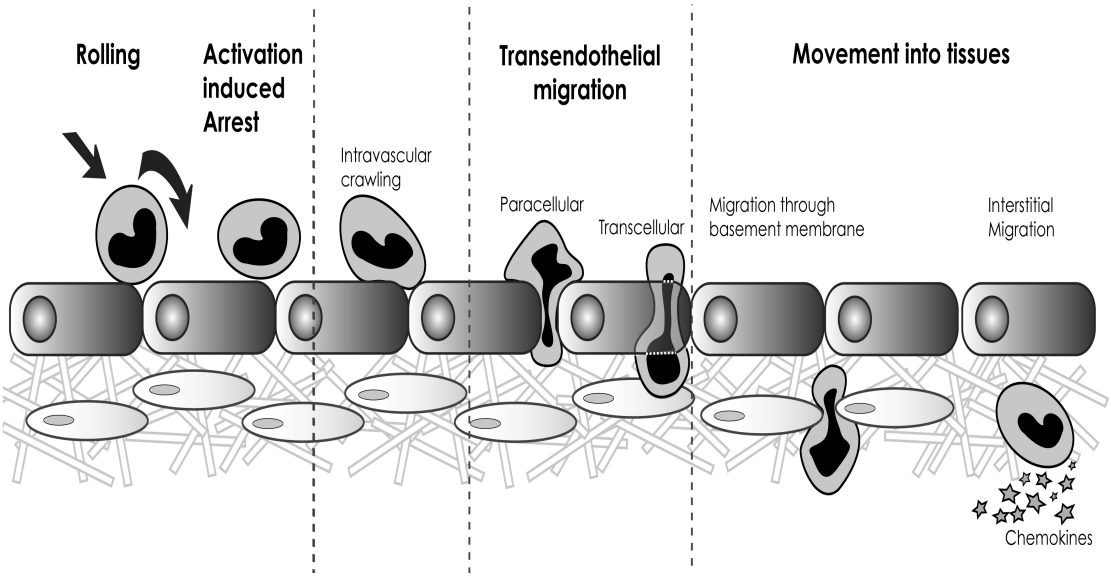


Fig. 1.4: The role of L-selectin (CD62L) in lymphocyte migration across blood vessel wall. Tethering and rolling of lymphocytes occurs initially through L-selectin molecules. L-selectin is pivotal for most of the steps where it's binding activate CC-chemokine receptor 7 (CCR7) and Leukocyte function associated-1 (LFA-1), which are required for further rolling and firm adhesion. L-selectin also results in subsequent activation of the integrin family for transmigration.
Ager, 2012

1.4. L-selectin (CD62L)

L-selectin is a C-type lectin receptor expressed by leukocytes. It is glycoprotein in nature composed of NH_2 terminus, C-type lectin domain, and epidermal growth factor domain, this is followed by two short consensus repeating unit, proximal membrane region, transcellular region and a cytoplasmic tail (Ivetic 2013). The history of L-selectin dates back to 1989 when the cDNA sequences of the three members of the selectin family were identified. L-selectin is coded by a gene called SELL, which encodes a 70KD protein and it is found on the chromosome 1 (Watson et al. 1990).

L-selectin is characterized as a peripheral lymph nodes homing receptor. $CD8^+$ T cells homing and migration across high endothelial venules (HEV) of lymph

nodes, which is essential for immune response strength, are regulated through L-selectin (Grailer et al. 2009). L-selectin is crucial for the initial tethering and rolling of $CD8^+$ T cell across the wall of blood vessels and, in particular, HEV of lymph nodes. Lymphocytes, in particularly $CD8^+$ T cells, home to peripheral lymph nodes, and following activation, they re-enter the bloodstream and migrate to sites of infection, which are all requirements of a potent immune cell.

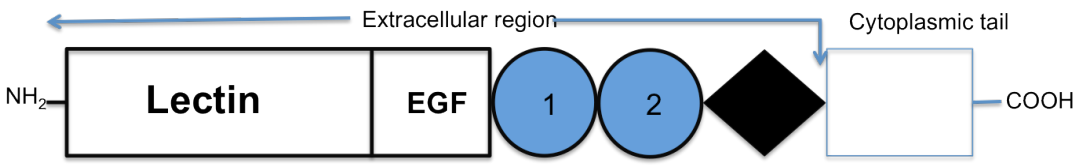


Fig. 1.5: L-selectin structure. L-selectin is expressed by all leukocytes and is responsible for the initial rolling and tethering of lymphocytes across the blood vessel wall and high endothelial venules. L-selectin comprises an N-terminus, followed by a lectin domain where most of it's ligands bind, epidermal growth factor-like domain, consensus repeating units, which vary in number among the selectin family members, a proximal membrane region, transmembrane domain and cytoplasmic tail.

Naive $CD8^+$ T lymphocytes express L-selectin on their surface in order to enter the lymph node through HEV using L-selectin-PNAd binding (Klinger et al. 2009). Injecting monoclonal antibodies specific for vascular addressin (PNAd) resulted in inhibition of the numbers of recruited lymphocyte to the peripheral lymph nodes without interfering with the homing of lymphocytes to the mucosal associated Peyer's patches in the gut (Streeter et al. 1988). PNAd, defines a mixture of

glycoproteins including GlyCAM-1 (Glycosylation Cell Adhesion Molecule-1) which is a soluble ligand and the cell surface ligands CD34 and podocalyxin-like protein (PCLP). To enter intestinal secondary lymphoid organs, $CD8^+$ T lymphocytes bind to MAdCAM-1 (Mucosal Addressin cell adhesion molecule-1)(Ley et al. 2007)(Grailer et al. 2009).

Using antibodies that target the lectin and epidermal growth factor domains of L-selectin, called MEL-14, resulted in reduction in the lymphocyte-endothelial cell interaction, which is necessary for lymphocyte recruitment to the lymph nodes (Bowen et al. 1990). Also deletion of the epidermal factor domain, which leaves the lectin domain alone, resulted in loss of MEL-14 binding. This suggests that MEL-14 binds the lectin domain of L-selectin in the presence of epidermal factor domain. Further studies, in which mice were deficient for L-selectin, through gene targeting, showed a severe reduction in the numbers of leukocytes in peripheral lymph nodes, further revealing that L-selectin is an obligate peripheral lymph node homing receptor (Arbornes et al. 1994).

However, in the absence of L-selectin, lymphocytes can use alternative mechanisms to enter lymph nodes through P-selectin expressed by activated platelets. P-selectin binds to PNAd on HEVs in a similar manner as L-selectin, and lymphocytes can undergo secondary tethering and rolling on captured platelets and other leukocytes to enter the lymph node (Diacovo et al. 1996).

Furthermore, the importance of L-selectin has also been shown in the context of leukocyte homeostasis, in particular $CD8^+$ T cells into lymphoid tissues. Abrogating the gene that codes for L-selectin in $CD8^+$ T cells or suppressing the mRNA (let-b7) of L-selectin by mycolactone resulted in a considerable loss of $CD8^+$ T lymphocyte homing to lymph nodes and accumulation in peripheral blood

(Guenin-macé et al. 2011). Mice, which lack lymphocytes (T and B cells) ($Rag^{-/-}$), are susceptible to homeostatic proliferation (HP) and L-selectin has a critical role in directing $CD8^+$ T cells to lymph nodes, where $CD8^+$ T cells undergo HP (Schuster et al. 2009).

1.4.1. L-selectin expression on the surface of activated $CD8^+$ T cells

Naïve $CD8^+$ T cells uniformly express a high level of L-selectin. An *in vitro* study has shown that upon $CD8^+$ T cell activation through TCR engagement with cognate peptide in the context of MHC class I on the surface of APC, L-selectin is shed on the surface of the of the $CD8^+$ T cells in the first 4 hours of activation by proteolytic cleavage and shedding. L-selectin is re-expressed within 48 hours due to an increase in the level of L-selectin mRNA. Following re-expression, L-selectin expression is markedly reduced between day 5-7 post activation *in vitro* due to L-selectin gene silencing (Chao et al. 1997).

L-selectin expression is controlled by the level of its mRNA transcription, translation and proteolysis of its full length structure i.e. two completely separate mechanisms of proteolytic cleavage and gene silencing control the expressions of L-selectin on the surface of $CD8^+$ T cells (Chao et al. 1997). Why, L-selectin expression is very tightly regulated on the surface of $CD8^+$ T cells and expression

varies between 0-8 days following activation, are questions, which are not fully understood.

Furthermore, the level of L-selectin expression on the surface of lymphocytes such as $CD4^+$ T cell is controlled by other mechanisms as well. It has been shown that viral proteins such as NEF and VPU, found in the Human Immunodeficiency Virus (HIV), are implicated in the down regulation of L-selectin. Both HIV proteins (NEF and VPU) were impeding the CD62L protein transportation onto the plasma membrane, retaining the CD62L protein in the perinuclear compartments. Increased CD62L down regulation were involved in the limitation of immune response against the virus (Vassena et al. 2015).

Although leukocyte migrations is a multi-cascade process and a number of cell adhesion molecules play a major role in the process, L-selectin is the key initiator and it is responsible for the first tethering and rolling of lymphocytes across the HEV in LN (Mora & Andrian 2006). The current dogma of leukocyte migration is naïve $CD8^+$ T cells express a high level of L-selectin on their surface and use it to enter the LN. Upon activation through TCR, L-selectin is shed on the surface allowing the $CD8^+$ T cells to enrich at non-lymphoid peripheral sites of infection and preventing them from re-entering the lymphoid tissue during the course of the infection (Lefrancois 2006). Whereas the *in vitro* study by (Chao et al. 1997), clearly showed that L-selectin is re-expressed on the surface of $CD8^+$ T cells 48 hours post activation.

1.4.2. L-selectin proteolytic cleavage by metalloproteinase ADAM family

A family of transmembrane proteinases called A Disintigrin and Metalloproteinase (ADAMs) play an important role in controlling the expression of various cell adhesion molecules on the surface of leukocytes. During the course of an infection; it is the responsibility of ADAMs to control migration and stationary patterns of leukocytes at various lymphoid and non-lymphoid tissues by controlling the expression of cell surface adhesion molecules.

ADAM is a family of proteins consists of around 19 members ADAM's main role in the immune system is the enzymatic cleavage of a numbers of cell adhesion molecules involved in leukocyte-endothelial cell interaction in the process of leukocyte migration (Edwards et al. 2008). ADAMs consists of a prodomain, metalloprotease, disintigrin-like, cysteine-rich and epidermal growth factor-like, transmembrane and cytoplasmic domains (Ager 2012). Various ADAMs that are responsible for cleaving various adhesion molecules have essential requirements in order to be able to perform their sheddase function, among the most important is structure of the substrate, substrate accessibility and the distance of the cleavage site from the cell membrane. Also, the ADAM structural conformation plays an important role in its sheddase activity. The C-shaped structure formed by the cysteine rich, and disintegrin domain pointed toward the metalloproteinase domain allows the ADAMs to easily engage with its substrate and perform its shaddase activity (Igarashi et al. 2007)(Wang et al. 2009).

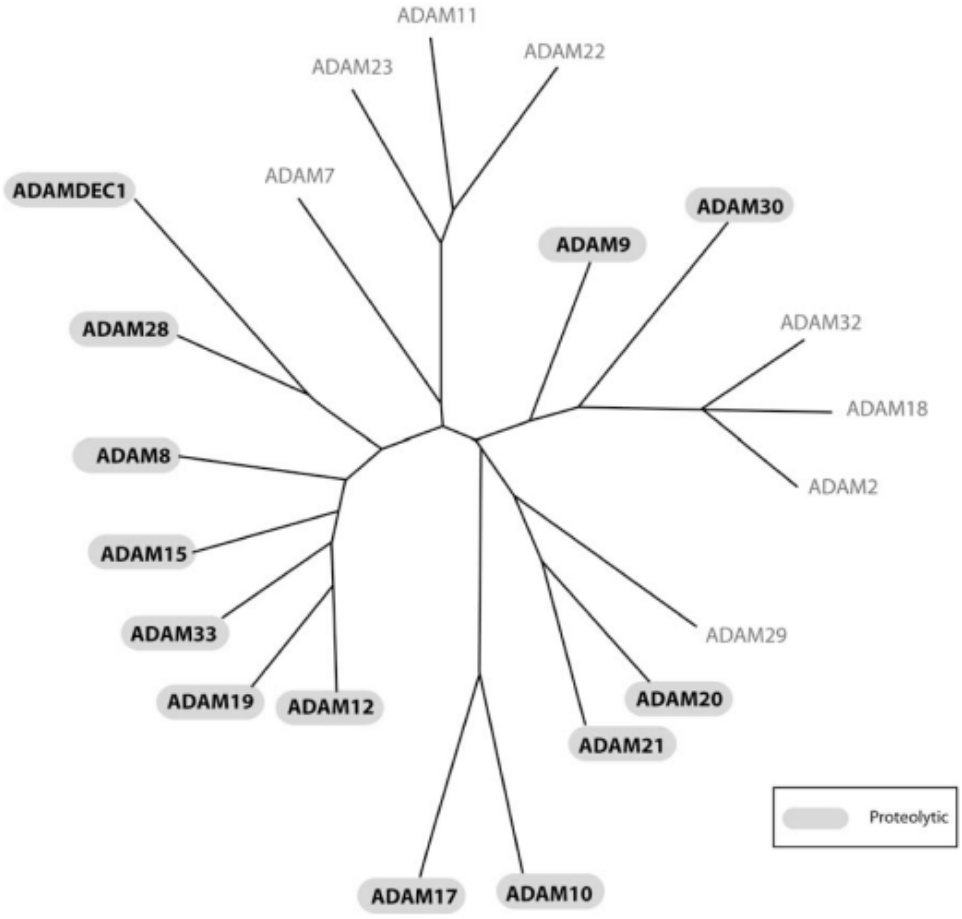


Figure 1.6: ADAM family. A Disintegrin and Metalloproteinase is a family of peptidases, which is mainly responsible for shedding extracellular membrane proteins.
Ager, 2012

ADAM-17 cleaves L-selectin on the surface of leukocytes, such as neutrophils (Wang et al. 2009). Intracellular signals following leukocyte activation will lead to the activation of the enzymatic activity of ADAM-17, however, extracellular activation of mature ADAM17 is another possible way of its activation through oxidative agent H_2O_2, which leads to ADAM-17 dependent shedding of L-selectin.

L-selectin shedding and its involvement in leukocyte migration were first studied in 1989. Neutrophils treated with chemotactic factor complement C5a or phorbol ester resulted in an increase in the level of cleaved L-selectin, soluble L-selectin (Kishimoto et al. 1989). The loss of L-selectin on the surface of neutrophils resulted in their inability to migrate efficiently to the site of infection (Jutila et al. 1989). In similar consistency with the neutrophil L-selectin shedding phenomena, lymphocytes lose L-selectin expression within 30 minutes upon treatment with phorbol ester (Jung & Dailey 1990).

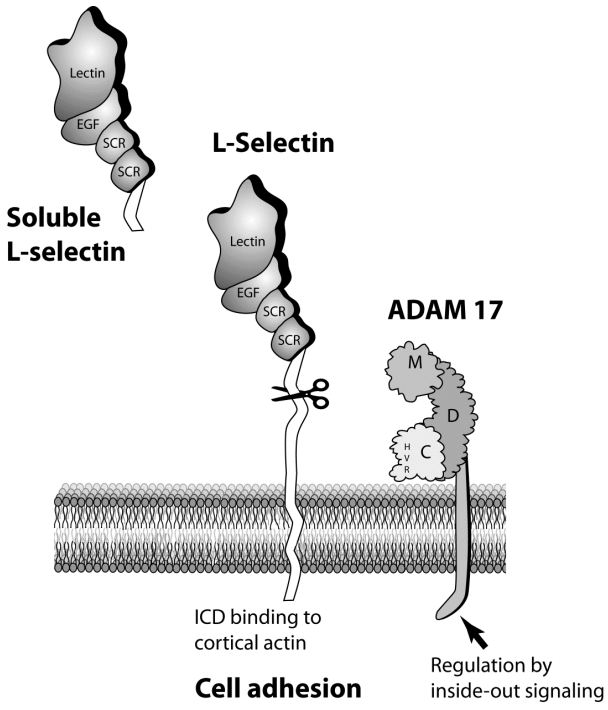


Fig. 1.7: The process of L-selectin shedding by ADAM 17. Metalloproteinase (M) domain together with the cysteine rich (C), hypervariable region (HVR) and disintegrin (D) region are responsible for forming a C shaped molecule structure to bind the membrane proximal region of L-selectin.
Ager, 2012

Other enzymes, such as recombinant human fibroblast collagenase (MMP-1) and to some extent stromelysin (MMP-3) can also cleave L-selectin from the surface of mouse leukocytes (Preece et al. 1996). Metalloproteinases, such as ADAMs, which are responsible for cleaving L-selectin upon leukocyte stimulation through phorbol ester, chemoattractants and L-selectin engagement, target the proximal membrane region of L-selectin, releasing a soluble fragment of L-selectin, which is consisting of lectin and epidermal growth factor domains (Preece et al. 1996). The amino acid specificity is not an absolute requirement for the proteolytic shedding of L-selectin to occur. A tertiary conformation of L-selectin with a certain distance of proximal membrane region from the plasma membrane allows the membrane-bound endoprotease to cleave L-selectin (Chen et al. 1995). Although the role of soluble L-selectin fragment released after its proteolytic shedding is not well understood, a study has shown that soluble L-selectin act as an adhesion buffer that have a distinct anti-adhesive function such as rapid dissociation of cell-cell interaction (Walcheck et al. 2003).

The proximal membrane region of L-selectin consists of 15 amino acids (Chen et al. 1995). Although the specific amino acid sequence of the proximal membrane region is not necessary for the L-selectin proteolytic shedding to occur since the introduction of alanine scanning mutation did not prevent L-selectin from shedding after PMA treatment. Also, alteration in the putative primary cleavage site (K283-S284) of human L-selectin resulted in a partial inhibition of shedding during activation-induced shedding (Chen et al. 1995), The length of the gap of the proximal membrane region from the cell membrane was the most critical in order for the cleavage enzyme to perform the cleavage action. The deletion of amino- or carboxy-terminal half of the proximal membrane region of L-selectin, as in ΔK-N or ΔM-N L-selectin transfected 300.19 cells, resulted in complete inhibition of L-selectin shedding and the release of soluble L-selectin into the

supernatant (Chen et al. 1995). Collectively, the structure of proximal membrane region and its position at the cell membrane is key in order for its sheddase to cleave it at the cell surface.

Since L-selectin is composed of a number of domains, L-selectin shedding by ADAM-17 or any other metalloproteinases at the proximal membrane region will leads to the release of soluble L-selectin into the circulation and leaving behind membrane retained fragment comprising of a shortened proximal membrane region, the transmembrane region and the cytoplasmic tail (Smalley & Ley 2005).

Consist with other type I integral membrane proteins, such as amyloid precursor protein (APP) expressed by neurons in the brain, in which after its primary proteolysis by β-secretase, the Aβ fragment is further processed by γ-secretase, which leads to the release of a cytoplasmic tail (Vorobyeva et al. 2014), L-selectin membrane retained fragment could be further processed by γ-secretase. Release of a cytoplasmic fragment that might be implicated in a number of signalling pathways has yet to be studied.

1.4.3. L-selectin gene transcriptional silencing

Upon $CD8^+$ T cell activation through its TCR, L-selectin is initially down-regulate by ectodomain proteolytic shedding. L-selectin has been shown to re-express during in vitro activation on the surface of $CD8^+$ T cells (Chao et al. 1997). After re-expression, L-selectin gradually down regulates again. This time the L-selectin down-regulation is controlled by the rate of transcription of its gene through the transcription factor Kruppel-like factor 2 (KLF2)(Sinclair et al. 2008).

Naïve and memory $CD8^+$ T cells maintain high level of L-selectin expression through continuous transcription of its gene in a rich IL-15 environment (Sinclair et al. 2008). Whereas, an IL-2 rich environment at the site of infection induces the inhibition of L-selectin gene transcription and effector $CD8^+$ T cells harvested from the site of infection are L-selectin negative (Mobley & Dailey 1992). IL-2 engaging with IL-2R (particularly CD25, IL-2 α-subunit) initiates a series of intracellular signals, which activate the phosphoinositide 3 kinase (PI3K)(Sinclair et al. 2008). PI3K is a family of enzymes that generate lipid messengers inside the cell and it is implicated in a number of cell functions. Among the most important are the activation and differentiation of T cells; collectively PI3K provides co-stimulation for T cell function (Okkenhaug & Vanhaesebroeck 2003). One of the in vivo studies in rats using PI3K blocking agents such as wortmannin or LY294002 has shown that PI3K blocking had a major immunosuppressive effect and also resulted in functional impairment to many organs (Gunther et al. 1989)(Fruman et al. 1999).

PI3K is classified into three major groups, Class I, II and III. PI3K require G-coupled receptors or tyrosine kinase receptors in order to be activated. Class I PI3K, which is involved in L-selectin gene silencing pathway, consists of two subunits, a catalytic subunit (p110) and a regulatory subunit (p85). The p110 subunit is further classified into a number of variants, p110α, p110β and p110δ. The p85 subunit is also classified into 5 different variants named, P85α, P55α, P50α, P85β and P55γ. One variant of each of catalytic and regulatory subunit forms a heterodimeric molecule to form a functional PI3K (Okkenhaug & Vanhaesebroeck 2003).

The function of PI3K is to phosphorylate phosphatidylinositol bisphosphate (PIP2) and form phosphatidylinositol triphosphate (PIP3). PIP3 together with the

mammalian target of rapamycin (mTOR) block the action of KLF2, which is responsible for the transcription of L-selectin gene (Sinclair et al. 2008). PI3K with mTOR, which is further blocking the action of KLF2, also leads to inhibition of the transcription of CCR7 and S1P1 gene. All three molecules (L-selectin, CCR7 and S1P1) are critical receptors in the process of leukocyte migration and exit into secondary lymphoid organs (LN)(Sinclair et al. 2008).

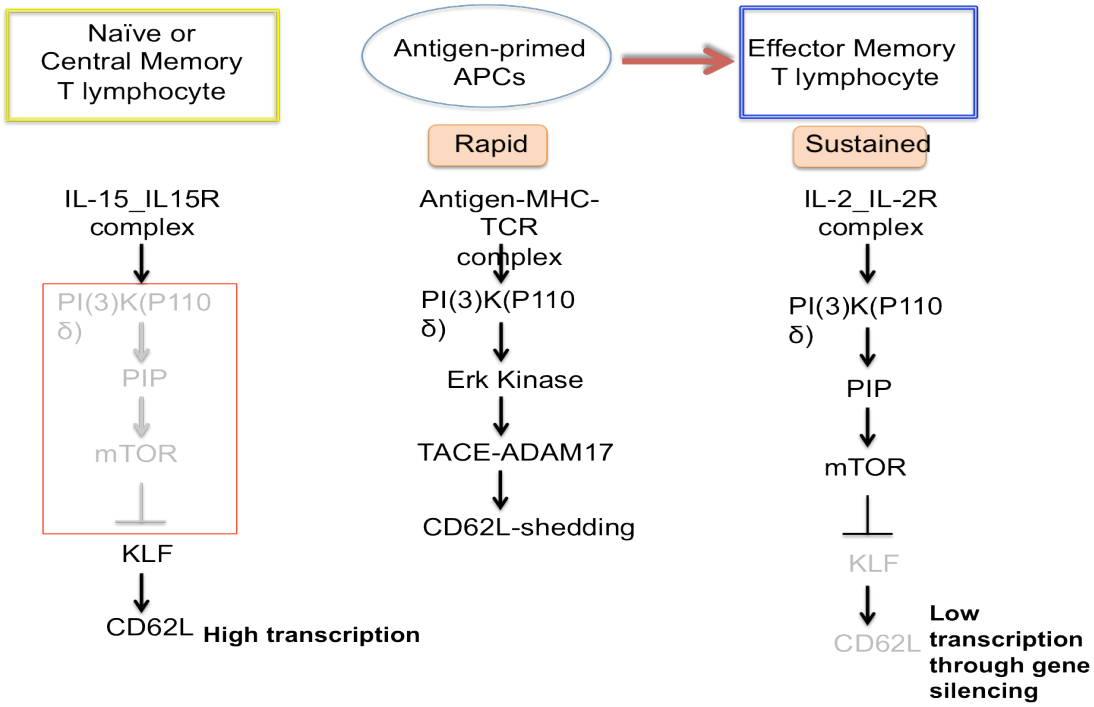


Fig. 1.8: Phosphoinositide-3-OH kinase (PI(3)K) signals together with the mammalian target of rapamycin (mTOR) account for the L-selectin expression status on the surface of various T lymphocyte subsets. Upon T lymphocyte activation, inactive ADAM-17 (TACE-ADAM17) converts to an active form and results in L-selectin shedding. L-selectin is re-expressed on the surface of central memory T lymphocytes in order to home to lymph nodes. But if the stimulus, which could be a virus or any other microorganism persists, PI3K activation is sustained in effector T lymphocytes and L-selectin gene will not be transcribed due to lack of KLF-2 expression.

Another pathway has also been implicated in controlling gene transcription of L-selectin and other adhesion molecules. Similar to PI3K and mTOR pathway, transcription factor forkhead box O 1 (FOXO1) is also functional in naïve T cells. FOXO1 in turn binds to KLF2 and allows the transcription of L-selectin and controls the level of L-selectin expression on the surface of naïve T cells. Inhibition of FOXO1 function is also controlled by downstream signalling of PI3K, which is activated only in naïve T cells primed with antigen (Fabre et al. 2008).

Although PI3K signalling controls these transcription factors which in turn control the level of L-selectin expression and lymphocyte migration behaviour, another role for PI3K is in controlling activated $CD8^+$ T cell homeostasis and the rate of proliferation and apoptosis. Another member of the FOXO transcription family, FOXO3, has been shown to control effector $CD8^+$ T cell fate. In mice, where $CD8^+$ T cells were deficient in FOXO3, there was a massive effector $CD8^+$ T cell accumulation during the effector expansion phase of acute LCMV infection. The increase in the effector $CD8^+$ T cells was not due to enhanced proliferation, but due to impairment in the rate of apoptosis (Sullivan et al. 2012).

1.4.4. L-selectin ligands

L-selectin was characterized as lymph nodes homing receptor on naïve $CD8^+$ T cells (Gallatin et al. 1987). Defining the ligands for L-selectin originated from the early studies where they observed a special affinity binding between the lymphocyte and the cryostat-cut section of lymph nodes *in vitro* (Stamper & Woodruff 1976). The molecular basis of this binding allowed (Gallatin et al. 1983) to define a molecule on the surface of lymphocytes that enable this lymphocyte

entry into the LN first, and then allowed the opportunity for others to describe the counterpart ligand for L-selectin on the vascular endothelium of specialized blood vessels (HEV) in the LN that complete the binding complex.

L-selectin, and other members of the selectin family, is able to recognizes a carbohydrate ligands on the cell surface because, all selectin members share a homology of N-terminal carbohydrate-recognition lectin domain. However, L-selectin binds a sulfated form of sialyl Lewis x with higher affinity (Varki 1994).

The first identified L-selectin ligand is the novel vascular addressin (commonly called Peripheral node addressin PNAd), which is well characterized to be involved in homing of T lymphocytes to peripheral lymph nodes (Streeter et al. 1988). PNAd is a complex name used to identify any glycoproteins that are reactive with L-selectin on the HEV within the lymph nodes. Among the most characterized members of the PNAd complex are GlyCAM-1, CD34 and Podocalyxin. GlyCAM-1 is a secreted protein and also called soluble L-selectin ligand, whereas CD34 and Podocalyxin are type 1 membrane proteins. All the three L-selectin ligands are glycoproteins and they have multiple O-linked glycans. The discovery of these glycoprotein ligands of L-selectins, containing silaic acid or Fuc at the termini of O-linked or N-linked oligosaccharides, were inspired by works which showed that treatment of lymph nodes with mixed glycosidase including sialidase inhibited the lymphocyte binding to HEV (Rosen 2004). Mucosal Addressin cell adhesion molecule-1 (MAdCAM-1) is originally identified as a ligand for α4β7 integrin. But cDNA cloning studies have shown that MAdCAM-1 has multiple functional sites, which can also support L-selectin binding (Berg et al. 1993).

There are conflicting literatures on the role of L-selectin in homing of lymphocytes to the site of infection and the presence of L-selectin ligands in peripheral tissues, which could support L-selectin dependent recruitment of lymphocytes into peripheral non-lymphoid tissues. First it has been shown that effector $CD8^+$ T cells express low level of L-selectin at the site of infection (Kaech et al. 2002; Richards et al. 2008) and second, short term homing studies showed that L-selectin is not a predominant receptor for homing of lymphocytes to the skin (Austrup et al. 1997; Hirata et al. 2002; Mobley & Dailey 1992; Mora & Andrian 2006). However, it has been shown that L-selectin controls the migration of lymphocytes into sites of chronic inflammation. HEV like structures present in the inflamed synovium during rheumatoid arthritis were present which might allow the engagement of L-selectin with molecules expressed on the surface of these HEV like structures and enhance the infiltration of lymphocytes to the site of infection (Jin et al. 2012).

Furthermore, transgenic mice expressing oncoprotein (Tag) in the islet β cells that elicit β cell hyperplasia with subsequent progression to tumor, showed that in the tumor surrounding area, there were ligands for L-selectin and α4β7, which support lymphocytic infiltration. Whereas L-selectin and α4β7 ligands were not expressed within the tumor, they were devoid of lymphocytic infiltration (Onrust et al. 1996).

Also, in patients with neuropathy, it has been shown that there were sulfoglucuronyl glycosphingolipids (SNGL) that allowed the absorption of L and P-selectin chimeric proteins but not E-selectin. The presence of these lipids in the brain might work as ligand for L-selectin on the surface of lymphocytes and allow the recruitment of lymphocytes into the brain tissues (Needham & Schnaar 1993). The role of L-selectin and its ligands were further demonstrated in the brain. It

has been shown that in a mouse model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), induced by treatment of these mice with Myelin oligodendrocyte glycoprotein peptide, disease did not develop in mice deficient in CD62L. They have also shown that CD62L is required for the induction of the EAE, but the T and non-T effector cells did not require CD62L to migrate to the central nervous system parenchyma (Li et al. 2006).

Finding ligands for L-selectin in the peripheral non-lymphoid tissues has been of interest to many scientists. Although the significant relevance of these ligands in the extravascular areas were not known yet, a number of ligands were identified in the past years. A research study has shown that there are ligands for L-selectin in the kidney distal straight tubules. Amino acid sequencing, immunoprecipitation and western blotting studies showed that the nature of these ligands for L-selectin in the kidney tubules is Versican. They confirmed that the L-selectin ligands, isolated from kidney tubule-derived cell line, bind to L-selectin chimeric protein (LEC-IgG) were blocked by using anti-L-selectin antibodies (MEL-14)(Kawashima et al. 1999).

1.5. Adaptive immune response against virus infection

The adaptive immune system orchestrates together to deal specifically with virus infections. Vertebrates born with severe defects in the adaptive immune system will die soon in case they were infected with viruses or any other pathogenic agents. Viruses are severely pathogenic because of their wide range of tissue tropism, pathologic lifecycles and their genomic DNA or RNA structure. Viruses are intracellular parasites, they utilize the infected cells to survive therefore interfere with the normal structure and function of the cell itself.

With the help of the innate immune response receptors such as TLR (Toll-like receptor), which detect viruses in extracellular compartments and NOD-like receptors (NLR) and AIM2-like receptors (ALRs) for intracellular viruses, Innate immune system cells are able to process the virus particles and seek the help from the adaptive immune system. Using a wide range of cytokines such as IL-6, IL1β and TNF-α, innate immune system facilitates the recruitment of the adaptive immune system cells to fight specifically against the infection. Both humoral and cellular arms of the adaptive immune system have important roles in anti-virus immunity (Aoshi et al. 2011). B cells mainly participate in secreting antibodies in response to a specific recognition of the virus particles and the secreted antibodies bind the virus and avoid its further function, infecting cells, and, therefore, avoid its further expansion. Whereas, T cells participate in fighting against the viruses by secreting a range of cytokines such as IFN-gamma to either kill virus infected cells or interfere with virus conformation and impair its replication.

Virus infection can be divided into 2 phases; first, the acute phase or primary infection, in which the NK cells and $CD8^+$ T cell arm of the adaptive immune system play important role in the elimination of the virus and infected cells. In the second, chronic phase, the virus either persists or keeps replicating and the immune system fails to control the infection. Cellular immunity during virus infection can also be classified into a number of phases; priming, activation and effector phase, which is related to the acute phase of the virus infection. Second, death phase, which is either related to the viral clearance or persistence phase of the infection. Generation and establishment of memory cells will follow mainly, if the virus cleared or even if it is reached the latent phase (Wherry & Ahmed 2004; Kalia et al. 2010).

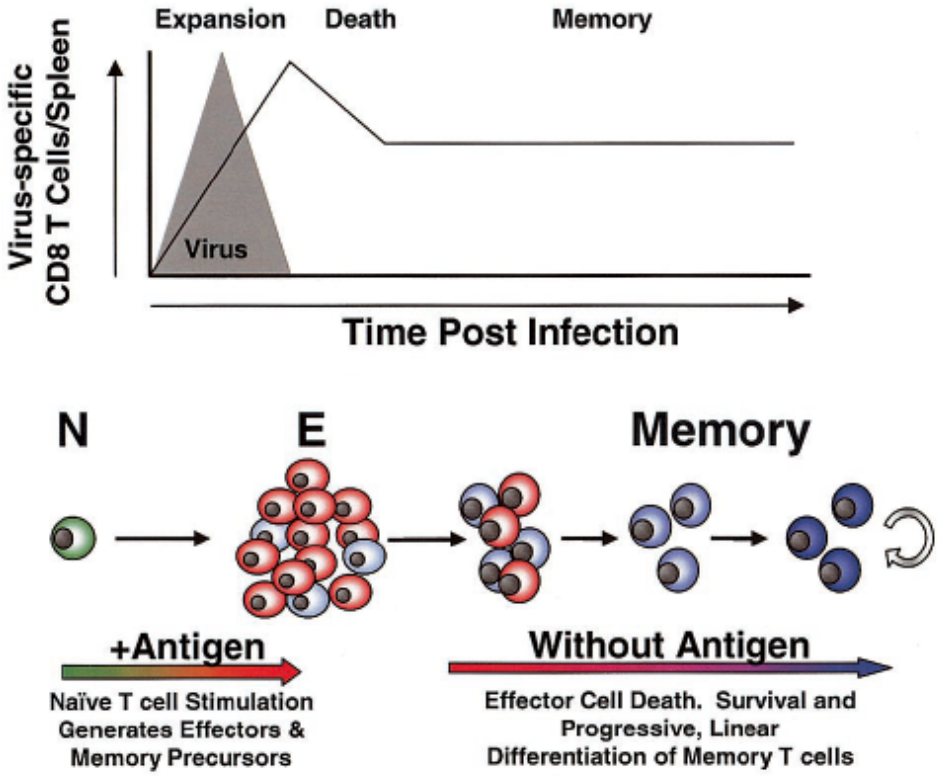


Figure 1.9: Time course of effector and memory CD8$^+$ T cells generation during a course of virus infection. Upon recognition of the virus peptide, naïve CD8$^+$ T cells activate and differentiate into effector CD8$^+$ T cells early during the primary course of the infection, then these cells undergo a contraction phase and cells who survive suring the virus infection, turn into memory CD8$^+$ T cells.
Ahmed, 2010

Leukocytes learn to fight against viruses during their development in the bone marrow (Hermesh et al. 2011). $CD8^+$ T cells play an important role in host defence against lymphocytic choriomeningitis virus (LCMV) infection (Althaus et al. 2007: Kotturi et al. 2007). $CD8^+$ T cells play an important role in both murine and man host defence against the *paramyxoviridae* family member respiratory syncytial virus (RSV). In a murine model of RSV infection, $CD8^+$ T cell depletion results in an increased persistence of the virus and adoptive transfer of primed $CD8^+$ T cells is enough to clear the virus (Cannon et al. 1987). Also in 60% of human donors infected with RSV, $CD8^+$ tetramer positive T cells were found against the immunodominant epitope (M) protein of the RSV virus (De Graaff et al. 2004).

In the presence of sufficient numbers of mature peripheral dendritic cells in the lung, protective immune response against influenza infection is $CD8^+$ T cell dependent (McGill et al. 2008). Also in mice, absence of $CD8^+$ T cells results in an increase in the lethal effects of influenza virus (Bender et al. 1992), whereas, depletion of $CD4^+$ T cells only results in a delay in viral clearance but full recovery (William Allan et al. 1990). $CD8^+$ T cells use their cell adhesion molecules and chemokine receptors in order to perform their function. $CD8^+$ T cells use CXCR3 to localize to virus-infected cells at the site of infection in the skin (Hickman et al. 2015). $CXCR4^+$ T cells, in the presence of IL-1β secreting leukocytes (Primarily macrophages), use CXCL12 chemokine to infiltrate blood brain barrier and contribute to the clearance of West Nile virus (Durrant et al. 2014).

1.5.1. Vaccinia virus

Vaccinia virus is a large, complex, double-stranded DNA virus. It belongs to the *Poxviridae* family. Among the most common member of the family is cowpox. Because it is a large enveloped virus, vaccinia virus replicates in the cytoplasm of the cells outside the nucleus. It has a 190kbp genome and it encodes for around 250 genes. These genes translate to provide the virus with the necessary enzymes and proteins to survive in the host (Tolonen et al. 2001). Once it is enters the cytoplasm 2 hours post infection, vaccinia virus starts DNA synthesis. At 6 hours post infection, all the virions start to assemble. Vaccinia virus produces 4 types of virions, which differ according to location, role and structure. Intracellular mature virion (IMV) is the most common and it is responsible for the spread between hosts. Other virions produced by vaccinia virus are the intracellular enveloped (IEV), cell-associated enveloped (CEV) and extracellular enveloped (EEV)(Smith et al. 2002).

The discovery of vaccination by Edward Jenner in late 17th century was a great achievement in the field of immunology. He immunized humans with the cowpox virus and provided protection upon infection with the smallpox virus. This pioneering experiment led other scientist to map different virus genomes and identify potential targets for virus eradication and successful vaccination. Heterologous viruses from the same family and recombinant viruses that express immunodominant epitopes from other viruses were used to understand cross-protection by the host immune system against different viruses.

Establishment of vaccinia virus infection in mice depends greatly on the dose of the virus and the route of virus inoculation (Briody 1959). A number of different

inbred strains of mice are susceptible to various strains of vaccinia virus. It has been shown that the neurovirulent, Western Reserve strain, killed mice when the virus was inoculated intranasally. The lung consolidation, a process in which liquid fills the alveoli of the lungs during pneumonia, seen in this experiment was accompanied by huge expansion of the virus, viremia and presence of the virus in the brain tissue at the terminal stages. In contrast, Copenhagen-glycerolated calf lymph Utrech, 1961 strain of vaccinia virus led to no detectable infection when inoculated intraperitoneally (Turner 1967).

1.5.1.1. How vaccinia virus establishes its infection in the body

Once the virus entered the body, mechanisms provided by the host help the virus to establish infection. In addition, viruses have evolved strategies that modulate several intracellular and extracellular signalling pathways that lead to a productive viral replication and viral spread. A research study has shown that the mitogen-activated protein kinase (MAPK) was up regulated during vaccinia virus infection. There was also enhanced expression of MAPK-negative regulator dual-specificity phosphatase 1 (DUSP1) during vaccinia virus infection. The presence or absence of DUSP1 did not have an effect of the modified vaccinia virus Ankara (MVA) since the virus has lost its ability to replicate efficiently in most mammalian cells (Cáceres et al. 2013).

Vaccinia virus uses other mechanisms to replicate and spread in the body during an acute infection. Macrophages are usually identified to provide a beneficial effect for the immune system as antigen presenting and phagocytic cells. During vaccinia virus infection, most of the macrophages such as granulocyte-macrophage colony-stimulating factor (GM-CSF)-polarized M1 and macrophage

colony-stimulating factor (GM-CSF)-polarized M2 are permissive for the generation of extracellular enveloped virion (EEV) of the virus (Sánchez-puig et al. 2004; Byrd et al. 2014).

1.5.1.2. Adaptive immune response against vaccinia virus infection

Once the virus has disseminated through viremia and infected various tissues in the body, based on the pathogenicity of the strain of the virus, the next stage of the infection with vaccinia virus is a strong cellular and humoral immune response. Although humoral immune response dominates the protection process (Xu et al. 2004), cellular immunity still provides protection in various situations based on the virus entry route (Goulding et al. 2014).

A number of proteins of the vaccinia virus are subject to the adaptive immune system response. Given this concern, defining candidate particles of virus that results in a strong adaptive immune response is important to improve vaccination strategy against other viruses and also it could help future eradication these viruses (Moutaftsi et al. 2006). H3L protein, an intracellular mature virion envelope protein, is one the main proteins targeted by the B cell arm of the adaptive immune system and high titer of antibodies against it protects the body against the lethal effect of vaccinia virus infection (Davies et al. 2005). Studies in mice using either passive transfer of serum containing anti-H3L antibodies or immunizing mice with H3L protein showed enhanced protection against the lethal effect of the wildtype Western Reserve strain of vaccinia virus (Davies et al. 2005). Another protein found in the vaccinia virus structure that leads to a strong neutralizing antibody response is L1 protein. Anti-L1 antibodies neutralize

vaccinia virus in an isotype and complement independent manner (Kaever et al. 2014).

Reports have shown that a vast range of vaccinia virus epitopes can also induce strong cellular immunity during acute infection. The route of vaccinia virus entry into the body has a great influence on the immunodominant epitopes presented to $CD8^+$ T cells (Lin et al. 2013). It has been shown that $B8R_{20}$ (TSYKFESV) epitope of vaccinia virus is the immunodominant epitope for the $CD8^+$ T cell response during intradermal (i.d.) and intraperitoneal (i.p.) route of virus entry (Tscharke et al. 2005; Moutaftsi et al. 2006; Lin et al. 2013).

Another immunodominant protein of vaccinia virus is A47 epitope. It is encoded by A47L gene, and it accounts for 60% of the $CD8^+$ T cells that are responsive against vaccinia virus (Tscharke et al. 2005; Yuen et al. 2010). Other epitopes from vaccinia virus were identified to influence a long lasting memory $CD8^+$ T cell response. Also, some of these memory cells provide a cross-reactive immunity for other viruses. $E7r_{130}$ and $a11r_{198}$ are two epitopes encode by the vaccinia virus large genome. It has been shown that $a11r_{198}$ provides cross-reactive $CD8^+$ T cell for LCMV infection, whereas, $E7r_{130}$ did not. Either epitopes influenced a good memory $CD8^+$ T cell pool that was the solely protective immunity against secondary challenge of the vaccinia virus infection (Cornberg et al. 2007).

Investigating the biological characterization of viruses is important to understand virus pathogenesis (Williamson et al. 1990). In order to study leukocyte migration during virus infections, it is important to identify which organs are involved in the pathogenesis of the virus. A virus that is inserted with genes from other viruses to produce a recombinant form of the virus is possible to change its virulence and pathogenesis (Williamson et al. 1990). Challenging DBA/2 (Dilute Brown Non

Agouti/2) mice i.n. with 10^6 pock forming units of WR vaccinia virus resulted in a lethal infection but mice were survived from the infection with inoculation of same amount of the recombinant vaccinia virus inserted with 340 genes of Epstein-Bar virus (Williamson et al. 1990).

Although recombinant vaccinia virus is not able to produce a lethal infection, it can be used as a good tool for vaccination. Low dose (10^5 PFU) recombinant vaccinia virus Ankara (MVA), led to the generation of large amount of virus-specific $CD8^+$ T cells that were protective against secondary challenge with mousepox virus ectromelia virus infection (ECTV). The effect of the memory $CD8^+$ T cell response in the vaccinated animals was measured by the complete disappearance of the symptoms and inability to detect the virus in various organs (Volz et al. 2014).

1.5.2. Influenza virus

Influenza virus is an RNA virus belonging to the family *Orthomyxoviridae*. It is classified into 3 major classes, Influenza class A, B and C. Influenza A is the most commonly described and it is responsible for most of the pandemic outbreaks that have occurred (Webster et al. 1992). Wild birds are the main natural host for the Influenza viruses. Mutations allow the virus to be transmitted to other animals, mainly poultry, and can also allow transmission to humans and cause devastating pandemic outbreaks (Hay et al. 2001). Influenza virus strain A has been widely used in experimental models of mice infection in order to understand the biological characteristics of the virus which helps to understand the pathogenesis of the virus in human and birds (Blazejewska et al. 2011).

An early study by Taylor, 1944, showed that Influenza virus A and B are almost structurally similar. Both viruses are mainly composed of protein, lipid, carbohydrates and nucleic acids. Chemical analysis revealed that lipid constitutes 24% of the Influenza A virus structure, whereas Influenza B virus has less lipid structure. Cholesterol, phospholipids, carbohydrates and nucleic acids account for the rest of the virus complex (Taylor 1944).

The influenza virus genome consists of eight segments of single-stranded negatively charged RNA. Influenza genome can encode 11 proteins, which participate in virus pathogenesis. Among the most common ones are the Hemagglutinin (HA), Neuraminidase (NA), Nucleoprotein (NP), polymerases PB1, PB2 and PA M1 and M2 proteins (Webster et al. 1992)(Ghedin et al. 2005). Influenza virus must have these proteins in order to be infective (Webster et al. 1992).

1.5.2.1. Influenza virus infection in human and role of pharmaceutically available inhibitors in the disease prevention

The Influenza genome undergoes continuous mutations, which makes it difficult for a single vaccination strategy to be fully successful and protect from the annual epidemics. Antigenic shift, which occurs much less frequently, is responsible for the creation of new strain to which humans will have less protective immunity and account for the seasonal pandemics (Ghedin et al. 2005). HA and NA are glycoproteins that are found on the surface of the influenza virus. Since they are expressed externally, they participate in the internalization process of the virus into the host cell.

In 1984, Justewicz et al. has shown that monoclonal antibodies that have the ability to neutralize hemagglutinin but not nucleoprotein and matrix protein of influenza A or B virus, reduced the infectivity of the virus which was measured by virus-induced cell-mediated cytotoxicity (Justewicz & Lecomte 1984). Also, It has been shown that a broad spectrum-neutralizing antibody CH65 binds to the receptor binding pockets of the HA protein and minimize the infectivity of 30 out of 36 H1N1 strains of influenza virus (Whittle et al. 2011). These monoclonal antibodies, which reduce the infectivity of influenza virus through the viral protein HA is mediated by first binding to the HA which prevents the virus attaching and being released into the target cell. Further mechanisms exploited by these antibodies that are important to the life cycle of the virus are preventing fusion between the viral and endosomal membrane, which occurs internally. Also, prevention of proteolytic activation of the HA is another mechanism of these broad spectra antibodies that neutralize the influenza virus (Brandenburg et al. 2013).

Young children, elderly people and people with naturally occurring morbidities are at higher risk of infection with seasonal influenza virus (Jefferson et al. 2010). Vaccination is still a successful approach to reduce the risk of influenza virus infection, which is recommended by most physicians (Moscona 2005). Another key important protein in the influenza virus is the neuraminidase (NA) protein on the surface of the virus. Although there is controversy on the effectiveness of NA inhibitors, beneficial effects of these inhibitors in the prevention of seasonal flu has been documented in some countries (Michiels et al. 2013). NA enables the newly formed virions to be released from infected cells and allow a further spread of the virus in the targeted body and even further between individuals (Moscona 2005). These NA inhibitors prevent the action of the influenza virus

neuraminidase enzymatic activity and, therefore, limit the spread of the virus. Two pharmaceutically available neuraminidase inhibitors are Oseltamivir, which can be inhaled because of the limited absorbability and Zanamivir, which can be taken orally (McNicholl & McNicholl 2001; Mckimm-breschkin 2012).

1.5.2.2. Influenza virus infection in mice and role of adaptive immune system in its clearance

The less common antigenic shift, which occurs occasionally, allows the influenza virus to adapt to a new host. This adaptation of the virus into a new host occur through reassortment in the co-infecting influenza A virus gene segments in conjunction with multiple adaptive mutations in various influenza A virus genes. The first important adaptation of the virus is the ability to be transmissible to the new host. The mouse-adapted Influenza virus A/Puerto Rico/8/34 has been used to infect guinea pigs to study the adaptation of the virus into the new host. During a number of nasal wash passaging, the influenza protein M1 and NP were the main determinants that mutated and allowed the virus to adapt into the new host, the guinea pig (Ince et al. 2013).

The role of cellular immunity in the clearance of influenza infection has been studied in mice in order to understand the mechanism of the viral clearance in humans. Although cytotoxic activity of $CD8^+$ T cells require time to develop, the vaccination strategy, using antibodies and inhibitors, to prevent the occurrence of the influenza infection still provides 40% protection (Michiels et al. 2011). Adaptive immune systems, including both humoral cellular immunity, play a complementary role to provide a strong immune response against influenza virus infection, but some parts of the adaptive immune system is more important in

direct clearance of the virus than others. Wu et al. 2010 have shown that chronically immunodeficient mice ($RAG2^{-/-}$) mice, which lacks recombination machinery and are devoid of mature T and B cells, challenged with mouse-adapted influenza virus A/PR/8/34 virus, had sustained viral clearance when compared with wild type mice. The $RAG2^{-/-}$ mice lost weight initially with similar kinetics to wildtype mice, but later, when the wildtype mice started to regain weight and survive the primary influenza virus infection, $RAG2^{-/-}$ mice lost further weight and culminated in death (Wu et al. 2010).

Although $CD4^+$ T cells are important for $CD8^+$ T cells in providing necessary cytokine storm for the $CD8^+$ T cell survival and functional enhancement during viral infection, monoclonal antibody mediated depletion of $CD4^+$ T cells, in mice that were challenged with HKx31 variant of H3N2 influenza A virus, had no effect on the viral load. The $CD4^+$ T cell depletion had little or no effect on the frequency of cytotoxic $CD8^+$ T cells, which dominated both mediastinal lymph node and lung during the infection (William Allan et al. 1990). Further studies confirmed that the role of $CD4^+$ T cells in primary influenza virus infection was inefficient in the absence of B and $CD8^+$ T cells (Mozdzanowska et al. 1997; Topham & Doherty 1998).

Further studies dissecting the participation of various subsets of cellular immunity in the clearance of influenza virus infection were of great importance. The role of B cells in primary influenza virus clearance is not very clear as it depends on the dose of the virus and the type of the virus. It has been shown that mice with a targeted mutation in immunoglobulin mu heavy chain or μKo mice, which lack mature B cells, were more susceptible to lethal influenza virus A/JAPAN/57 infection than wildtype C57BL/6 mice, whereas, using lower virus dose, B lymphocyte deficient mice survived the infection (Graham & Braciale 1997). Also,

adoptive transfer of virus-specific $CD8^+$ T cells, but not $CD4^+$ T cells, prevented the lethal effect of influenza virus A/JAPAN/57 infection in B-lymphocyte deficient mice (Graham & Braciale 1997). Further studies also showed that B cells contributed to the recovery of mice from lethal influenza virus A/PR8/34 infection in a T helper cell independent manner. μKo mice transferred with splenocytes from T helper cell-depleted mice reduced mortality to 0% (Mozdzanowska et al. 1997).

The concepts of designing vaccines that promote the $CD8^+$ T cell arm of the adaptive immune system are always interesting. This is mainly because antibody B cell-mediated protection against influenza virus infection is narrow with regards to viral strain specificity (Epstein et al. 1998). A short report has shown that $CD8^+$ T cell deficient mice lacking class I major histocompatibility complex, showed significantly delayed virus clearance after challenge with non-lethal form of the influenza virus A/Port Chalmers/1/73 (H3N2). Furthermore, challenging these $CD8^+$ T cell deficient mice with a more lethal form of the influenza virus A/PR8/34 resulted in high mortality rate (Bender et al. 1992). The role of the $CD8^+$ T cell response in influenza virus clearance seems to be virus subtype dependent, because it has been shown that mice lacking $CD8^+$ T cells, either through antibody depletion or lacking MHC class I, can eventually recover from influenza virus HKx31 (H3N2) infection (Eichelberger et al. 1991).

Furthermore, a number of studies using adoptive cell transfer of virus-specific $CD8^+$ T cells have shown that virus-specific CTL can protect mice from the lethal effect of the influenza virus A/WSN infection (McKenzie 1978). Non-glycosylated internal NP protein from influenza A virus serves as a potent target antigen for a cross-reactive T cell clone. Adoptive transfers of cytotoxic Influenza A virus NP-specific cross-reactive T cell clone BA4 and T9/13 were able to protect BALB/c

mice by reducing the detectable viral titers following influenza virus A/JAPAN and HKx31 infection, respectively (Taylor & Askonas 1986). Also, during infection with virulent influenza A/PR8/34 infection, adoptive transfer of B4 T cell clone was able to protect C57BL/6 mice from the lethal effect of the infection (Taylor & Askonas 1986). The effect of the T9/13 NP-specific cytotoxic T cell clone was further determined by histological analysis of lung tissues from BALB/c mice infected with influenza virus A/X31. BALB/c mice, which received T9/13 cells, showed much reduced epithelial abnormalities compared with the control mice at day 6 post infection (Mackenzie et al. 1989).

Similar to the antibody response against various subtypes and variants of influenza virus infection, T cell clones against influenza virus are specific too, since each clone has its TCR. It has been shown that simultaneous challenge of mice with lethal doses of two different influenza virus subtypes at the same time will lead to failure of the recovery and protection by adoptive transfer of subtype-specific T cell clone, whereas the subtype-specific T cell clone was protective, if the mice were challenged with their target influenza virus (Lukacher et al. 1984).

1.6. Role of co-stimulation during lymphocyte activation

$CD8^+$ T cell response against viruses and cancer is a critical part of the cellular immune system. Although $CD8^+$ T cell requires activation through target antigen presented in the context of MHC class I antigen-presenting cells, $CD8^+$ T cells will not be optimally activated without co-stimulatory signals (McAdam et al. 1998). The most common $CD8^+$ T cell co-stimulation molecules are the CD28 molecules expressed by T cells and the B7 (CD80) molecules expressed by antigen presenting cells (Harding & Allison 1993). The engagement of CD28 and CD80

during T cell activation provides co-stimulatory signals for further T cell activation and also for T cell survival by secretion of cytokines such as IL-2 (Boise et al. 1995).

Originally, during $CD8^+$ T cell activation through TCR, the co-stimulatory signals provided by engagement of CD28 on T cells with CD80 on APCs were thought to be an absolute necessity for T cell activation otherwise T cell will become anergic, which is an unresponsive T cell that fails to display certain functional properties (Schwartz 1990). Whereas, it has been shown that $CD28^{-/-}$ mice triggered a robust cytotoxic $CD8^+$ T cell response when infected with lymphocytic choriomeningitis virus (Shahinian et al. 1993). Furthermore, naïve $CD8^+$ T cell were fully activated in the presence of signal 1 only which is the engagement of TCR with its cognate peptide, as indicated by up- regulation of the activation makers, CD69, CD25 and CD44 (Pardigon et al. 1998).

1.7. Role of L-selectin in co-stimulation during leukocyte activation

The immune system has evolved to compensate for missing molecules and signals to some extent. It can adapt to the environment or find an alternative to organize its function. In the absence of CD28 engagement with CD80 co-stimulation, T cells can benefit from other molecules for co-stimulation such as ICAM-1, 2 and 3, LFA-3, VCAM-1, OX40L and other tumor necrosis factor family members (Croft & Dubey 1997).

Similar to other cell adhesion molecules, L-selectin has been implicated in intracellular signalling pathways upon engagement with its ligands (Stadtmann et

al. 2013). A recent study has shown that engagement of L-selectin with monoclonal antibodies or endothelial cell expressed ligand enhances chemotaxis of T cells to secondary lymphoid chemokine (Subramanian et al. 2012). L-selectin on the surface of neutrophils engaging with PSGL-1 on the surface of another neutrophil or other leukocytes lead to a series of intracellular signalling that activate LFA-1 integrin. The L-selectin-PSGL-1 complex dependent activation of integrin regulates rolling and recruitment of neutrophils under *in vitro* flow conditions (Stadtmann et al. 2013). Also, on the surface of lymphocytes, L-selectin ligation by monoclonal antibodies has been shown to provide signals that enhance cell-cell interaction and lymphocyte recruitment (Steeber et al. 1997). Furthermore, it has been shown that L-selectin on the surface of freshly isolated human lymphocytes increases the surface expression of chemokine receptor, CXCR4 upon ligation with its ligands such as sulfatide or fucoidan, whereas, L-selectin ligation with these ligands did not have any effect on the surface expression of CCR7, CCR5 and β_2 integrin. This suggests that L-selectin co-stimulation allows a selective enhancement in the expression of cell adhesion molecules, which will allow a selective recruitment of cells to various parts of the body (Ding et al. 2003).

Intracellularly, a number of cytosolic partners have been reported to associate with L-selectin molecules. Among these partners is alpha-actinin, which binds to the carboxy terminal 11 amino acids of the L-selectin cytoplasmic tail. Alpha-actinin further connects L-selectin to the actin cytoskeleton of the cell (Pavalko et al. 1995). Although the alpha-actinin connection between L-selectin and cytoskeleton is required to control leukocyte rolling, which is adhesion under flow conditions, the role of α-actinin in L-selectin dependent intracellular signalling and co-stimulation is not clear (Ivetic 2013).

Other molecules associated with L-selectin tail are Calmodulin (CaM) (Gifford et al. 2012) and Ezrin-Radixin-Moesin (ERM) (Ivetic et al. 2002). L-selectin naturally occurs as monomeric molecules in the lipid bilayer cell membrane. Upon engaging with its ligand, L-selectin clusters and forms a dimeric molecule (Dwir et al. 2002). Then, this L-selectin dimerization allows further engagement of Ras activator, Son of Sevenless (SOS), associated ion with ERM proteins and the small GTPase, Ras, associated with CaM. A study by Brenner et al., 1996, showed that upon L-selectin engagement by antibodies or glycomimetics, a series of signalling events were initiated through activation of Ras and eventually resulted in an increase in the level of free radical O_2 synthesis in Jurkat T cells.

Another important signalling cascade initiated through L-selectin occurs due to the presence of two serine residues in its tail. PKC including PKC isozyme θ and ι were found on the non-phosphorylated tail of L-selectin (Kilian et al. 2004). Following lymphocyte activation by cross-linking of CD3 or through TCR, PKC θ has been shown to be associated with PKC α and both were associated with L-selectin tail, which ae phosphorylate the serine residues on L-selectin tail (Murakawa et al. 1992). In steady state, CaM cytosolic partner, binds L-selectin tail and prevent PKC phosphorylation of the serine residues. Serine phosphorylation in the cytoplasmic tail can be reversed by protein phosphatase 2A. Also, dephosphorylation is controlled by the protein phosphatase 2A inhibitor Phap II, collectively CaM and phosphatases and PKC are responsible for the signals initiated and terminated from the tail of L-selectin (Fig 1.10)(Wedepohl et al. 2012).

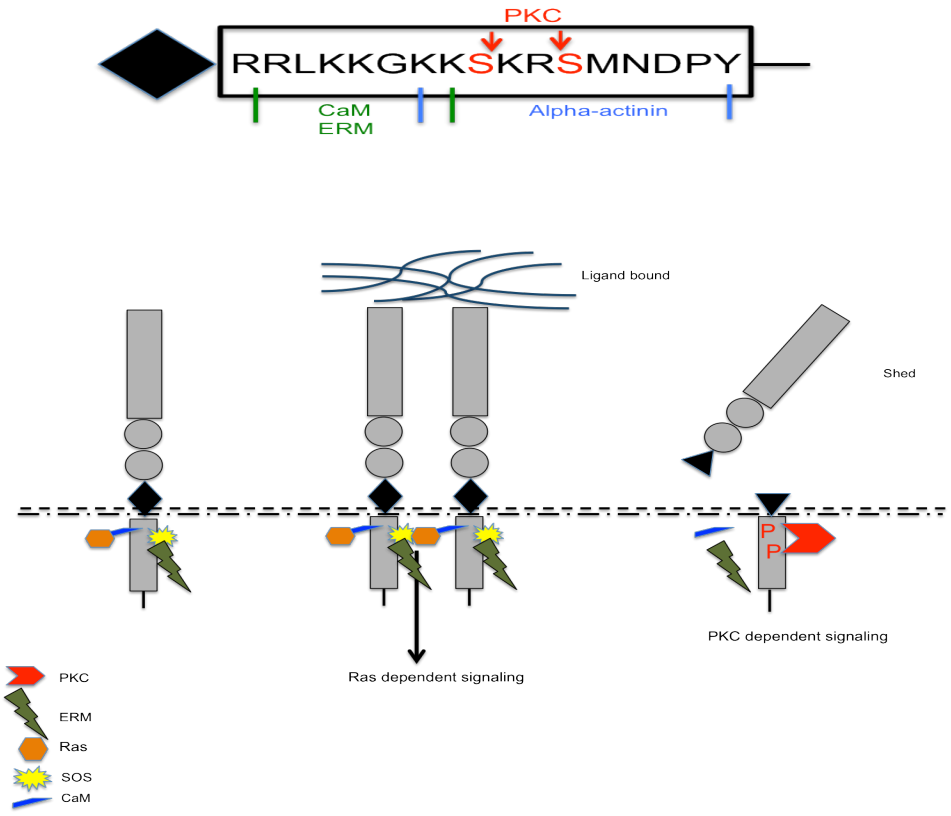


Fig. 1.10: Mouse L-selectin tail structure. (A) Cytoplasmic tail of L-selectin consists of 17 amino acids. The tail is critical for the function of L-selectin. L-selectin tail contains Alpha actinin, CaM and ERM binding sites, which are important functional molecules involved in the signaling pathways required to control leukocyte-endothelial cell interaction. (B) Upon $CD8^+$ T cell activation and clustering of L-selectin molecules by ligand, ERM associates with Ras exchange factor, son of sevenless (SOS) and together with CaM it is hypothesized that L-selectin forms clusters leading to Ras dependent signaling, which is considered essential for transendothelial migration of leukocytes. After Ras dependent signaling, these molecules are released from L-selectin tail allowing Protein Kinase C (PKC) to phosphorylate the serine residues in the tail, which leads to PKC dependent signaling essential for interstitial chemotaxis.

Chapter Two

2. Material and Methods

2.1. Mice

Transgenic mice homozygous for mutant L-selectin on a C56BL/6 (B6) L-selectin deficient background (LΔP) were crossed with mice homozygous for transgenic F5 TCR on a B6 L-selectin deficient background ($F5^{+/+}$L-$sel^{-/-}$) to generate F5/LΔP mice as described previously (Galkina et al. 2007; Galkina et al. 2003). F5/B6 mice, which express matched levels of F5 TCR and similar levels of L-selectin as F5/LΔP mice, were generated by crossing homozygous F5B6 mice ($F5^{+/+}$L-$sel^{+/+}$) with B6 and used as controls. Mice, which were homozygous for F5 TCR and mutant L-selectin (F5LΔP) were generated by crossing two heterozygous F5/LΔP mice and genotype their offspring to identify homozygous F5LΔP mice for further breeding. F5L-$sel^{-/-}$ mice, which were homozygous for F5 TCR and deficient in L-selectin were generated by crossing homozygous F5 TCR mice with L-selectin deficient mice (Table 1 and 2).

Thy 1.1 (CD90.1) and Rag1 and 2 $^{-/-}$ mice on a B6 background were bred in house. B6 recipient mice (CD90.2) were purchased from Harlan or Charles River Laboratories. All experiments were conducted according to institutional guidelines and U.K. home office regulation.

Table 2.1: The breeding strategy to generate mice with various F5, mutant L-selectin, wildtype L-selectin and L-selectin knockout status.

Parent 1	Parent 2	Progeny
LΔP	F5	F5/LΔP
F5	B6	F5B6
F5	L-sel$^{-/-}$	F5L-sel$^{-/-}$
F5B6	B6	F5/B6
F5/LΔP	F5/LΔP	F5LΔP

Table 2.1: The homozygosity or heterozygosity status of the F5 transgenic TCR, the levels of wildtype L-selectin and the presence or absence of mutant L-selectin LΔP.

Mice	F5	L-selectin	LΔP
F5/LΔP	+/-	-/-	+/-
F5LΔP	+/+	-/-	+/+
F5/B6	+/-	+/+	-
F5B6	+/+	+/+	-
F5L-sel$^{-/-}$	+/+	-/-	-

2.2. Viruses

2.2.1. Recombinant vaccinia virus (vaccNP)

Recombinant vaccinia virus expressing nucleoprotein peptide $NP_{366-374}$ from influenza A strain E61-13-H17 virus (vaccNP)(Cerundolo et al. 1997) was a kind gift from Alan Townsend.

2.2.1.1. Infecting mice with recombinant vaccinia virus (vaccNP)

Mice were inoculated with $2x10^6$ PFU vaccNP; briefly, mice were manually restrained on their back, and vaccNP in 400µl phosphate buffered saline (PBS) (stock $5x10^6$/ml) were injected intraperitoneally using a 1ml syringe. The mice were kept in scantainer cages with a negative airflow in order to comply with health and safety regulations.

2.2.1.2. Recombinant vaccinia virus (vaccNP) titer

For recombinant vaccinia virus (vaccNP), ovary, liver, heart, and lung from infected mice were harvested at indicated time points and viral titers were determined using a plaque assay on 70-80% confluent TK- cells as described previously (Jones et al. 2003). Briefly, $1x10^5$ TK- cells were re-suspended in 1ml complete DMEM media [DMEM media (Gibco-Invitrogen, Carlsbad, USA) added with 10% fetal calf serum FCS (Gibco-Invitrogen, Carlsbad, USA), penicillin-streptomycin, L-glutamine, non-essential amino acids (life technologies-Invitrogen, Carlsbad, USA)] and plated in a 24 well plates and incubated at 37°C 5% CO_2 for 24 hours, when the cells reached 70-80% confluent. Interested organs from vaccNP infected mice were homogenized with a sonicator (IKA T10

electric tissue homogenizer) and serially diluted from 1:10 to 1:1000,000 and TK- cells were incubated with 200µl of the diluted ovary homogenates for 2 hours. 1ml complete DMEM media was added and the plate incubated overnight and stained with 0.01% crystal violet in 4% formaldehyde for 3 hours. The plates were washed with water and plaques counted using an inverted microscope. Total pfu was calculated based on the serial dilution, well with 30-300 pfu were considered countable.

2.2.2. Influenza A virus

Influenza A virus strain A/PuertoRico/8/34 (PR8, H1N1) and (H17, H3N2) was obtained from the National Institute for Medical Research (London, UK). Stocks were stored in -70°C.

2.2.2.1. Infecting mice with Influenza A virus (H17 and PR8)

Mice were inoculated with 50 FFU of either PR8 or H17; briefly, mice were put under general anesthesia and manually restrained on their back, 50 FFU Influenza virus in 50µl were inoculated drop wise into nostrils in a split dose of 25µl each using a Gilson pipette fitted with a small tip, ensuring each drop was inhaled. The mice were kept in scantainer cages with a negative airflow in order to comply with health and safety regulations.

2.2.2.2. Influenza strain A virus titer (H17 and PR8)

Lungs from mice infected with PR8 or H17 virus were harvested at day 8 post infection and viral load determined using a virus titration assay as previously described (Bachmann et al. 1999) but with minor modifications. Briefly, lungs

were homogenized in 1ml serum free IMDM media and serial 10–fold dilutions of lung homogenates were incubated with 1.4 x 10^5 trypsinized Luc9.1 clone of Madin Darby canine kidney (MDCK) cells (Hossain et al. 2010) and plated on a 24-well plate. After 4 hours, a methyl cellulose overlay was added to prevent virus transmission between MDCK cells and plates incubated for 48 hours at 37^{o}C in 5% CO_2. The plates were washed with PBS and fixed with 4% formaldehyde for 30 minutes and then permeabilized with 1% triton X100 for 30 minutes. Following permeabilization, MDCK cells were incubated with a monoclonal antibody specific for the PR8 haemagglutinin, stained with horseradish peroxidase-conjugated secondary antibodies (Biorad, USA) and the plates developed using AEC (3-Amino-9-ethylcarbazole) reagents (Cat no. SLBG7194V, Sigma, USA). Each stained, influenza-infected MDCK cell was recorded immediately after staining as a single focus forming unit (ffu) and the total ffu per lung calculated.

2.3. Cell isolation

2.3.1. $CD8^+$ T lymphocyte isolation using Miltenyi magnetic technology

Mice were culled by schedule 1 (CO_2 Euthanasia) and spleen and/or lymph node cell suspension was prepared by mashing the spleen or lymph node through a 70µm cell strainer. Red blood cell lysis was performed for spleen cell suspension by adding 5ml (1X) RBC lysis buffer (10X stock, Biolegend) to the cells and incubating on ice for 5 minutes with occasional shaking. Cells were resuspended in MACS buffer (PBS, 0.5% FCS, 2mM EDTA) and $CD8^+$ T lymphocytes were purified by positive or negative selection using magnetic bead MACS LS column (Miltenyi Biotech, Bergisch Gladbach, Germany). For positive selection, 10^7 cells were re-suspended in 90µL MACS Buffer and mixed with 10µL antibody/10^7cells

(Microbeads conjugated to monoclonal anti-mouse CD8a), the tube mixed well in order to allow the equal labeling of cells with their counterpart antibodies in the suspension. After 15 minutes incubation at 4-8°C, the cell suspension was washed and up to 10^8 cells were re-suspended in 500µL MACS buffer. Then sorting step performed by placing the LS column in the dedicated magnet and the column washed with 3ml MACS buffer. The cell suspension was applied to the column and unlabelled cells, which passed through the column were collected. The column was washed three times with 3ml MACS buffer and following that the column was removed from the magnetic field and 5ml MACS buffer layered on top. Labeled cells ($CD8^+$ T cells) were collected by placing the plunger on top of the column and pushing hard to expel all the labeled cells.

2.3.2. $CD8^+$ T lymphocyte isolation using Stem Cell Technology

The $CD8^+$ T cells were alternatively purified using magnetic cell sorting technique from Stem Cell Technology (Vancouver, Canada) according to manufacturer's instruction. Briefly, spleen and/or lymph nodes from desired donor mice were harvested. The mouse was culled by schedule 1 (CO_2 Euthanasia), then, single cell suspensions were prepared by mashing the tissue through 70µm cell strainer and red blood cells lysed using 1x red blood cell lysis buffer (Biolegend, UK). $1x10^8$ lymphocytes were spun down and re-suspended in 1 ml cell sorting buffer and incubated with 50µl rat serum and 50µl antibody cocktail for 10 minutes, after that, the cells were incubated with 125µl bead microspheres for 2.5 minutes, then the cell suspension brought up to 2.5 ml in total and the cells were placed in the purple magnet (STEM CELL Ltd, Vancouver, Canada) for further 2.5 minutes. After incubation, the desired $CD8^+$ T cells were poured into a tube by inverting the cells in the tube with the magnet in one direction. The purity of the cells then

checked by staining for $CD8^+$ T cells using flow cytometry and cells were considered pure by achieving > 95-98% $CD8^+$ T cells staining.

2.4. Adoptive transfer of Naïve $CD8^+$ T cells

Naïve $CD8^+$ T cells were isolated and purified from donor mice and adoptively transferred via tail vein into recipient mice. Mice were maintained in the heating chamber for 20-30 minutes in order to dilate the tail vein. Mice were restrained in specially built mouse restrainer in order to allow easy access to the tail vein. Prior to injection, the cells were washed with saline or PBS 3 times and desired numbers of cells, according to various experiments, were re-suspended in saline or PBS and injected into the tail using a 29g insulin syringe. After injection low pressure was applied on the injection site to cease the bleeding and to avoid any loss of the injected samples.

2.5. Preparation of mouse tissues for donor and host cell analysis

For all in vivo experiments, at the end point, mice were euthanized by CO_2 asphyxiation in a rising concentration chamber. The schedule 1 death was confirmed by dislocation of the neck at cervical region. The tissues were then removed and placed in vials containing PBS and placed on ice and transferred to the laboratory for further processing.

2.5.1. Ovary

Ovaries were excised from the peritoneal cavity and placed in vials containing PBS on ice until further processing. The ovaries were mashed through a 70µm cell strainer using a plunger of a 2ml syringe. The cell strainer was washed 3-4

times with ice-cold PBS containing 2%FCS to collect all isolated cells, the cell suspension was centrifuged at 400g for 5 minutes, the supernatant discarded and the cell pellet re-suspended in PBS for further analysis.

2.5.2. Lung

Lung was excised from the thoracic cavity and transferred to vials containing 5% FCS, 1mg/ml collagenase D (Roche, USA), 50µg/ml DNase (SIGMA-ALDRICH, USA) and 5mM $CaCl_2$ and placed on ice until further processing. The lungs then transferred to the laboratory and incubated for 1 hour at 37°C, after incubation, lungs were mashed through 70µm cell strainer using the plunger of 2ml syringe and isolated cells collected as described above. Cell strainer was washed 3-4 times with cold PBS to allow all the cells to go through. The cell suspension then centrifuged 400g for 5 minutes. Supernatant was discarded and the cell pellet were re-suspended in 5ml 1X RBC lyses buffer for 5 minutes on ice to lyse the red blood cells. The reaction was then stopped by adding 20 ml PBS. The cell suspension then centrifuged and the cell pellet was re-suspended in PBS for donor and host cell analysis.

2.5.3. Lymph nodes

Lymph nodes (axillary, brachial, inguinal, mediastinal, lumbar and mesenteric) were excised from their location and transferred into vials containing PBS and placed on ice. Single cell suspension was prepared similarly to ovary samples.

2.5.4. Spleen

The spleen was excised from the peritoneal cavity and transferred into vials containing PBS and placed on ice. Single cell suspension was prepared by mashing the lymph node through a 70μm cells strainer using a plunge of a 2ml syringe, The cell suspension was then centrifuged 400g for 5 minutes. Supernatant was discarded and the cell pellet was re-suspended in 5ml 1X RBC lyses buffer for 5 minutes on ice to lyse the red blood cells. The reaction then stopped by adding 20 ml PBS. The cell suspension was then centrifuged and the cell pellet was re-suspended in PBS for donor and host cell analysis.

2.6. MEL-14 blocking strategy

To inhibit L-selectin function, 250 μg of monoclonal antibody to mouse L-selectin (MEL-14, IgG2a)(BioXcell, West Lebanon, NH, USA) or rat IgG2a isotype control antibodies MAC193 (Price et al. 1997) or MAC 219 (Forsyth et al. 2000) were injected i.v. and/or i.p. either once or daily commencing 52 h post vaccNP or Influenza A virus infection. Donor cells were harvested from the ovary, ovdLN and spleen 5 days post infection during vaccNP infection to assess L-selectin involvement in the recruitment of these cells into the site of infection and donor cells were harvested from lung, mediastinal LN and spleen 4 and 8 days post infection during Influenza A infection.

2.7. $CD8^+$ T cell culture (Activation and Differentiation)

$CD8^+$ T cells were sorted by positive selection using Miltenyi technique as described previously. $CD8^+$ T cells were re-suspended in complete DMEM medium (Gibco-Invitrogen, Carlsbad, USA) added with 10% fetal calf serum FCS

(Gibco-Invitrogen, Carlsbad, USA), 100IU/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate (life technologies-Invitrogen, Carlsbad, USA) and 60µM 2β mercaptoethanol (2Me) (Sigma-Aldrich, St Louis, USA). CD8+ T cells were plated at $2x10^6$ cells/well in a 24 well plate in 1ml complete DMEM media. CD8- T cell fraction ($CD4^+$ T lymphocytes, B lymphocytes, NK, NKT cells) from positive selection sorting or unsorted splenocytes were used as antigen presenting cells (APCs). $6x10^6$ APCs were resuspended in 1 ml complete DMEM media and incubated with 5µg freshly prepared NP68 peptide (stock at 1mg/ml) for 1 hour at 37°C. APCs were irradiated at 3000 centri Grays to inhibit the growth of those cells in the culture. After that, $6x10^6$ APCs in 1 ml media, loaded with NP68 peptide, were added to the $CD8^+$T cells cultures totalling 2ml media/well. The cells were incubated at 37°C and 5% CO_2 for 2 days and then 1 ml of the medium was replaced with fresh complete DMEM medium added with 2x 360 IU/ml human recombinant IL-2 (hrIL-2) to induce proliferation. The cells were kept in the culture for a desired length of time, up to 8 days.

2.8. Short term competitive homing study

F5LΔP and F5L-$sel^{-/-}$ $CD8^+$ T cells were isolated and cultured as described above. Cells were harvested at day 7 post activation in culture, mixed 1:1 and a total of 5.58 x 10^6 cells (2.76 x 10^6 F5LΔP and 2.82 x 10^6 F5L-$sel^{-/-}$ determined using Cytocount beads) injected i.v. into 4 vaccNP infected $CD90.1^+$ B6 mice (at day 3 post infection). After 3 hours, ovaries, ovdLNs, axillary lymph nodes, inguinal lymph nodes, mediastinal lymph node, spleen, peripheral blood were harvested, stained with CD8, CD90.2, and L-selectin and analyzed by flow cytometry for numbers and the L-selectin expression on donor $CD8^+$, $CD90.2^+$ cells.

2.9. Labeling cells with fluorescent conjugated dye

2.9.1. Assessing Donor $CD8^+$ T cell proliferation *in vivo,* using CFSE

In order to assess adoptively transferred donor $CD8^+$ T cell proliferation, naïve $CD8^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-selectin F5/LΔP were isolated and purified. 10^7 Cells were re-suspended in 1ml PBS and mixed 1:1 with 1ml 10 μM CFSE in PBS to give a final concentration of 5μM CFSE. The cells were incubated with CFSE for 10 minutes at room temperature in the dark, the reaction was quenched by adding 10ml ice cold FCS. The cells then were washed extensively 3-4 times with ice-cold PBS to remove the excess dye and re-suspended in normal saline and injected i.v. at 2 x 10^6 cells/mouse. 24 hours later, mice were inoculated with 2 x 10^6 vaccNP i.p. and at days 1 and 2 post infection, various lymphoid tissues were harvested to assess donor cell proliferation by flow cytometry (FACS Canto II BD) based on the CFSE dye dilution.

2.9.2. Labeling cells with EdU dye to assess proliferation *in vivo*

The proliferation of donor virus specific $CD8^+$ T cells during the course of the virus infection was also assessed through the incorporation of the DNA dye 5-Ethynyl-2-deoxyuridine (EdU)(Life Technology, USA). Mice were injected with 1mg EdU dye i.p. one day before organ harvest. Single cell suspensions were prepared as described above. Cells were stained for appropriate extracellular

markers and fixed with 4% formaldehyde (FA). Cells were then permeabilized with saponin buffer, and incorporated DNA dye EdU was detected through the Click-it plus EdU Alexa Fluor 647 flow cytometry assay kit according to manufacturer's instructions (Life Technology, USA). Briefly, the cells were incubated with a 50µl cocktail of 0.25µl Alexa Fluor 647, 1µl copper sulphate ($CuSO_4$), 1x Click-iT reaction buffer and 43.75µl 1x Click-iT EdU buffer additive. Cells were incubated with the cocktail for 20 minutes at room temperature in the dark, then washed twice and re-suspended in 2%FCS PBS buffer.

2.9.3. Assessment of $CD8^+$ T cell proliferation *in vitro*

In order to assess $CD8^+$ T cell proliferation *in vitro*, naïve $CD8^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-selectin F5/LΔP were isolated and purified as described in section 2.3.1., cells were re-suspended in PBS and mixed 1:1 with 4 µM CFSE in PBS to give final concentration of 2µM CFSE. The cells were incubated with CFSE for 10 minutes at room temperature in the dark, the reaction was quenched by adding 10ml ice cold FCS. The cells then were washed extensively 3-4 times with ice-cold PBS to remove the excess dye and re-suspended in complete DMEM. Cells then cultured as described in section 2.7. At days 4, 5 and 6 post culture, cells were harvested to assess $CD8^+$ T cell proliferation by flow cytometry (FACS Canto II BD) based on CFSE dye dilution.

2.9.4. Extracellular staining

Cells were added to a 96 well U-bottom plate well. Cells were re-suspended and washed in PBS 2% FCS. Most of the steps were performed on ice to avoid internalization of the surface receptors, except for CCR7 staining which was

performed at 37°C for 30 minutes. Cells were stained for live/dead exclusion with aqua fluor amcyan (life Technology, USA) and incubated for 30 minutes at room temperature in the dark. After that, they were washed with PBS 2% FCS. The cells were then blocked with PBS, 5% Rat serum and 2% FCS and staining antibodies were added and incubated for 30 minutes at 4-8°C. Cells were again washed with PBS 2% FCS. Later, cells were fixed with 4% FA for 15 minutes at room temperature in the dark. Finally, cells were washed and re-suspended in 200µl PBS 2% FCS and examined by Flow cytometry (FACS Canto II BD). FlowJo 9.7.6. was used to analyze the results (Table 2.3).

2.9.5. Intracellular staining for IL-2, TNF-alpha, IFN-gamma and granzyme B

$CD8^+$ T lymphocytes, which were isolated and cultured for either 6 or 8 days, or $CD8^+$ T cells harvested from ovary and/or spleen of vaccNP infected mice, were harvested and re-stimulated with NP68 2µg/ml for 4 hours in the presence of 2µg/ml brefeldin A (Sigma- Aldrich, USA), which was added to the cells in order to stop the transportation of secreted cytokines by Golgi apparatus. After incubation, the cells were stained for appropriate extracellular surface molecules and fixed with FA 4%. Permeabilisation of the cells was carried out with saponin buffer at room temperature for 10 minutes, and then the cells were stained intracellularly with fluorescence-conjugated cytokines antibodies for 20 minutes at room temperature. The cells were washed and analyzed using flow cytometry (FACS Canto II BD)(Table 2.4).

2.9.6. Intracellular staining for CD107a

$CD8^+$ T lymphocytes were isolated and cultured as previously described. $CD8^+$ T cells were re-stimulated with NP68 peptide 2µg/ml and incubated at 37°C. Prior

to incubation, anti-CD107a FITC (Biolegend, UK) was added to the cell suspension. After 1 hour of incubation, 0.7µg/ml monensin (Golgi Stop) was added to stop recycling of the mobilized granules. The cells were further incubated for 4 hours at 37°C. After incubation, the cells were washed with PBS 2% FCS and stained for appropriate extracellular surface molecules and fixed with FA 4%. The cells were washed and analyzed using flow cytometry (FACS Canto II BD)(Table 2.4).

Table 2.3: Details of antibodies used for detection of cell surface-expressed proteins and markers by flow cytometry.

Cell marker	Fluorophore	Clone	Dilution	Company
αCD8a	PerCP Cy5.5	53-6.7	1:250	BD Bioscience
αCD8a	APC-Cy7	53-6.7	1:250	eBioscience
αCD62L	PE	MEL-14	1:100	R&D
αCD62L	PE-Cy7	MEL-14	1:500	Biolegend
αCD44	FITC	IM7	1:500	Biolegend
αCD44	APC-Cy7	IM7	1:500	Biolegend
αCCR7	APC	4B12	1:200	eBioscience
αCD69	APC	H1.2F3	1:200	Biolegend
Streptavidin	PE	-	1:50	Life Technology
αCD90.1	PE-Cy7	OX-7	1:200	Biolegend
αCD90.2	Pacific Blue	53-2.1	1:200	Biolegend
αVβ11	FITC	KT-11	1:500	Biolegend

Table 2.4: Details of antibodies used for detection of intracellular cytokine secretion by flow cytometry.

Cell marker	Fluorophore	Clone	Dilution	Company
αIL-2	Pacific Blue	JES6-5H4	1:200	
αTNF-α	PE-Cy7	TN3-19	1:200	eBioscience
αIFN-γ	PerCP Cy5.5	XMG1.2	1:200	Biolegend
αGranzyme B	FITC	GB-11	1:20	Biolegend
αCD107a	FITC	LAMP-1	1:200	Biolegend

2.10. Acquisition using FACS Canto II Flow Cytometer and data analysis

The flow cytometer BD FACS Canto II was used to acquire data throughout the whole study. Compensation was achieved by using antibody capture beads (BD Biosciences), which were singly stained with individual fluorochrome conjugated monoclonal anti-mouse antibodies used in each experimental staining panels. Fluorescence minus one (FMO), which is a flow cytometry staining control that contains all the fluorochromes in the staining panel except for the one that is going to be measured, was used to identify the positive gate for each stain. The absolute number of donor cells was counted using Cytocount bead (Dako, Denmark). Briefly, single cell suspensions were added with 10µl cytocount beads containing 1093 bead/µl. The numbers of donor cells were achieved using the following formula; the number of cells counted divided by the number of beads counted and then multiply with the bead number and dilution factor. Data was analyzed using Flow Jo software version 9.7.6. (TreeStar Inc, Ashland, OR).

2.11. Reagents and consumables

2.11.1. Media and associated reagents

Dulbecco's modified Eagle's minimal essential media (DMEM), Roswell Park Memorial Institute medium (RPMI), phosphate buffered saline (PBS), Hank's balanced salt solution (HBSS), L-glutamine, penicillin, streptomycin, sodium pyruvate and 0.5% trypsin in HBSS were purchased from Gibco, Invitrogen [Paisley, U.K.].

2.11.2. Distilled Water (dH_2O)

Millipore, Milli-Q system, H_2O was distilled by Millipore reverse osmosis system followed by filtration through two ions exchange resin columns.

2.11.3. Foetal calf serum

Foetal calf serum (FCS) was purchased from Sigma-Aldrich and PAA Laboratories Limited [Pasching, Austria], and unless done so by the supplier, it was heat inactivated at 56^{o}C for 30 minutes.

2.11.4. Interleukin-2 (IL-2)

Recombinant human IL-2 was purchased as 'Proleukin' from Chiron Limited [Harefield, U.K.] and reconstituted to 1mg/ml under sterile conditions in ddH_20, diluted to 20ng/ml in RPMI and stored in 1ml aliquots at -70^{o}C. For continuous use, aliquots were thawed and stored at 4^{o}C.

2.11.5. NP68 peptide

The NP68 peptide (ASNENMDAM)(Peptide Synthetics, Hampshire, UK), from the Influenza A virus nucleoprotein A/NP/60/68 (also called $NP_{366-374}$) binds to H-$2D^b$ and is recognized by the cytotoxic T cell receptor (F5 TCR). The peptide was obtained as a dry powder and reconstituted in PBS to make a 1mg/ml stock, which then was aliquoted and stored at -20^oC.

2.11.6. NP34 monomer

The NP34 monomer (ASNENMETM), from Influenza A virus (strain A/Puerto Rico/8/1934 H1N1) nucleoprotein ($NP_{366-374}$), binds to H-$2D^b$ was supplied by NIH tetramer facility (National Institute of Health, USA).

2.11.7. Collagenase D

Collagenase D was purchased from Roche, USA as a dry powder. It was dissolved in HBSS buffer at 50mg/ml, stocks were stored in -20°C until use.

2.11.8. DNase

DNase was purchased from Sigma Aldrich, USA as a dry powder. It dissolved in 0.15M NaCl at 100mg/ml. Stocks were stored in -20°C until use.

2.11.9. $CaCl_2$

$CaCl_2$ was purchased from Sigma Aldrich, USA as a granule. 0.5M solutions were prepared by dissolving 7.3 g $CaCl_2$ in 100ml ddH_2O.

2.11.10. Sodium citrate

Sodium citrate was purchased as a dry powder. 0.38 g Sodium citrate was dissolved in 100ml PBS. It was used as an anticoagulant in a 1:1 mix to collect blood either through heart puncture or tail bleeds for genotyping of donor $CD8^+$ T cell analysis during the course of the viral infection.

2.11.11. Red Blood cell lysis buffer

Red blood cell lysis buffer (10X stock) was purchased from Biolegend, UK. It was used at 1X concentration by diluting with sterile ddH_2O. It was used to lyse red blood cells in a single cell suspension from spleen, lung and peripheral blood.

2.11.12. AEC (3-Amino-9-ethylcarbazole)

AEC powder was purchased from Sigma Aldrich, USA. 25 mg was dissolved in 2.5 ml DMF (Dimethyl Formamide). This solution was then added to 47.5 ml of 50mM acetate buffer (pH 5.0). AEC solution was used as a substrate for the horseradish peroxidase enzyme during the influenza titer assay, which provides a brownish colour to detect the virus-infected cells. Just before use, 25 µl 30% hydrogen peroxide (H_2O_2) was added to the AEC solution.

2.11.13. Rat Serum

Rat serum was purchased from (Sigma Aldrich, USA) and aliquots of 250µl stored at -20°C until use. A cocktail of 5% rat serum plus 2% FCS in PBS was used as Fc blocking agent during cell staining for cell surface-expressed proteins for flow cytometry analysis.

2.11.14. Formaldehyde (FA)

Formaldehyde 37-40% was diluted in PBS to a final concentration of 4%. FA 4% was used to fix cells either for flow cytometry analysis or to fix TK- or MDCK cells in viral titer assays.

2.11.15. β-mercaptoethanol

2.11.16. MACS buffer

MACS buffer was prepared fresh before starting the magnetic purification of $CD8^+$ T cells using MACS technology. The cocktail was composed of PBS, 2mM EDTA and FCS.

2.11.17. Saponin Buffer

Suponin powder was purchased from (Sigma-Aldrich, USA). To make up 100ml, 0.5g suponin powder was dissolved in PBS and supplemented with 0.05% sodium azide and 0.05% FCS.

2.12. Cell membrane dye

2.12.1. CFSE

Carboxyfluorescein diacetate Succinyl Ester (CFSE) powder was purchased from (Invitrogen, USA) and suspended in Dimethyl sulfoxide (DMSO) to give a stock solution of 10mM. It was stored in 10µl aliquots at -20°C. The aliquots were thawed and used once for an experiment and were not refrozen.

2.12.2. Aqua live dye

Aqua live dye was purchased from Invitrogen (USA) and suspended in DMSO according to manufacturer instruction, and then aliquots of 10µl were stored in -20°C until use.

2.13. Cell lines

2.13.1. Thymidine Kinase deficient (TK-) cells

TK- cells, which are mouse fibroblast deficient in thymidine kinase enzyme, were used for the recombinant vaccinia virus (VaccNP) titer.

2.13.2. MDCK luc9.1

Madin-Darby Canine Kidney (MDCK) cells were a kind gift from Dr. M. Jaber Hossain (Influenza Division, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA). MDCK cells were used for the influenza strain A (PR8, H1N1) virus titer.

2.14. Cryopreservation of TK- and MDCK cells

TK- or MDCK cells, which were 90-100% confluent, were frozen down for further and later use. Excess medium was removed by pipetting and the cells washed with 0.9% saline to remove excess media. 0.5% Trypsin-EDTA solution was added and incubated at 37°C 5% CO_2. Cells were examined macroscopically and microscopically to ensure detachment. Cells were washed with PBS and re-

suspended in complete FCS for viability count. 2 x 10^7 viable cells were re-suspended in 1ml FCS and mixed with freeze mix (80% FCS mixed with 20% DMSO) at a ratio of 1:1 (volume/volume). 1ml was added to the previously labeled cryovial and placed into Nalgene cryo freezing container (Merc Laboratories supplies, Dorset, UK) and kept overnight at -80°C. Cryovials were transferred into liquid nitrogen and kept until further use.

2.15. Thawing up TK- and MDCK cells

A cryovial of either TK- or MDCK cells were removed from the liquid nitrogen and placed in a container with dry ice and brought to the laboratory. The cryovial placed in the water bath for 1-2 minutes in order to thaw quickly. pre-warmed complete DMEM medium was added to a T75 flask, the TK- or MDCK cells transferred to the T75 flask and incubated for 24 hours in order to reach 90-100% confluency.

Chapter three

3. Role of L-selectin in homing of activated $CD8^+$ T cells to sites of infection.

3.1. Introduction

"Leukocyte migration is the heart of the adaptive immune system" (Andrian & Mackay 2000). The strength of the adaptive immune system against viral infection is inextricably linked to the number of the cytotoxic effector $CD8^+$ T cells at the site of infection (Andrian & Mackay, 2000; Masopust & Schenkel, 2013). Although mechanisms of activation and differentiation controlling the generation of effector $CD8^+$ T cells are important, the migration of these effector $CD8^+$ T cells from the their site of priming to the site of infection is equally important for a successful immune response.

The kinetics of L-selectin expression on the surface of $CD8^+$ T cells during their activation through TCR at the time of viral infection has not been studied in detail. If L-selectin molecules on the surface of naïve $CD8^+$ T cells is controlling the homing of these cells into the peripheral LN, whether re-expressed L-selectin on the surface of activated $CD8^+$ T cells have any role in homing into the site of infection would be of great interest. In this chapter, the role of L-selectin molecules will be studied with regards to their participation in homing of activated $CD8^+$ T cells to sites of infection. The role of L-selectin will be investigated by comparing $CD8^+$ T cells expressing wildtype L-selectin with $CD8^+$ T cells with a specific mutation that do not express L-selectin (Ko). Further to understand the role of L-selectin in homing of activated $CD8^+$ T cells, wildtype L-selectin expressing $CD8^+$ T cells will be compared with $CD8^+$ T cells that have been genetically manipulated to constantly express L-selectin (LΔP).

3.2. Hypothesis

L-selectin expression is variable during $CD8^+$ T cell activation due to ectodomain shedding and gene silencing. Our hypothesis is, if various levels of L-selectin on the surface of activated $CD8^+$ T cell is controlling homing of these cells into sites of viral infection, recruitment will be enhanced in constant L-selectin expression (LΔP), moderate recruitment in (WT; B6) and recruitment will be abrogated for $CD8^+$ T cells that do not express L-selectin (Ko).

3.3. Results

3.3.1. Mouse model of viral infection; recombinant vaccinia virus expressing NP68 peptide

The mouse adapted Western Reserve (WR) strain of vaccinia virus engineered to express nucleoprotein peptide $NP_{366-374}$ from Influenza A strain E61-13-H17 was used in these studies and will be referred to as vaccNP. $CD8^+$ T cells expressing cognate F5 TCR for H2-D^b $NP_{366-374}$ were used to determine the role of L-selectin in $CD8^+$ T cell activation and homing following primary infection with vaccNP. Genetic modification of vaccinia virus with NP peptide could alter the virulence and pathogenicity of the virus; therefore, we first determined the sites of vaccNP infection after intraperitoneal administration to C57BL/6 (B6) mice. Viral titers were determined in visceral organs such as ovary, spleen, liver, heart and lung at day 5 post virus infection (Fig. 3A). As described previously, high viral titers were found in the ovaries of C57BL/6 mice, but in contrast to the parental WR strain (Zhao et al. 2011), virus was not detected in other visceral organs (Fig. 3.1B).

Furthermore, to confirm the site of infection, histochemical studies were performed to compare ovaries from vaccNP infected mice with non-infected control. The analysis at day 5 post infection revealed a dense cellular infiltrate with signs of tissue destruction and necrosis in infected ovaries, but not in non-infected ovaries (Fig. 3.1C).

3.3.2. Mediastinal LN is the site of $CD8^+$ T cells priming during recombinant vaccinia virus infection

One of the critical steps in the adaptive immune response against viruses and other pathogens is the antigen presentation to the $CD8^+$ T cells by the APCs in the secondary lymphoid tissues draining the site of pathogen entry and sites of infection. The sites of antigen presentation to $CD8^+$ T cells during vaccNP infection were considered important in order to study the kinetics of $CD8^+$ T cell priming and activation during the primary course of the infection. To identify the site of $CD8^+$ T cells priming during vaccNP infection, CFSE labeled naïve F5 $CD8^+$ T cells were adoptively transferred into C57BL/6 mice. 24 hours later, mice were challenged with vaccNP. Draining and non-draining lymph nodes (LN), spleen and ovaries were harvested 1 and 2 days post-infection and CFSE-labelled T cells analyzed by flow cytometry. CFSE-labeled T cells were present in all lymphoid organs analyzed. T cell priming as indicated by CFSE dilution was not detected until 2 days post infection and was only seen in the mediastinal LNs (Fig. 3.2). Though vaccinia virus replicates in ovaries, naïve or activated F5 $CD8^+$ T cells were not detectable in ovaries and there was no evidence of priming in ovary draining LN (ovdLN) during the first 2 days following vaccinia infection. Therefore F5 $CD8^+$ T cell priming to vaccNP occurs within the first 2 days and is restricted to a single LN, the mediastinal LN.

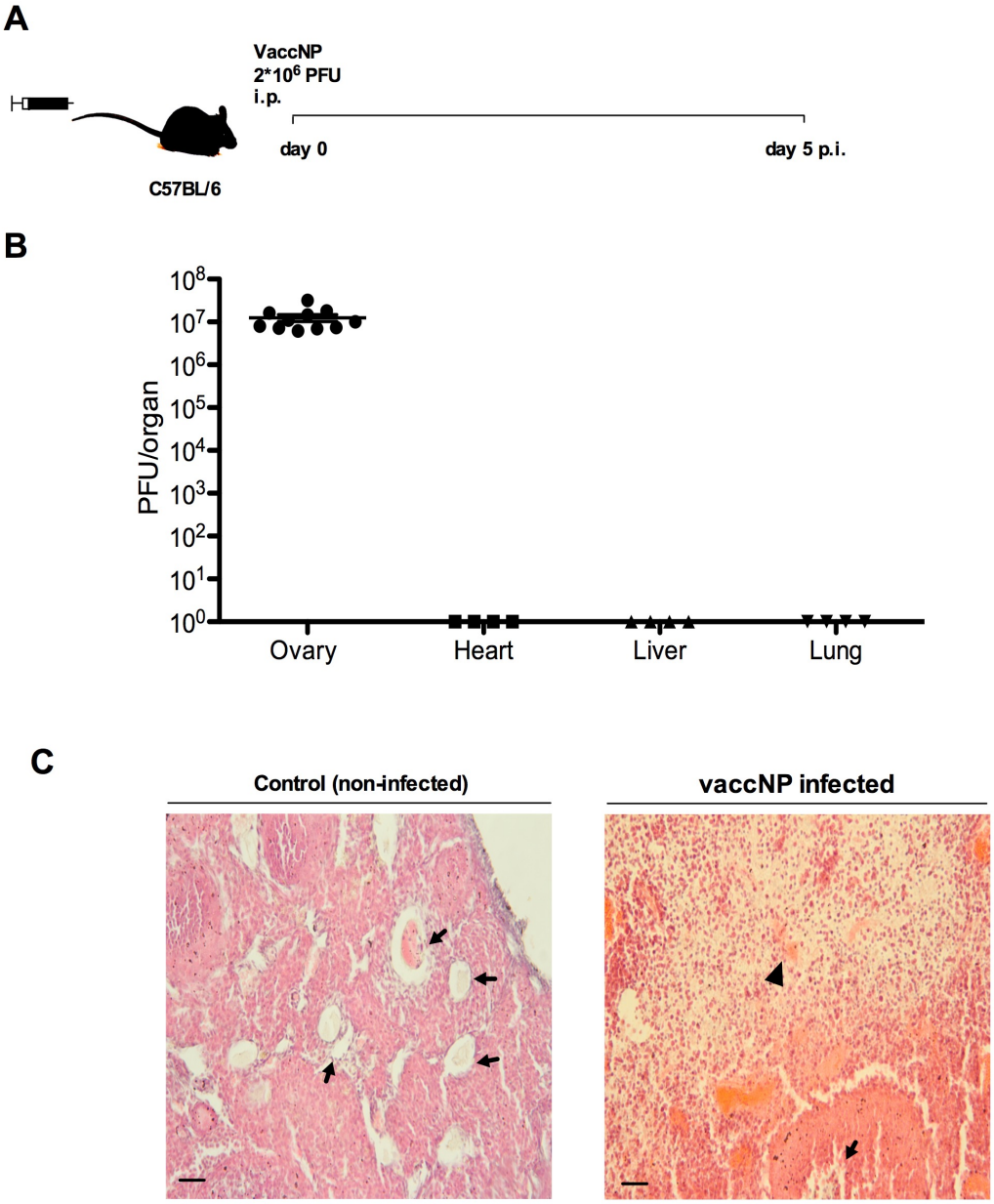


Figure 3.1: Ovaries are the major sites of recombinant vaccinia virus replication and infection. (A) Naïve C57BL/6 mice were inoculated i.p. with 2 x 10^6 pfu recombinant vaccinia virus expressing NP68 peptide (vaccNP). 5 days later, ovary, heart, liver and lungs were harvested and viral titers in homogenized tissues determined by plaque assay. **(B)** Viral titers were compared in various internal organs. High titres were only found in the ovaries. Bars show median value of the viral titre and each symbol represents a single mouse (n=4-12). **(C)** Histological studies using Haematoxylin and Eosin (H&E) staining of vaccNP infected ovaries compared with non-infected control further revealed ovarian tissue destruction and lymphocyte infiltration. Developing follicles (arrows) and cellular infiltrates (arrowheads) are marked. Results show H&E staining of one representative mouse from a single experiment (n=3).

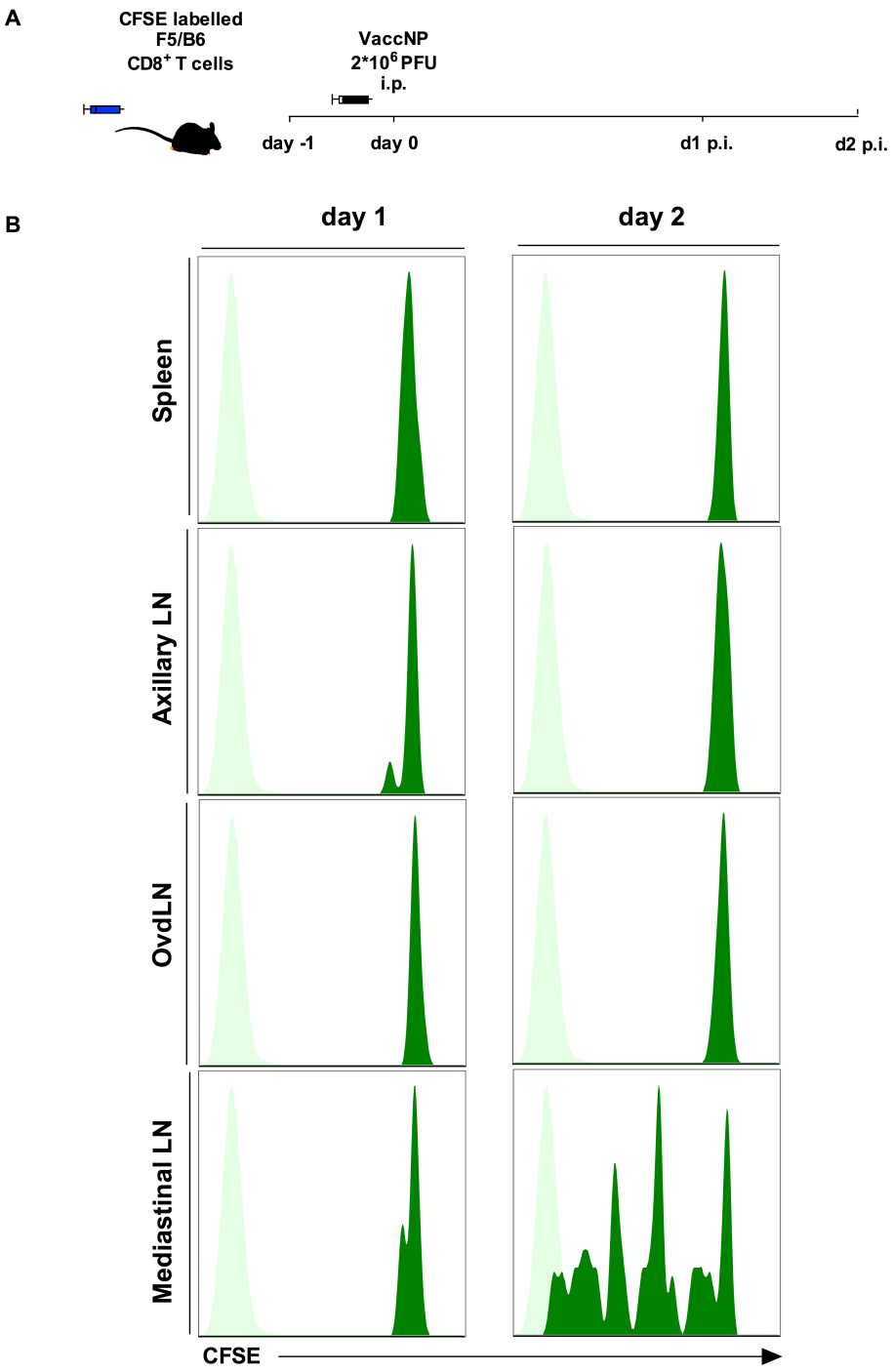


Figure 3.2: Mediastinal LN is the site of CD8$^+$ T cell priming to recombinant vaccinia virus following i.p. inoculation. (A) Naïve CD90.2$^+$ CD8$^+$ T cells co-expressing transgenic F5 TCR and wildtype L-selectin were isolated and stained with 10μM CFSE, and a total of 2 x 10^6 cells were adoptively transferred into naïve CD90.1 B6 recipient mice. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. At days 1 and 2 post infection, various lymphoid tissues were harvested for CD8$^+$ T cell priming analysis. **(B)** CFSE labelling of F5 CD8$^+$ T cells in mediastinal, axillary and ovary draining LN and spleen of vaccNP infected mice (solid green) were compared with control unlabelled cells (solid light green). Results show CFSE staining of F5 CD8$^+$ T cells in one representative infected mouse in a single experiment (n=3).

3.3.3. Kinetics of L-selectin expression on the surface of $CD8^+$ T cells during primary viral infection

Analysis of donor cell recovered from the site of priming (mediastinal LN) at day 1 p.i. showed that F5/B6 $CD8^+$ T cells downregulated L-selectin. This has been previously reported during *in vitro* activation of T cells that initial loss of L-selectin is due to ectodomain shedding and proteolysis. Then, the following day (day 2 p.i.), activated F5/B6 $CD8^+$ T cells in mediastinal LN fully re-expressed L-selectin. Activated F5/B6 $CD8^+$ T cells in the peripheral circulation at day 3 p.i., still expressed L-selectin and the first populations of activated F5/B6 $CD8^+$ T cells entering the site of infection were also L-selectin positive. Virus specific $CD8^+$ T cells started to down-regulate L-selectin from day 5 p.i. and $CD8^+$ T cells were almost fully L-selectin negative on day 8 p.i. (Fig. 3.3). This finding clearly illustrating that L-selectin undergoes cyclical expression on the surface of $CD8^+$ T cells upon activation *in vivo* concurrent with what have been found during T cell activation *in vitro*.

The cyclical expression of L-selectin on the surface of endogenously generated virus specific $CD8^+$ T cells was also investigated. Upon influenza A virus infection, virus specific CD8+ T cells harvested from Med LN at day 2 p.i. expressed only 30% L-selectin. Then they started to re-express their L-seletin (94%) by day 4 p.i.. Virus specific $CD8^+$ T cells left the sequentially left the priming site (MedcLN) and entered the bloodstream started to downregulate their L-selectin on the surface (55%) and the first population of virus specific $CD8^+$ T cells infiltrated the site of infection (lung) was also expressed significant amount of L-selectin (54%) at day 4 p.i.. The virus specific $CD8^+$ T cells harvested at day 8 p.i. was almost fully downregulated L-selectin on their surface (3%)(Fig. 3.4).

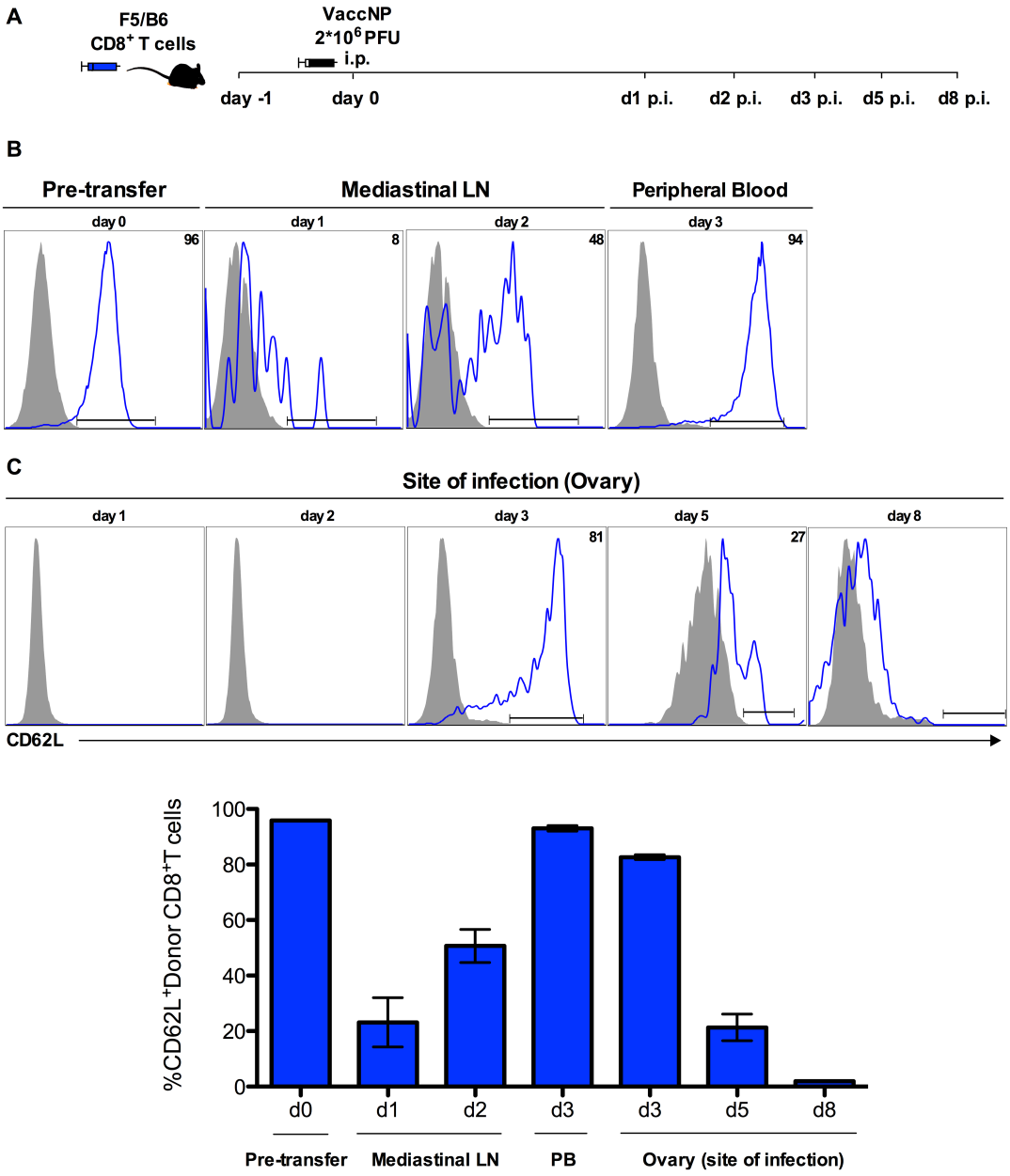


Figure 3.3: Cyclical expression of L-selectin during primary recombinant vaccinia virus infection. (A) Naive CD90.2$^+$ or CD90.1$^+$ CD8$^+$ T cells co-expressing transgenic F5 TCR and wildtype L-selectin were isolated and a total of 2 x 10^6 were adoptively transferred into CD90.1 or CD90.2$^+$ recipient B6 mice. 24 hours later, mice were inoculated with 2 x 10^6 vaccNP. At days 1, 2, 3, 5 and 8 post infection, mediastinal LN (site of CD8$^+$ T cell priming), peripheral blood, ovary (site of infection) were harvested for donor cells analysis. **(B)** Representative histogram plots showing the % CD62L$^+$ donor virus specific CD8$^+$ T cells at various times and location during primary vaccNP infection. **(C)** Bar chart showing mean $\pm$ standard error of mean (SEM) of % CD62L$^+$ donor CD8$^+$ T cells of 3 mice in a single experiment.

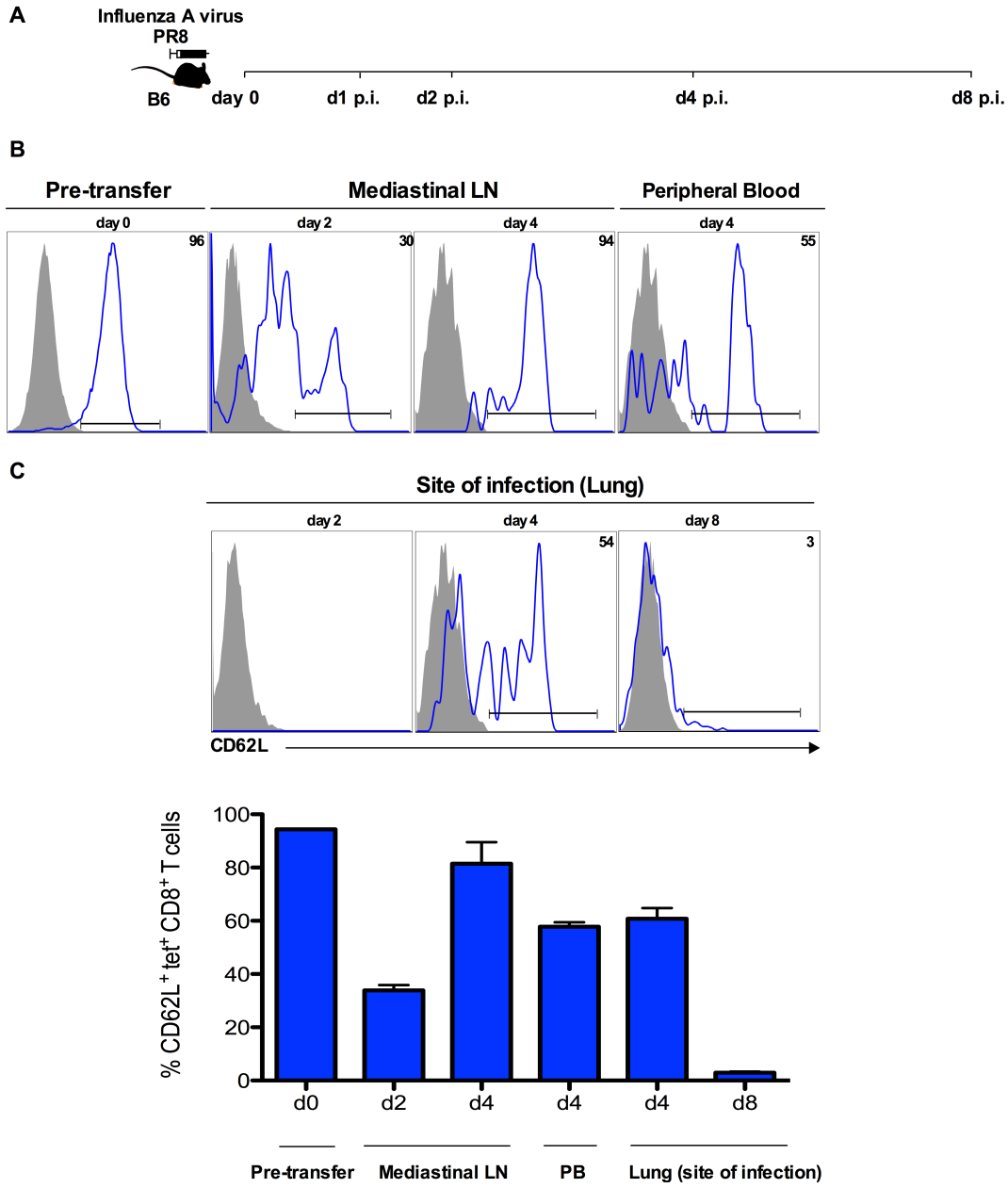


Figure 3.4: Cyclical expression of L-selectin during primary influenza A virus infection. (A) Naive B6 mice were infected with 50 ffu influenza A virus (PR8). At days 2, 4 and 8 post infection, mediastinal LN (site of $CD8^+$ T cell priming), peripheral blood, lung (site of infection) were harvested for virus specific $CD8^+$ T cell analysis. **(B)** Representative histogram plots showing the % $CD62L^+$ $tetramer^+$ $CD8^+$ T cells at various times and location during primary influenza A virus infection. **(C)** Bar chart showing mean ± standard error of mean (SEM) of % $CD62L^+$ $tetramer^+$ $CD8^+$ T cells of 4 mice in a single experiment.

3.3.4. Recruitment of $CD8^+$ T cells to sites of recombinant vaccinia virus infection is L-selectin dependent

To test the hypothesis whether L-selectin is involved in homing of activated $CD8^+$ T cells to site of virus infection. Also, if L-selectin expression is manipulated through introducing a non-cleavable mutant form of the molecule into the cells, $CD8^+$ T cells could home better to sites of infection. Naïve $CD8^+$ T cells co-expresing F5 TCR and either wildtype (F5/B6) or mutant L-selectin (F5/LΔP) (Fig 3.5) were adoptively transferred into naïve B6 mice. 24 hours later mice were challenged with vaccNP. At days 5, 8 and 12, sites of vaccNP infection (ovary), ovary draining lymph nodes (ovdLN), and spleen were harvested to study the recruitment of $CD8^+$ T cells to sites of infection and whether mutant L-selectin could promote homing. $CD8^+$ T cells started to infiltrate to sites of vaccNP infection from day 3 post infection and onward (Fig. 3.2). At days 5 and 8 post infection, donor cell enumeration analysis showed that there was enrichment of F5/LΔP over F5/B6 $CD8^+$ T cells (Figure 3.6), which reveal that there were L-selectin dependent homing of the $CD8^+$ T cells to the site of vaccNP infection and constant expression of L-selectin as in mutant L-selectin expressing cells, were promoting the recruitment. On day 12 p.i., there was no longer any statistical significant difference in the numbers of F5/LΔP and F5/B6 $CD8^+$ T cells In the infected ovaries. In the ovdLN, although the difference were not statistically significant, there were more F5/LΔP $CD8^+$ T cells compared to F5/B6 cells at days 5 and 8 but not at day 12 (Figure 3.6). We used the spleen as a negative control as homing of $CD8^+$ T cells is not L-selectin dependent (Arbornes et al. 1994). There were no differences in the numbers of the F5/LΔP and F5/B6 $CD8^+$ T cells in the spleen at any time point analyzed during vaccNP infection (Fig 3.6).

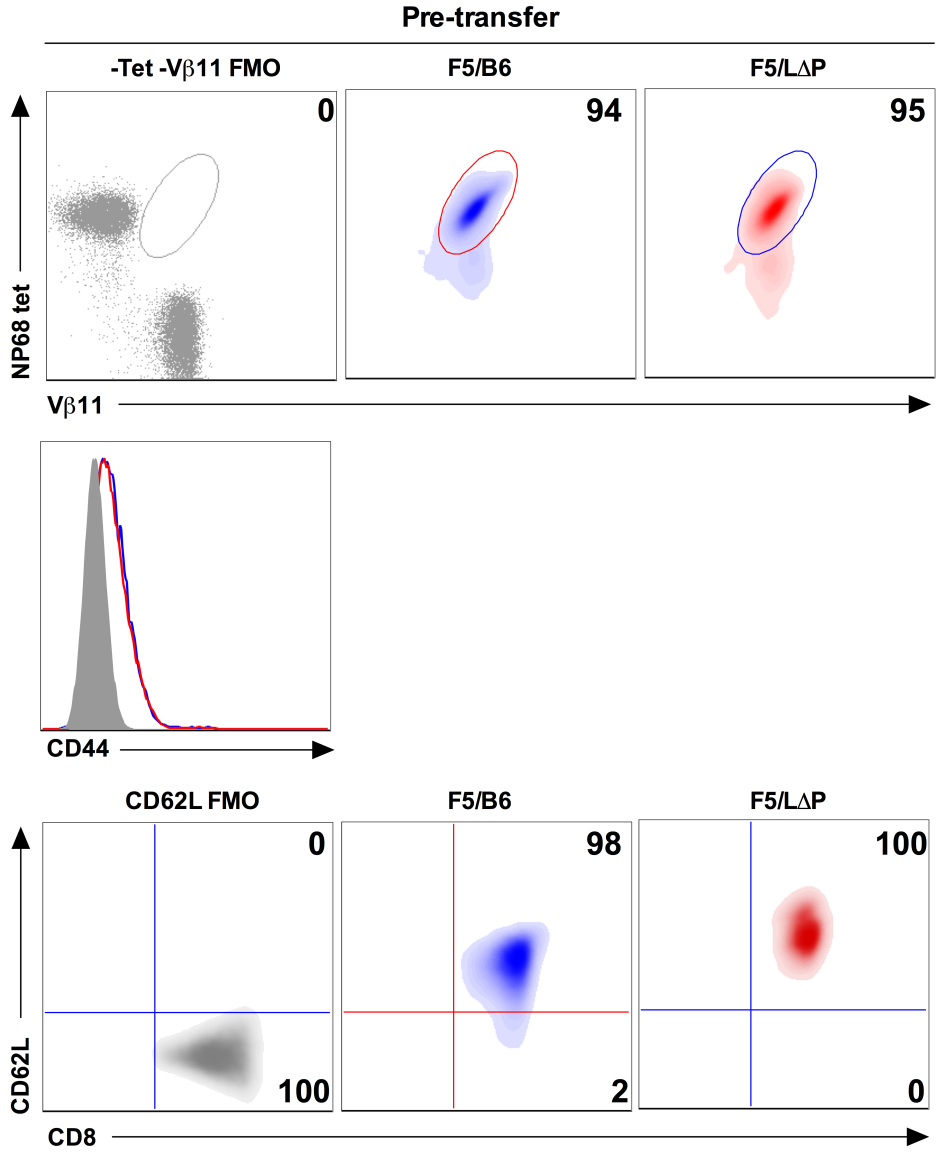


Figure 3.5: Phenotypic characteristics of naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype or mutant L-selectin. Spleen and/or lymph nodes from F5/B6 mice, in which CD8$^+$ T cells co-express transgenic F5 TCR and wildtype L-selectin or F5/LΔP mice in which CD8$^+$ T cells co-express F5 TCR and mutant L-selectin were harvested, and then mashed through 70 μM cell strainer and CD8$^+$ T cells were isolated using magnetic cell sorting technique. After isolation and purification, cells were stained for CD8α, NP68 tetramer, Vβ11 (KT-11), CD44 and CD62L to assess their naivety. Representative bivariate density plots shows CD8, NP68 tetramer and Vβ11 triple positive cells of F5/B6 (Blue) and F5/LΔP (Red) compared with their FMO (light grey). Overlaid histograms show level of CD44 expression, bivariate density plots show CD8 and CD62L double positive compared with CD62L FMO.

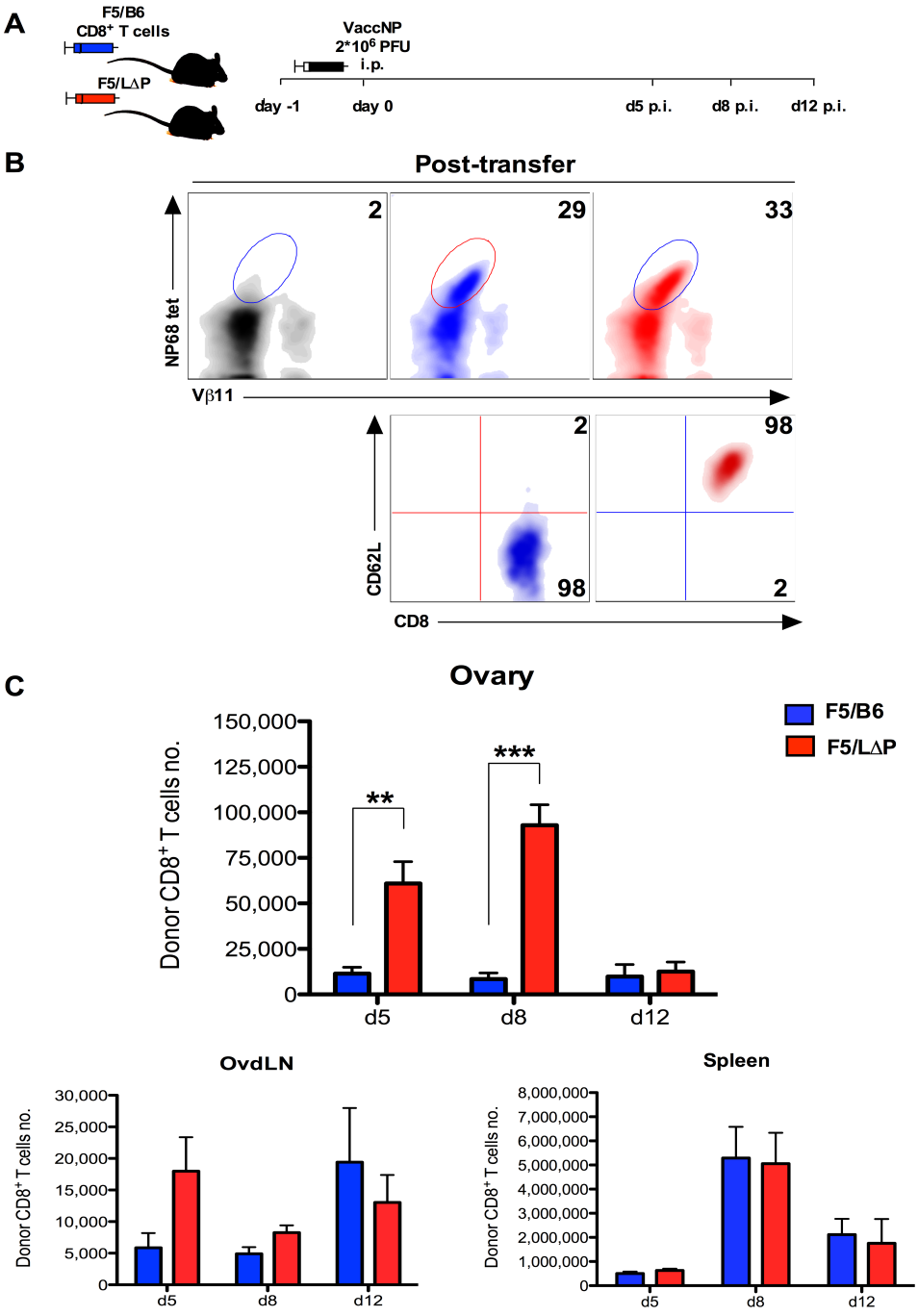


Figure 3.6: L-selectin dependent homing of CD8$^+$ T cells to sites of recombinant vaccinia virus infection. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype L-selectin, (F5/B6) or mutant L-selectin (F5/LΔP) were isolated and adoptively transferred into B6 mice. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. At days 5, 8 and 12 post infection, ovary, ovdLN and spleen were harvested and analyzed for homed donor CD8$^+$ T cells. **(B)** Donor cells were identified as CD8, NP68 tetramer and Vβ11 triple positive cells. Density plots shows F5/B6 (Blue) and F5/LΔP (Red) donor cells and their CD62L expression. **(C)** Absolute numbers of homed donor F5/B6 and F5/LΔP CD8$^+$ T cells in ovary, ovdLN and spleen at 5, 8 and 12 days post infection. Results are mean ± SEM (n=3). ** P<0.001, *** P<0.0001, Two-Way Anova.

3.3.5. L-selectin does not influence priming, activation and differentiation of virus-specific $CD8^+$ T cells

Although naïve $CD8^+$ T cells expressing mutant or wild type L-selectin showed equal ability to home to lymph nodes (Galkina et al. 2003), enrichment of $CD8^+$ T cells expressing mutant L-selectin in the ovaries of vaccinia virus infected mice raised the possibility that the presence of mutant L–selectin may co-stimulate the activation and differentiation of $CD8^+$ T cells resulting in the generation of increased numbers of effector cells. This question was tested by determining the kinetics of T cell activation at the site of priming in the mediastinal LN and the time course of T cell differentiation during the course of viral infection. The early activation antigen CD69 was upregulated by day 1 and downregulated by day 2 on wildtype F5/B6 $CD8^+$ T cells in mediastinal LN. The kinetics of CD69 upregulation and downregulation on F5/LΔP $CD8^+$ T cell were similar, suggesting that expression of mutant L-selectin did not alter the timing of interactions with antigen presenting cells at the site of priming (Fig 3.7). To determine whether L-selectin affects the differentiation of virus-specific $CD8^+$ T cells, expression of the late activation antigen CD44 on donor cells was measured. Donor cells were harvested from the ovary, ovdLNs and spleen at 5, 8 and 12 days following virus administration. In comparison with pre-transferred naïve cells, CD44 expression on F5/B6 cells increased 20-fold by day 5 and was maintained at this level to day 12 in the ovary and draining LN. F5/LΔP cells up-regulated CD44 to a similar extent and with identical kinetics indicating that both cell types activated and differentiated in a similar manner. Furthermore, the kinetics of CD44 upregulation in the spleen were also unaffected by expression of mutant L-selectin, thus collectively demonstrating that L-selectin did not influence T cell activation (Fig. 3.8).

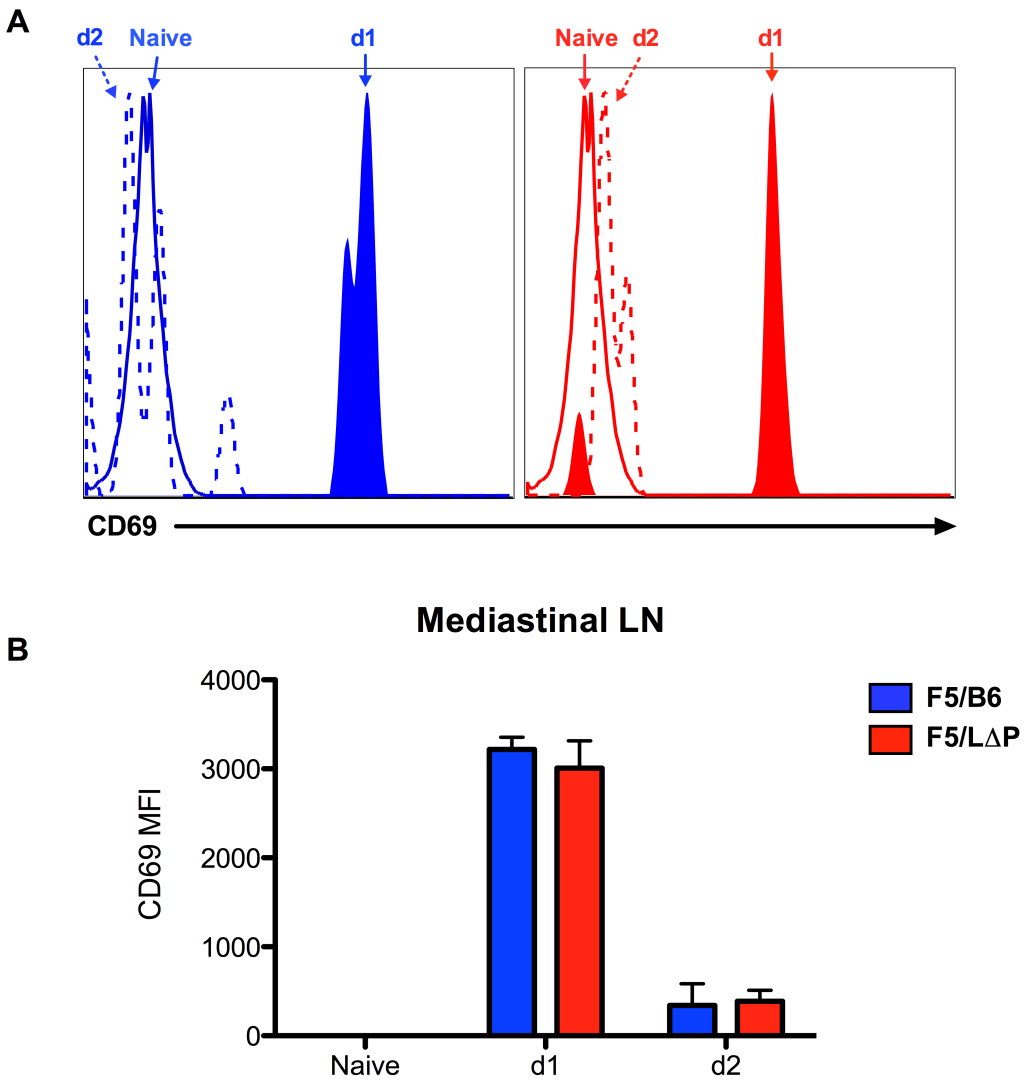


Figure 3.7: L-selectin expression does not influence the activation of virus-specific CD8$^+$ T cells. Naïve CD90.2$^+$ CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype L-selectin F5/B6 or mutant L-selectin F5/LΔP were isolated and adoptively transferred into CD90.1 B6 mice. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. At days 1 and 2 post infection, mediastinal LN (site of CD8$^+$ T cell priming) was harvested for donor CD8$^+$ T cells analysis. (A) Representative histograms show the low level of the early activation marker, CD69, by naïve CD8$^+$ T cells from F5/B6 (Blue) and F5/LΔP (Red), the up-regulation of CD69 at day 1 post virus inoculation and subsequent down-regulation of CD69 at day 2. (B) Bar chart shows the kinetics of CD69 up-regulation and down-regulation at day 1 and day 2 by F5/B6 and F5/LΔP CD8$^+$ T cells during vaccNP infection. Results are mean $\pm$ SEM (n=3).

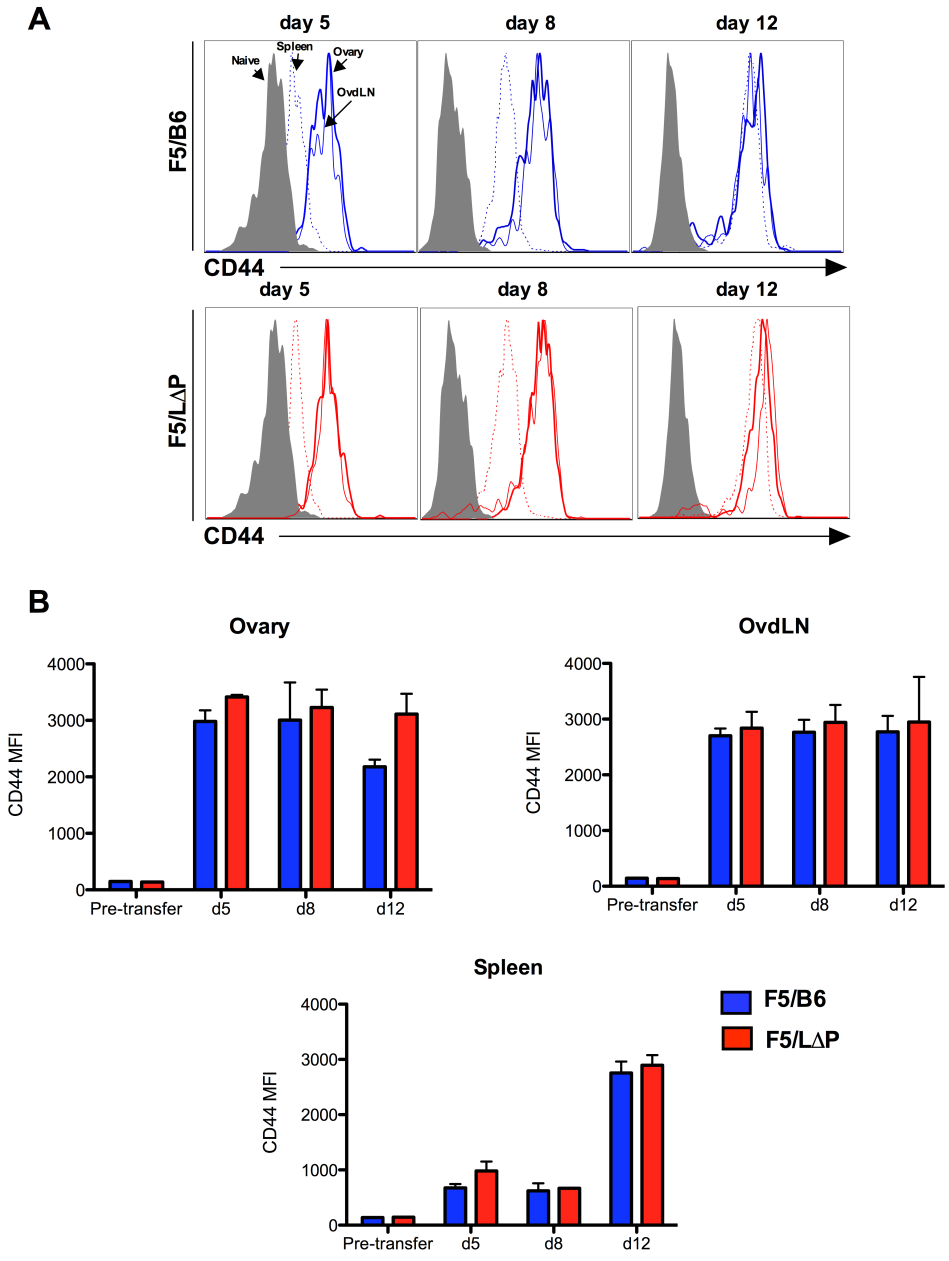


Figure 3.8: L-selectin expression does not influence the differentiation of virus-specific CD8$^+$ T cells during recombinant vaccinia virus infection. Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-selectin F5/LΔP were isolated and adoptively transferred into B6 mice. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. At days 5, 8 and 12 post infection, ovary, ovdLN and spleen were harvested for homed donor CD8$^+$ T cell analysis. **(A)** Representative histograms show the low level of CD44 expression by naïve pre-transferred cells and the up-regulation of CD44 on effector CD8$^+$ T cells differentiation of F5/B6 (Blue) and F5/LΔP (Red) during primary vaccNP infection at days 5, 8 and 12 post infection in the ovary, ovdLN and spleen. **(C)** Bar charts show the mean ± SEM of CD44 expression (n=3).

3.3.6. The impact of L-selectin on co-stimulating $CD8^+$ T cell proliferation

During viral infection, a naïve $CD8^+$ T cell, from a specific clone, recognizes the viral peptide, and it enters the activation and expansion phase. This phase is responsible to generate large number of effector cells to fight the infection. It has been shown that type I transmembrane protein 4BB-1, influence $CD8^+$ T cell proliferation (Shuford et al. 1997). L-selectin is a type I transmembrane glycoprotein and to test whether L-selectin influence on $CD8^+$ T cells proliferation and also, presence of mutant L-selectin could strength the co-stimulation. Using a dilution sensitive dye (CFSE), the proliferation of F5/B6 $CD8^+$ T cells was compared with the F5/LΔP cells. Furthermore, Enrichment of mutant L-selectin expressing $CD8^+$ T cells at the site of vaccNP infection brought up another possibility that these cells proliferate faster. Adoptive transfer of CFSE labeled naïve F5/B6 and F5/LDP $CD8^+$ T cells into B6 mice, and challenging the mice 24 hours later with vaccNP tested these questions. In the first day following the vaccNP infection, as shown in fig 3.7, both F5/B6 and F5/LΔP $CD8^+$ T cells were primed similarly in the mediastinal LN, as both upregulated the early activation antigen CD69 in a similar manner, but neither cell type had entered the proliferation cycle. Whereas at day 2 post viral challenge, both F5/B6 and F5/LΔP had entered the proliferation cycle but in contrast to our hypothesis, F5/B6 $CD8^+$ T cells expressing wildtype L-selectin, entered the proliferation cycle earlier indicated by more CFSE dilution and higher division index than F5/LΔP cells. This finding indicates that the signal, which is co-stimulated proliferation, was provided by the regulated expression of the wildtype L-selectin rather than a constant expression. Therefore enrichment in the numbers of F5/LΔP $CD8^+$ T cells at the site of vaccNP infection was not due to hyper-proliferation of these cells (Fig 3.9).

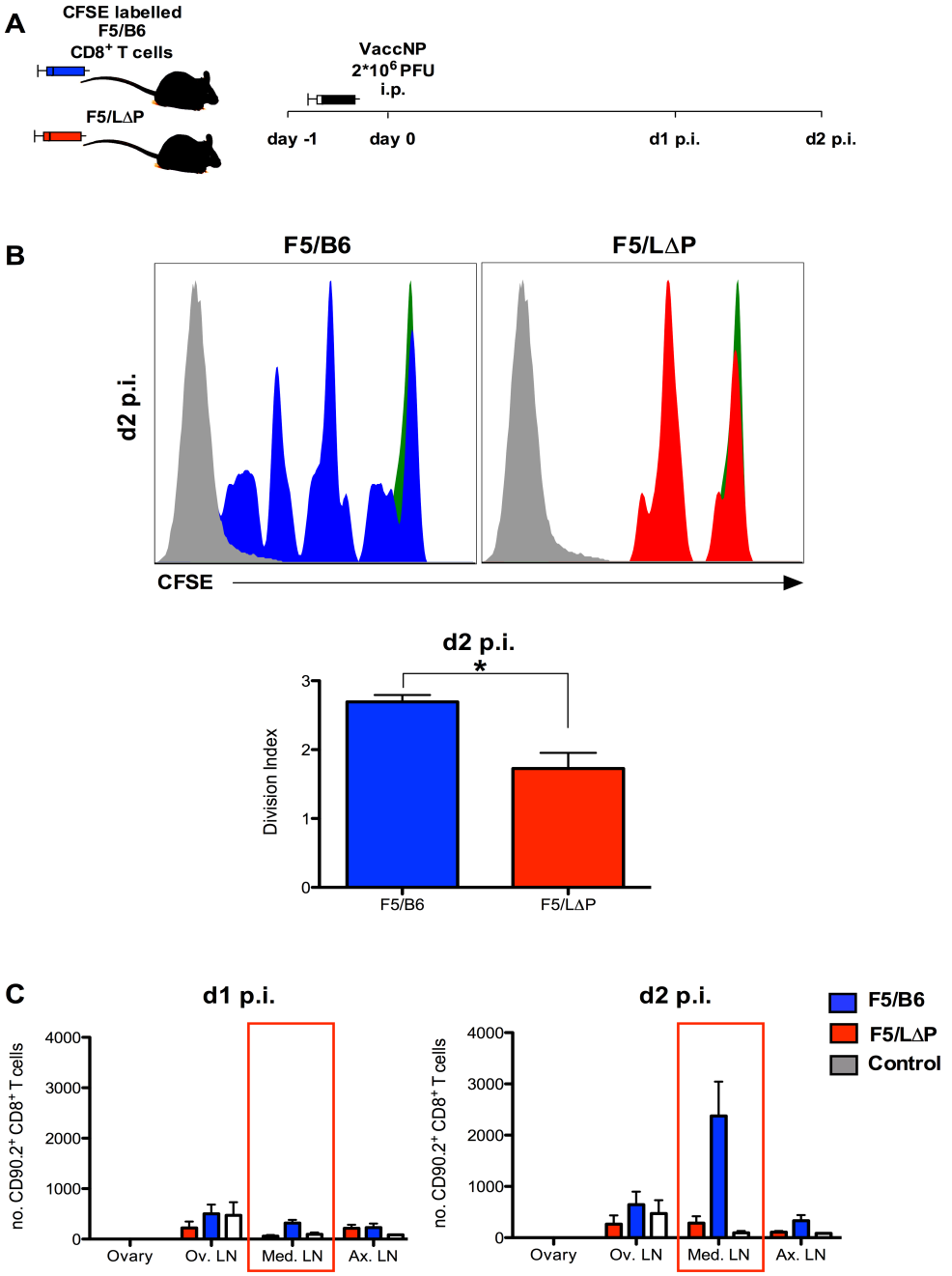


Figure 3.9: The impact of L-selectin on CD8$^+$ T cell proliferation. (A) Naïve CD90.2$^+$ CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-selectin F5/LΔP were isolated and stained with 10μM CFSE, and a total of 2 x 10^6 cells were adoptively transferred into naïve CD90.1 B6 recipient mice. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. At days 1 and 2 post infection, mediastinal LN were harvested for CD8$^+$ T cell proliferation analysis. **(B)** Representative histogram plots showing the CFSE dilution of F5/B6 and F5/LΔP CD8$^+$ T cells in the mediastinal LN at day 2 post vaccNP infection (unpaired t-test). **(C)** The absolute numbers of donor F5/B6 and F5/LΔP CD8$^+$ T cells further revealing the early entry of F5/B6 CD8$^+$ T cells compared with F5/LΔP CD8$^+$ T cells and cells in the control mice with no virus infection in the mediastinal LN at day 2 post infection. Results are mean ± SEM (n=3).

3.3.7. Recruitment of $CD8^+$ T cells to site of recombinant vaccinia virus infection was abrogated in the absence of L-selectin

To understand the role of L-selectin in homing of activated $CD8^+$ T cells to sites of viral infection further, the homing ability of $CD8^+$ T cells expressing wildtype L-selectin F5B6 was compared with L-selectin ko F5L-$sel^{-/-}$ $CD8^+$ T cells. The enrichment of $CD8^+$ T cells expressing mutant L-selectin over wildtype, raised the question whether the homing of L-selectin ko $CD8^+$ T cells to sites of infection will be abrogated. The same approach was used in which naïve F5B6 or F5L-$sel^{-/-}$ $CD8^+$ T cells were adoptively transferred to B6 mice, 24 hours later; mice were challenged with vaccNP. At day 5 p.i., ovary, ovdLN and spleen were harvested for donor cell analysis. Interestingly, there were very low numbers of F5L-$sel^{-/-}$ $CD8^+$ T cells at the site of vaccNP infection when compared with F5B6 $CD8^+$ T cells. We also revealed that homing of $CD8^+$ T cells to ovdLN is fully L-selectin dependent, as there was a significant difference between the numbers of F5L-$sel^{-/-}$ and F5B6 $CD8^+$ T cells. As described above, homing of $CD8^+$ T cells to spleen is not L-selectin dependent and there were no differences between the numbers of F5L-$sel^{-/-}$ and F5B6 $CD8^+$ T cells in the spleen (Fig 3.10).

Furthermore, CD44 up-regulation was also compared between wildtype L-selectin expressing $CD8^+$ T cells and L-$sel^{-/-}$ cells. Both cell types upregulated CD44 in a similar manner at day 5 post infection in the ovary, ovdLN and spleen of vaccNP infected mice (Fig. 3.11), which further illustrates that the presence of L-selectin constantly (mutant L-selectin), variably (wildtype) or its absence (L-selectin ko) did not impact $CD8^+$ T cell activation and differentiation which, in turn, supports a role for L-selectin in the homing ability of these cells.

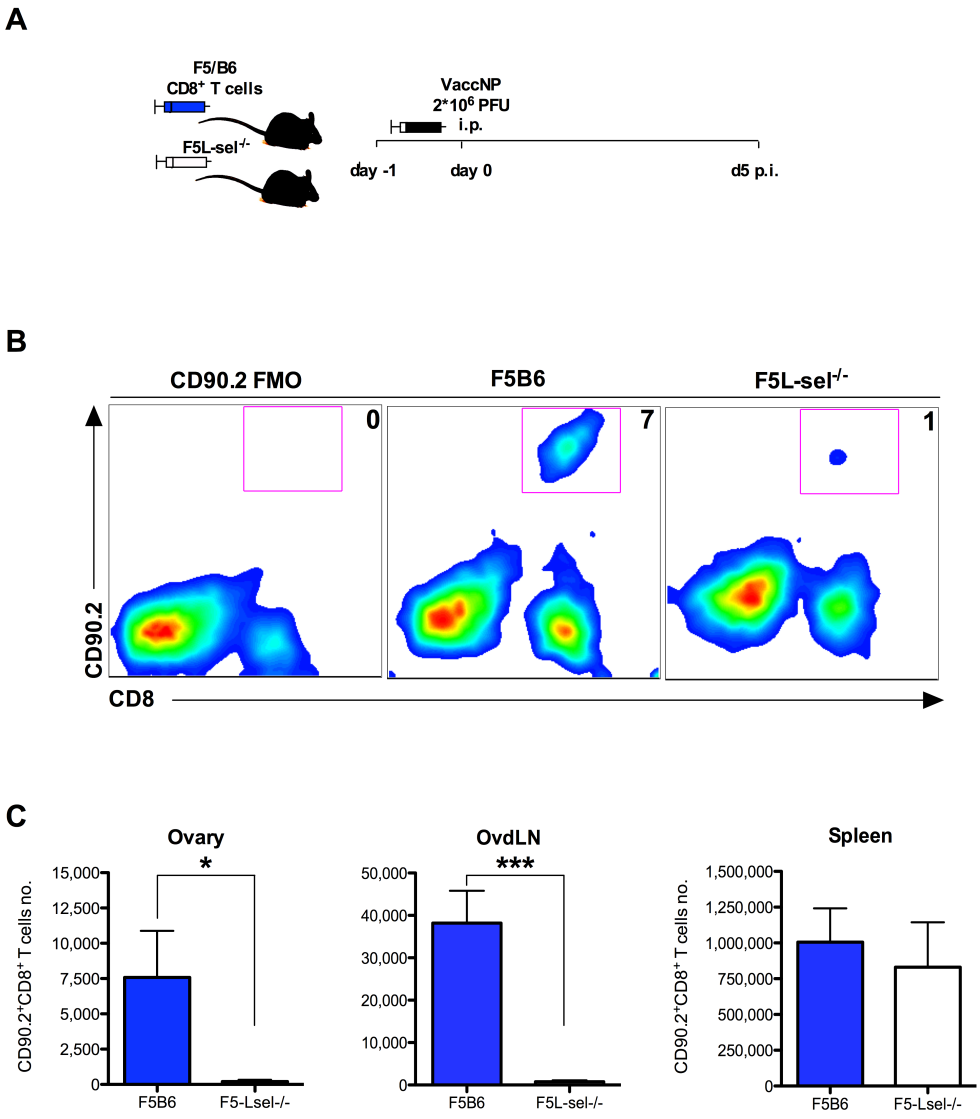


Figure 3.10: Enrichment of CD8$^+$ T cells at sites of recombinant vaccinia virus infection is L-selectin dependent. (A) Naïve CD90.2$^+$ CD8$^+$ T cells co-expressing transgenic F5 TCR and wildtype L-selectin (F5B6) or CD8$^+$ T cells express F5 TCR and knockout for L-selectin (F5L-sel$^{-/-}$) were isolated and adoptively transferred into CD90.1 B6 mice. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. At day 5 post infection, ovary, ovdLN and spleen were harvested and analyzed for homed donor CD8$^+$ T cells. **(B)** Representative bivariate plots shows donor CD90.2$^+$ CD8$^+$ T cells in the ovary of mice receiving F5B6 and F5L-sel$^{-/-}$ CD8$^+$ T cells at day 5 post infection. (C) Bar charts show absolute numbers of F5B6 and F5L-sel$^{-/-}$ CD8$^+$ T cells in the ovary, ovdLN and spleen at day 5 post infection. Results are mean $\pm$ SEM from two in dependent experiment (n=6)(unpaired student t-test).

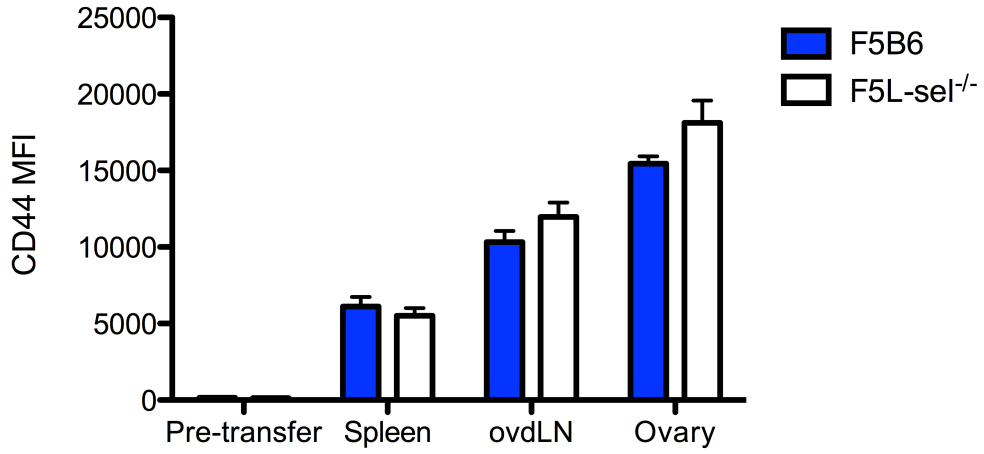


Figure 3.11: L-selectin expression does not influence the differentiation of virus-specific CD8$^+$ T cells during recombinant vaccinia virus infection. Naïve CD90.2$^+$ CD8$^+$ T cells co-expressing transgenic F5 TCR and wildtype L-selectin F5B6 or CD8$^+$ T cells expressing F5 TCR and knockout for L-selectin F5L-sel$^{-/-}$ were isolated and adoptively transferred into CD90.1 B6 mice. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. At day 5 post infection, ovary, ovdLN and spleen were harvested for donor CD8+ T cells analysis. Bar chart shows the similar kinetics of CD44 up-regulation by F5B6 (Blue) and F5L-sel$^{-/-}$ (White) CD8$^+$ T cells during vaccNP infection at day 5 from the ovary, ovdLN and spleen. Results are mean $\pm$ SEM (n=6).

3.3.8. MEL-14, L-selectin specific antibody, blocked homing of $CD8^+$ T cells to sites of infection

Taking into consideration that L-selectin was re-expressed by activated $CD8^+$ T cells once they leave the sites of priming and activation in the draining LN, and if L-selectin was involved in homing of $CD8^+$ T cells to sties of infection, we hypothesized that $CD8^+$ T cell homing to sites of infection would be abrogated by antibodies reactive to L-selectin. MEL-14 antibody binds to the lectin domain of L-selectin and prevents it from engaging with its ligands, thus blocking all known functions of L-selectin (Gallatin et al. 1987).

Based on the sites of priming and kinetics of $CD8^+$ T cell activation (Fig 3.1), naïve $CD8^+$ T cells were allowed to home and be primed and activated in the mediastinal LN before starting treatment with MEL-14. F5/LΔP $CD8^+$ T cells were transferred to naïve B6 mice and one day later, mice were inoculated with vaccNP. Mice were injected with 250 µg MEL-14 or isotype control antibodies 52 hours following virus inoculation, and donor cell analysis was performed day 5 post infection. Strikingly, MEL-14 reduced the number of donor $CD8^+$ T cells in infected ovaries by 85% compared to mice treated with control antibody. MEL-14 also reduced the number of donor $CD8^+$ T cells in the draining LNs by 80% but had no effect on donor $CD8^+$ T cell numbers in the spleen. Also, to exclude the possibility that MEL-14 blocked the activation and differentiation of the F5/LΔP donor $CD8^+$ T cells after the treatment, the activation status of F5/LΔP $CD8^+$ T cells was assessed by the up-regulation of CD44. Donor cells in both, MEL-14 and isotype control treated mice, up-regulated CD44 in a similar manner (Fig 3.12).

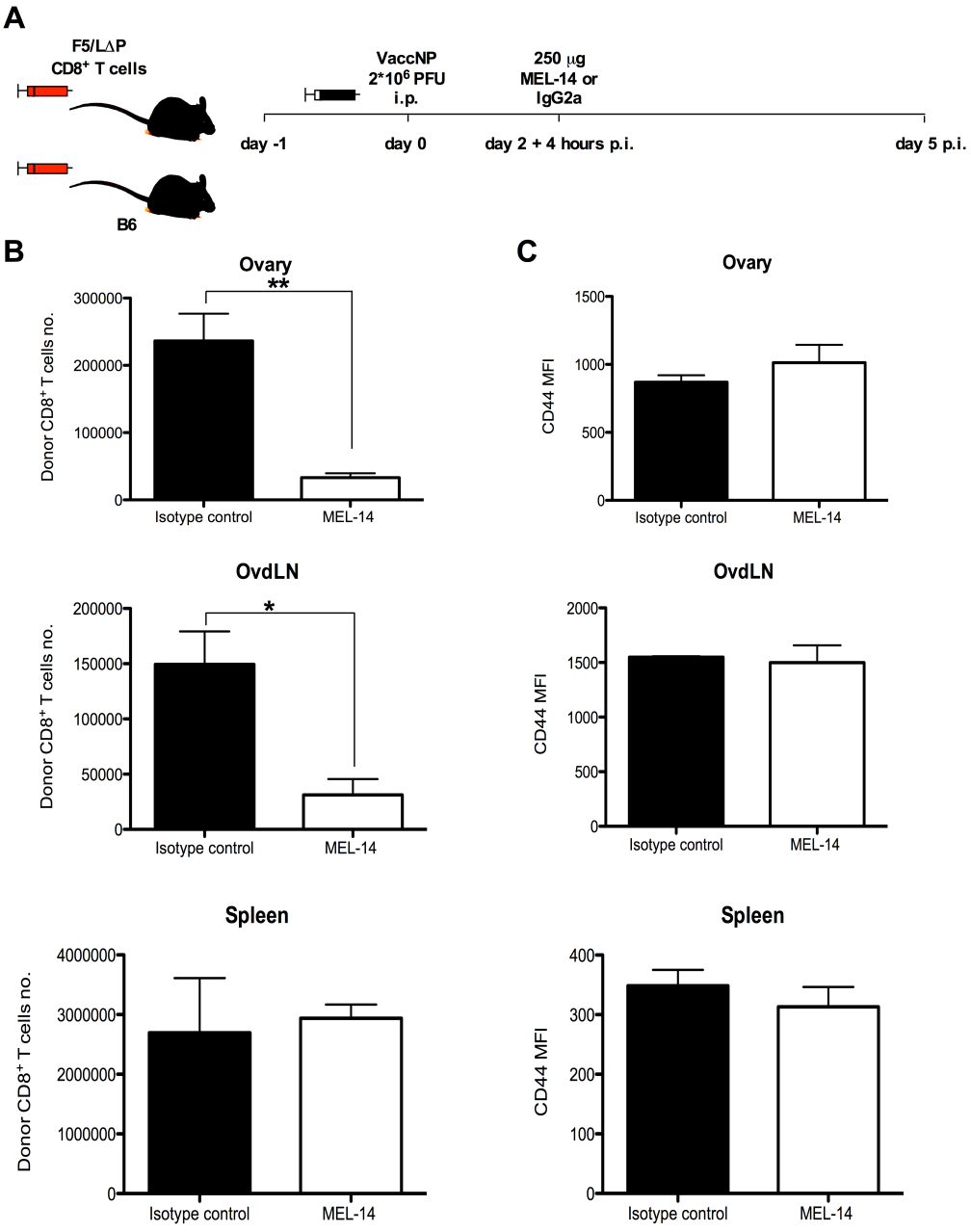


Figure 3.12: MEL-14 antibodies blocked homing of CD8$^+$ T cells to the site of vaccNP infection. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and mutant L-selectin (F5/LΔP) were isolated and adoptively transferred into B6 mice. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. 52 hours following virus inoculation, 250μg MEL-14 or IgG2a isotype control antibodies were injected in split doses of 125μg i.p. and 125μg i.v., and at day 5 post infection, ovary, ovdLN and spleen were harvested for homed donor cell analysis. **(B)** Donor CD8$^+$ T cells were identified as in figure 3.5., the bar charts showing the absolute numbers of homed donor CD8$^+$ T cells in mice that received either MEL-14 (black bar) or isotype control antibodies (white bar) in the ovary, ovdLN and spleen at 5 days post infection. **(C)** The bar charts show the level of CD44 expression by donor CD8$^+$ T cells in mice receiving either MEL-14 or isotype control antibodies in ovary, ovdLN and spleen at day 5 post infection. Results are mean ± SEM (n=3). * P<0.05, Un-paired student t test.

3.3.9. A short-term, competitive homing study further reveals the role of L-selectin in homing of activated CD8$^+$ T cells to site of infection

A competitive short-term homing experiment was conducted using *in vitro* activated CD8$^+$ T cells sufficient and deficient in L-selectin to understand the direct involvement of L-selectin in homing of activated CD8$^+$ T cells to site of virus infection. As described in the material and methods, d7 *in vitro* or d5 *in vivo* activated CD8$^+$ T cells from F5LΔP or F5L-sel$^{-/-}$ mice were mixed 1:1 and adoptively transferred into CD90.1 recipient mice, which have been infected with vaccNP 3 days before. Shortly after 3 hours, ovary, ovdLN, spleen and a number of peripheral and mucosal LN were harvested for donor cell analysis.

At 3 hours post-transfer, L-selectin expressing CD8$^+$ T cells were highly enriched over L-selectin deficient cells in the virally infected ovaries. The ratio of L-selectin positive and negative T cells circulating in peripheral blood remained 1:1 at this time point showing that the altered ratio in the ovaries was not simply due to depletion of L-selectin deficient T cells from the circulation. L-selectin positive CD8$^+$ T cells represented nearly all donor cells in the ovdLNs, and also in two peripheral LNs known to be L-selectin dependent, the axillary and inguinal LNs (Arbornes et al. 1994). L-selectin positive CD8$^+$ T cells were not enriched in the mediastinal LN, which further confirms that L-selectin is not a dominant homing receptor for this LN. The ratio of L-selectin positive to L-selectin negative CD8$^+$ T cells in the spleen was similar to the pre-transfer population since homing to the spleen is independent of L-selectin (Arbornes et al. 1994). The enrichment of L-selectin expressing activated CD8$^+$ T cells in the recently recruited cell population in the ovary further demonstrates clearly that L-selectin controls recruitment of activated CD8$^+$ T cells from the bloodstream into virus-infected tissues (Fig 3.13) (Fig 3.14).

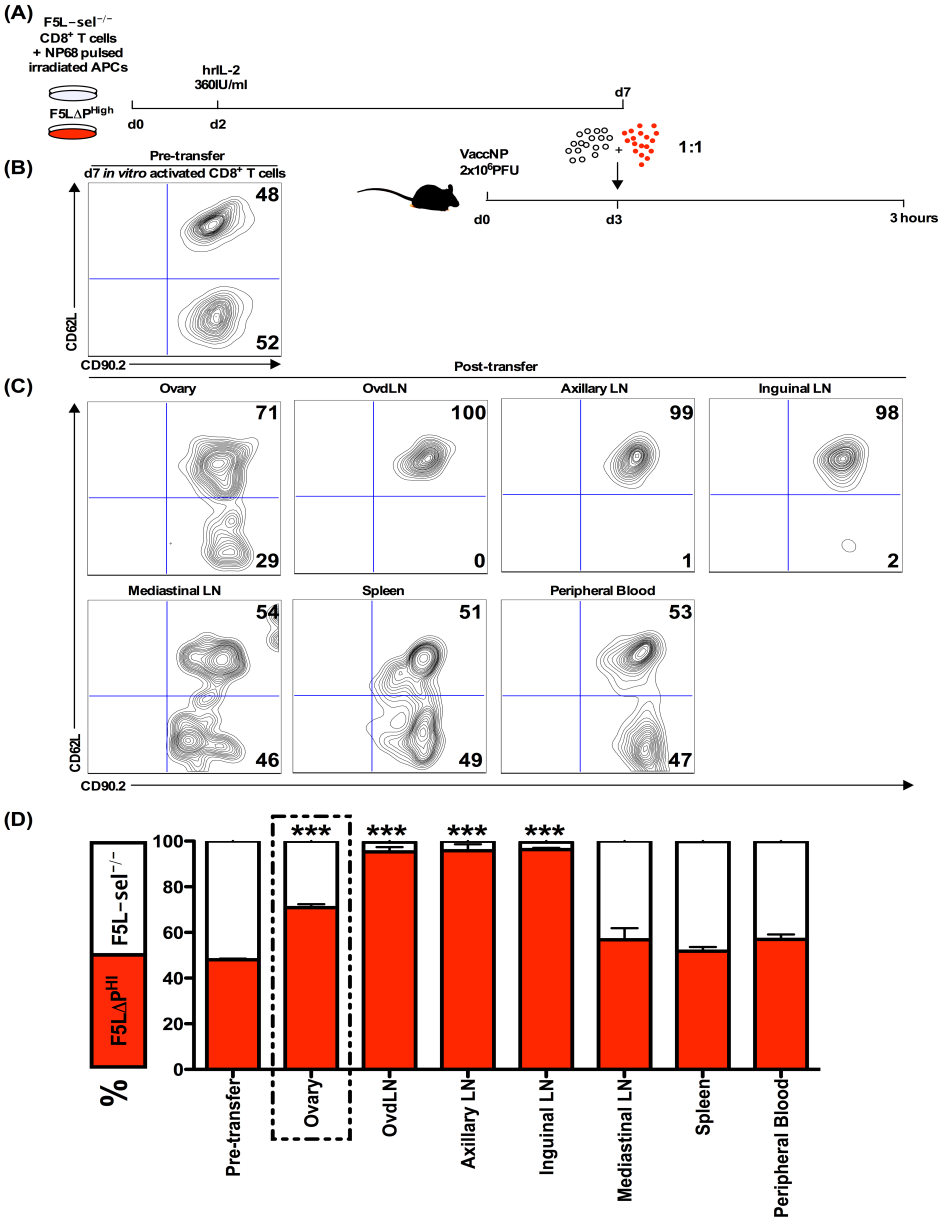


Figure 3.13: in vitro activated L-selectin positive CD8$^+$ T cells compete with the L-selectin deficient cells to home to sites of recombinant vaccinia virus infection. (A) Naïve CD90.2$^+$ CD8$^+$ T cells co-express transgenic F5 TCR and either mutant L-selectin (F5LΔP) or knockout for L-selectin (F5L-sel$^{-/-}$) were isolated and cultured in vitro for 7 days in the presence of NP68 pulsed irradiated antigen presenting cells and IL-2, meanwhile, naïve CD90.1 B6 mice were inoculated with 2 x 10^6 vaccNP, On day 3 post vaccNP infection, mice were adoptively transferred with 1:1 mix of activated F5LΔP and F5L-sel$^{-/-}$ CD8$^+$ T cells. 3 hours post T cells transfer, mice were euthanized and organs harvested for a competitive short-term homing of the F5LΔP^{HI} and F5L-sel$^{-/-}$ CD8$^+$ T cells analysis. **(B and C)** The representative bivariate plot shows percentage F5LΔP (CD90.2$^+$ CD62L$^+$) and F5L-sel$^{-/-}$ (CD90.2$^+$ CD62L$^-$) CD8$^+$ T cells pre-transfer and post-transfer in the ovary, ovdLN, axillary, inguinal, mediastinal LN and spleen also in the peripheral blood at the time of harvest. **(D)** The chart shows the mean + SEM (n=4)(Bonferroni test).

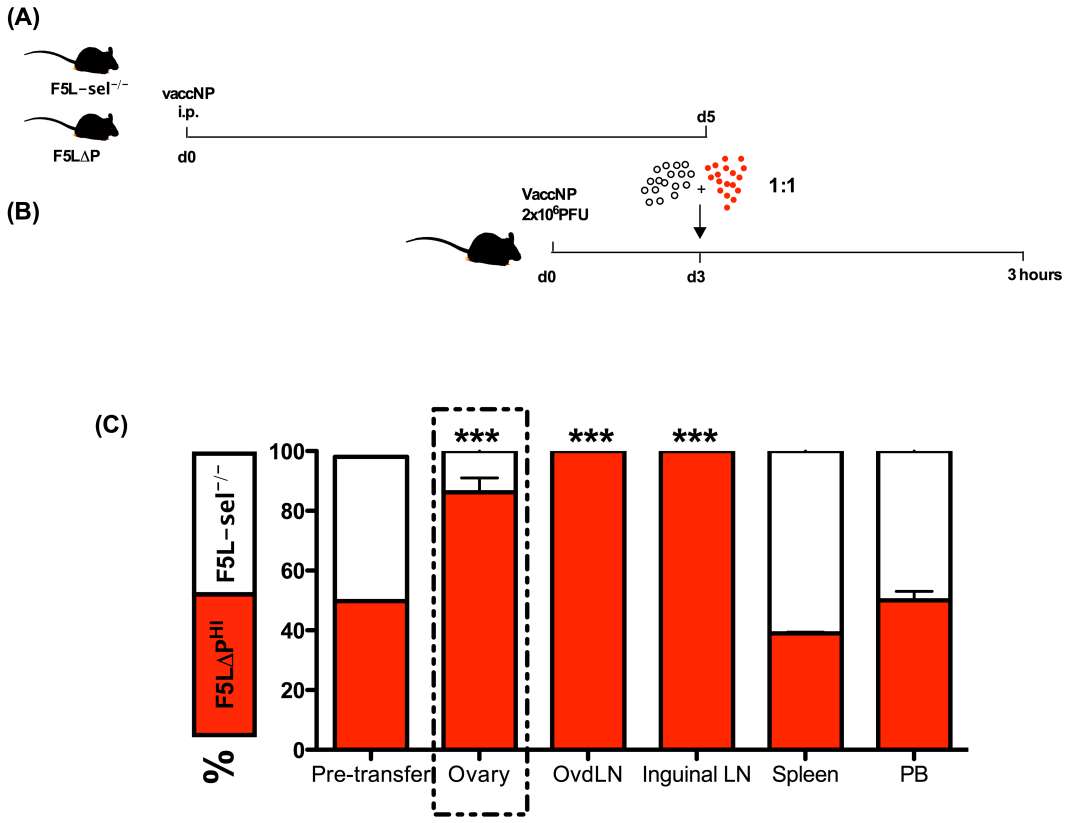


Figure 3.14: in vivo activated L-selectin positive CD8$^+$ T cell compete with the L-selectin deficient cells to home to sites of recombinant vaccinia virus infection. (A) Naïve F5LΔP and F5L-sel$^{-/-}$ mice were inoculated with 2 x 10^6 vaccNP, at day 5 post infection, mice were euthanized and CD8$^+$ T cells were isolated from spleen. Mean while, CD90.1 B6 mice were inoculated with 2 x 10^6 vaccNP. On day 3 post vaccNP infection, mice were adoptively transferred with 1:1 mix of in vivo activated F5LΔP and F5L-sel$^{-/-}$ CD8$^+$ T cells. 3 hours post T cells transfer, mice were euthanized and organs harvested for a competitive short-term homing of the F5LΔP and F5L-sel$^{-/-}$ CD8$^+$ T cells analysis. **(B)** The chart shows the mean ± SEM of percentage F5LΔP (CD90.2$^+$ CD62L$^+$) and F5L-sel$^{-/-}$ (CD90.2$^+$ CD62L$^-$) CD8$^+$ T cells in the ovary, ovdLN, inguinal LN, mediastinal LN and spleen also in the peripheral blood at the time of harvest. (n=4) (Bonferroni test)

3.3.10. L-selectin dependent homing of virus specific $CD8^+$ T cells to site of Influenza virus infection

$CD8^+$ T cells play an important role during respiratory virus infections. Influenza infection in particular, during both primary and secondary infection, requires a strong $CD8^+$ T cell response. Since we have shown that early-activated $CD8^+$ T cells express L-selectin, the role of L-selectin in homing of virus specific $CD8^+$ T cell to sites of infection, other than ovary, became of interest. To understand the involvement of L-selectin in homing of virus specific $CD8^+$ T cells to sites of influenza virus infection, naïve polyclonal B6 and LΔP mice were challenged with 50 pfu influenza strain A (H1N1, PR8) virus and 8 days later, mice were euthanized and lungs and spleen were harvested for analysis of endogenous virus-specific $CD8^+$ T cells. Consistent with the recombinant vaccinia virus adoptive transfer model, the numbers of endogenously produced NP34-tetramer specific $CD8^+$ T cells were higher in the lungs at day 8 post infection in the polyclonal LΔP mutant L-selectin expressing mice compared with wildtype L-selectin B6 mice (Fig 3.15).

Also to confirm that the enrichment in the numbers of virus specific $CD8^+$ T cells expressing mutant L-selectin in the influenza infected lungs of LΔP mice comparing to wildtype L-selectin expressing in B6 mice was not due to hyper-differentiation, up-regulation of the late activation antigen CD44 was measured. Virus specific $CD8^+$ T cells harvested from LΔP and B6 mice at day 8 post infection from the lungs and spleen, upregulated CD44 in a similar manner. Similar to recombinant vaccinia virus infection, CD44 up-regulation was lower on virus specific $CD8^+$ T cells harvested from spleen compared to the cells harvested from lungs (Fig 3.16).

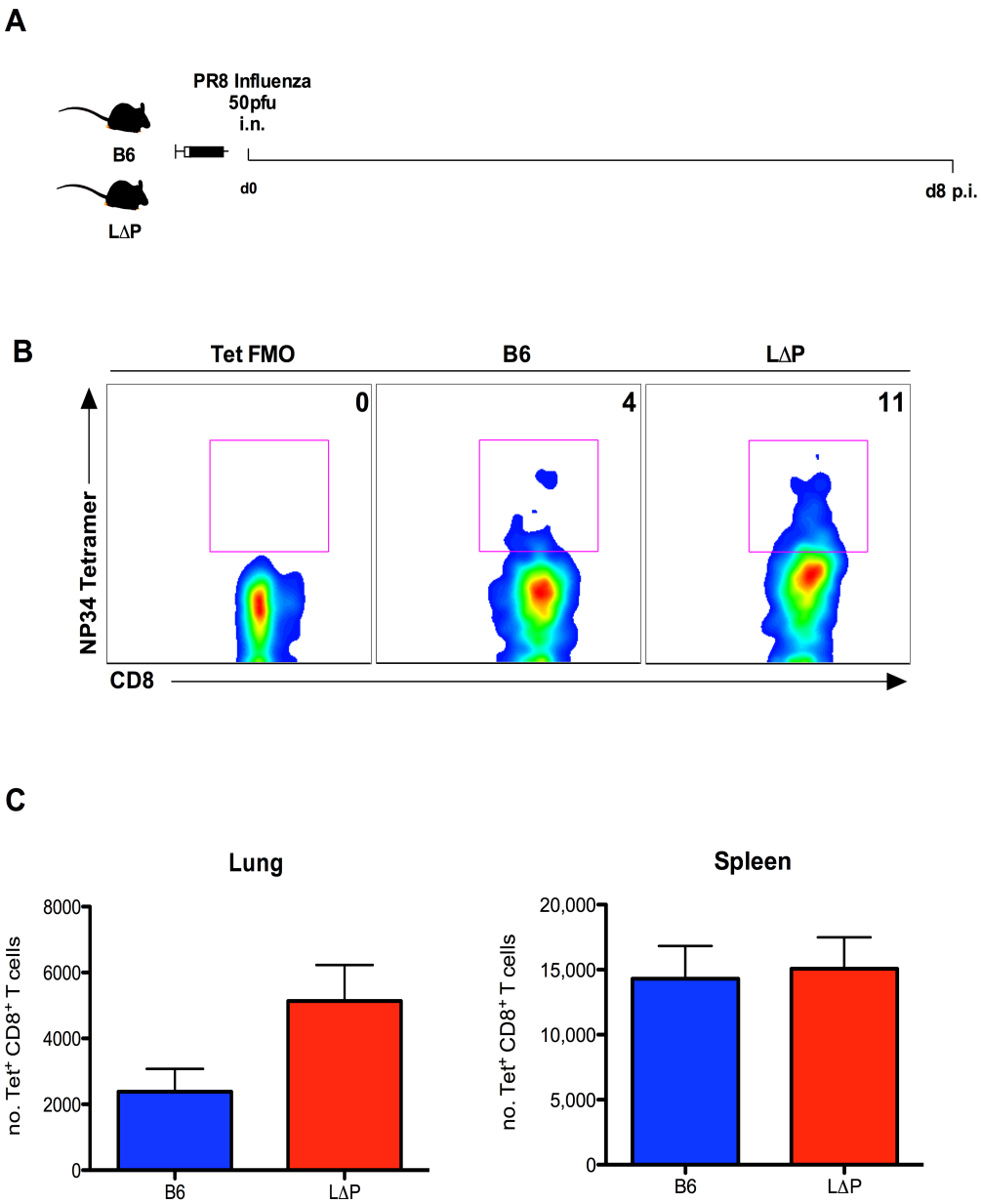


Figure 3.15: L-selectin dependent homing of CD8$^+$ T cells to site of influenza virus infection. (A) Naïve B6 and LΔP mice were challenged with 50 pfu influenza strain A (PR8, H1N1) virus. 8 days later, mice were euthanized and lung, spleen were harvested for analysis of endogenously generated virus specific CD8$^+$ T cells. **(B)** The representative bivariate plots show the % NP34 tetramer$^+$ CD8$^+$ T cells in the lungs of B6 and LΔP mice at day 8 post influenza infection. **(C)** The bar charts show the absolute numbers of NP34 tetramer$^+$ CD8$^+$ T cells in the lungs and spleen of B6 and LΔP mice at day 8 post influenza infection. Results are mean $\pm$ SEM (n= 4).

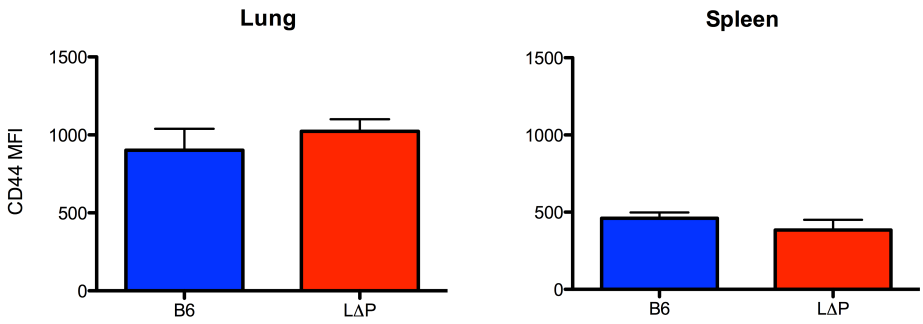


Figure 3.16: L-selectin expression does not influence the differentiation of virus-specific CD8$^+$ T cells during influenza infection. Naïve B6 and LΔP mice were challenged with 50 pfu influenza strain A (PR8, H1N1) virus. 8 days later, mice were euthanized and lungs and spleens were harvested for analysis of endogenously generated virus specific CD8$^+$ T cells. Bar charts show similar levels of CD44 expression on virus specific CD8$^+$ T cells in B6 and LΔP mice in the lung and spleen at day 8 post influenza infection. Results are mean $\pm$ SEM (n-4).

3.3.11. MEL-14 blocked homing of endogenously generated virus specific $CD8^+$ T cells to site of influenza virus infection

To understand the role of wildtype L-selectin in homing of virus specific $CD8^+$ T cells to site of virus infection, wildtype B6 mice were infected with 50 pfu influenza strain A (H1N1, PR8) virus and 52 hours later, when the $CD8^+$ T cells were already primed in the mediastinal LN (Yoon et al. 2007), mice were injected with either MEL-14 or isotype IgG2a control antibodies. At days 4 and 8 post infection, lung, mediastinal LN and spleen were harvested and analyzed for endogenously produced virus specific $CD8^+$ T cells. MEL-14 reduced the number of endogenously generated virus specific $CD8^+$ T cells in the lungs at day 4 post infection by 40%. By day 8 post infection, MEL-14 resulted in a much greater reduction of 70% in the numbers of the virus specific $CD8^+$ T cells in the lungs. Whereas, in the mediastinal LN, which is the major site of T cell priming following intranasal influenza administration (Yoon et al. 2007). The numbers of the virus specific $CD8^+$ T cells were not affected by MEL-14 treatment at both days 4 and 8 post infection, which confirms that homing of CD8+ T cell to this particular mucosal LN is not L-selectin dependent. Also, in the spleen, the numbers of virus specific $CD8^+$ T cells were not affected by MEL-14 treatment as shown during vaccNP infection (Fig 3.17). Furthermore, to determine whether MEL-14 affected the differentiation of virus specific $CD8^+$ T cells at various locations during the course of primary influenza infection, CD44 expression was measured. Virus specific $CD8^+$ T cells in both MEL-14 and isotype control antibody treated mice up-regulated CD44 in a similar manner in the lung, mediastinal LN and spleen (Fig 3.18). These findings further support the hypothesis that L-selectin is involved in homing of virus specific $CD8^+$ T cells to sites of infection but not in differentiation.

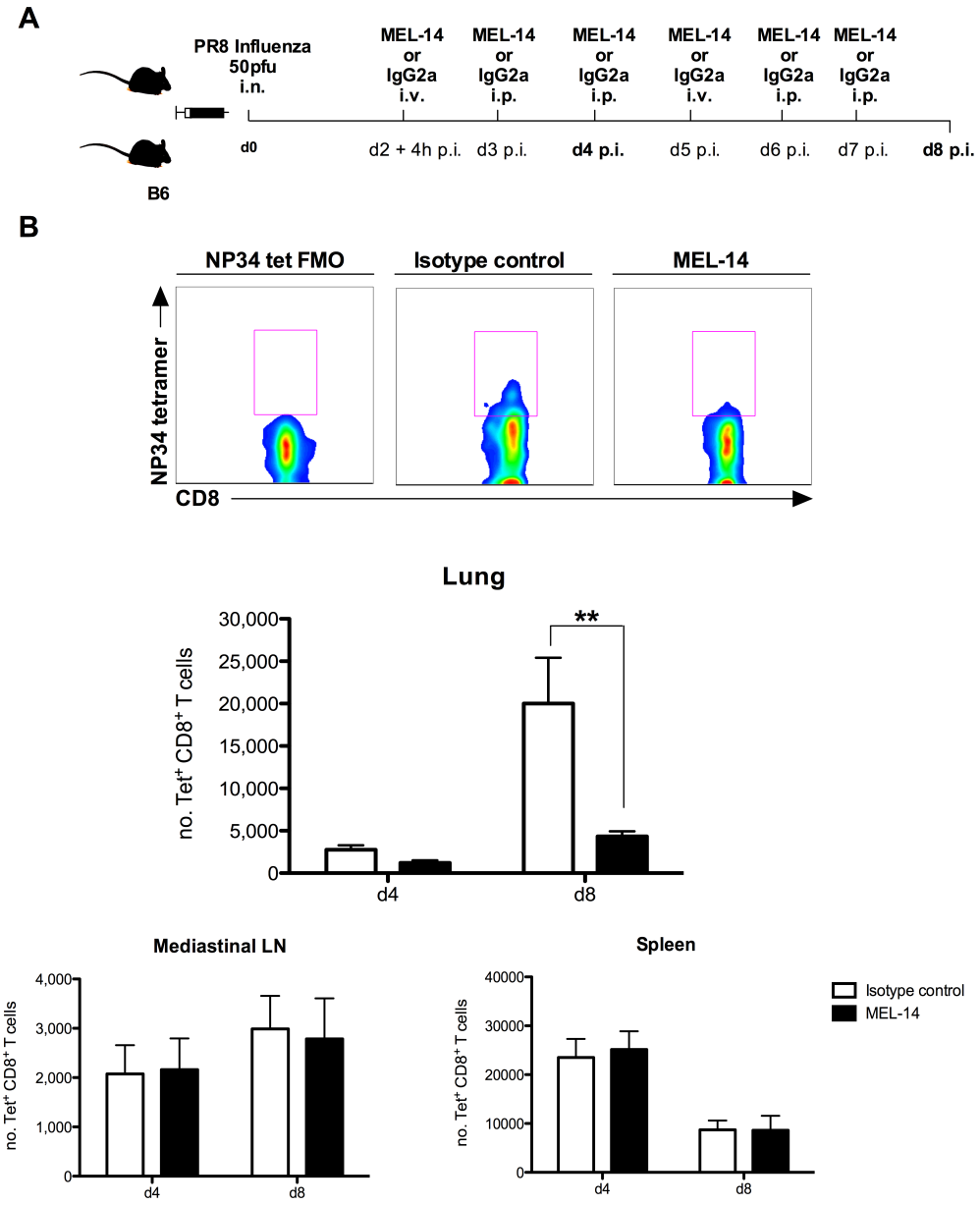


Figure 3.17: MEL-14 antibodies blocked homing of endogenously generated virus specific CD8$^+$ T cells influenza virus infected lungs. (A) Naïve B6 mice were challenged with 50 pfu influenza strain A (PR8, H1N1) virus intranasally. 52 hours following virus administration, mice were injected with 200μg MEL-14 or isotype IgG2a control i.v., followed by daily injection of MEL-14 and control antibodies i.p. At days 4 and 8 post infection, mice were euthanized and lungs (site of infection), mediastinal LN (site of CD8$^+$ T cell priming) and spleen were harvested for analysis of endogenously generated virus specific CD8$^+$ T cells. **(B)** Bar charts show absolute numbers of tetramer$^+$ CD8$^+$ T cells in the lung, mediastinal LN and spleen of mice treated with either MEL-14 or isotype control antibodies at day 4 and 8 post infection. Results are mean ± SEM (n=5). ** P< 0.001, Two-way ANOVA.

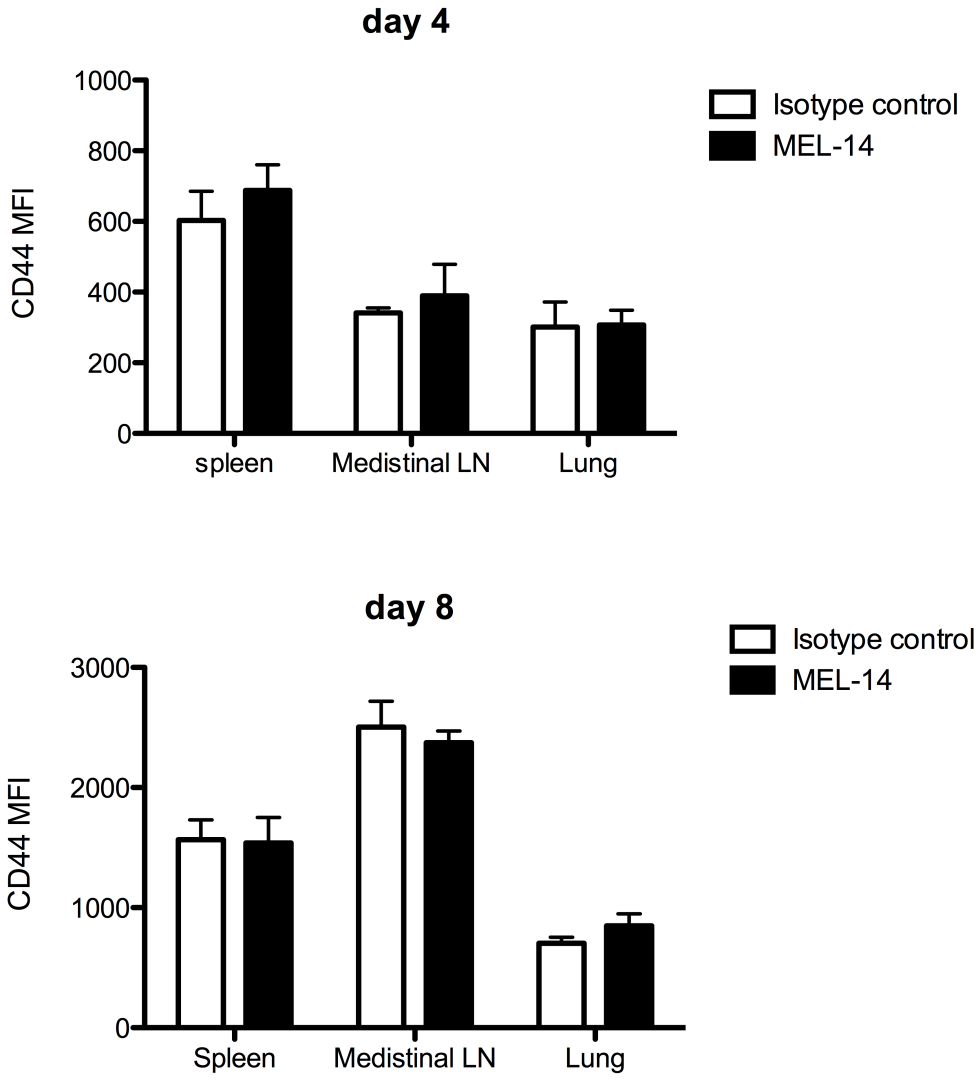


Figure 3.18: Differentiation status of virus specific $CD8^+$ T cells during primary influenza virus infection. Naïve B6 mice were challenged with 50 pfu influenza strain A (PR8, H1N1) virus intranasally. 52 hours following virus administration, mice were injected with 200μg MEL-14 or isotype IgG2a control i.v., followed by daily injection of MEL-14 and control antibodies. At days 4 and 8 post infection, mice were euthanized and lung (site of infection), mediastinal LN (site of $CD8^+$ T cells priming) and spleen were harvested for homed endogenously generated virus specific $CD8^+$ T cells analysis. Bar charts show CD44 upregulation by virus specific $CD8^+$ T cells at **(A)** day 4 and **(B)** day 8 post infection in lung, mediastinal LN and spleen. Results are mean + SEM (n=5).

Chapter four

4. The role of L-selectin in anti-virus immunity

4.1. Introduction

The role of $CD8^+$ T cells in protection against primary virus infection is well characterized. $CD8^+$ T cells can directly kill the virus-infected cells or they can secrete cytokines in order to modify the virus replication system. Effector lymphocyte communication with the virus or virus-infected cells is key in a successful immune response. Also, the number of effector $CD8^+$ T cells at the site of infection during virus infection determines the outcome. The level of cell adhesion molecules and their counterpart ligands on the infected cells and the lymphocytes determine the efficacy of the recruitment and therefore the number of effector cells at the site of infection. Improvement in effector $CD8^+$ T cell generation and migration during an adaptive immune response against virus and cancer is one of the main concerns in the field of immunology.

During virus infection, non-lymphoid infected tissues start to express various cell adhesion molecules, which allows the recruitment of virus-specific lymphocytes from the bloodstream to the site of infection (Roe et al. 2014), however the contributions of individual adhesion molecules to viral immunity have not been studied in detail. In this chapter, the role of L-selectin in conferring anti-virus immunity will be illustrated.

4.2. Hypothesis

The level of L-selectin on virus-specific $CD8^+$ T cells controls anti-viral immunity.

4.3. Results

4.3.1. Kinetics of recombinant vaccinia virus infection in C57BL/6 mice

To test the hypothesis that L-selectin dependent homing of $CD8^+$ T cells confers anti-virus immunity, the kinetics of the vaccNP infection in B6 mice and the components of the immune system that participate in viral clearance were dissected. Although it has been shown that clearance of wildtype Western Reserve (WR) vaccinia virus infection in B6 mice is $CD4^+$ T cell and MHC class II dependent (Xu et al. 2004), CD8+ T cells show site dependent activation and generate effector and memory $CD8^+$ T cells during the course of a primary vaccinia virus infection (Laouar et al. 2008)(Xiao et al. 2007).

Viruses such as HIV-1 use mechanisms of L-selectin downregulation from the surface of $CD4^+$ T cells, such as binding to proteins NEF and VPU (Vassena et al. 2015), to limit immune responses during the course of their infection. Vaccinia virus might adopt a similar mechanism to limit the numbers of $CD8^+$ T cells recruited to sites of infection. Therefore, the immune system might find alternative pathways to control vaccinia virus infection, which are independent of $CD8^+$ T cells.

The kinetic of vaccNP infection in B6 mice was studied by challenging naïve B6 mice with 2×10^6 pfu vaccNP. Since ovary is the main site of virus infection, fig 3.1, the virus was allowed to replicate and infect the host, and at days 5, 8 and 12 post infection, mice were euthanized and the site of virus replication and infection (ovary) was excised for viral titer by plaque assay (Fig. 4.1A). At the time points analyzed, vaccNP infection was highest titer at day 5 post infection. Viral titres fell

between day 5 and day 8 post infection by 2 logs, which reveals that the immune system started to clear vaccNP infection. Furthermore, at day 12 post infection, though the virus was not completely cleared from the ovaries, further signs of viral clearance were noticeable as the titer fell between day 8 and day 12 post infection (Fig. 4.1B).

4.3.2. Recombinant vaccinia virus infection in immunodeficient mice

After studying the kinetics of vaccNP infection in B6 mice, the role of lymphocytes and in particular, $CD8^+$ T cells, in the clearance of vaccNP infection was of great interest. In order to test to what extent $CD8^+$ T cells are participating in vaccNP clearance, vaccNP infection was studied in various types of $CD8^+$ T cell depleted mice.

First, immune competent B6 mice were manipulated to be acutely $CD8^+$ T cells deficient. Mice were sub-lethally irradiated to eliminate circulating lymphocytes (Unsinger et al. 2009) and prepare the host for adoptive transfer of purified naïve CD8+ T cells from donor mice. We addressed a question that if sub-lethal dose of irradiation is able to eliminate the host circulating lymphocytes for a minimum of 2-3 weeks, the adoptive transfer of naïve $CD8^+$ T cells into these irradiated mice will help to understand the participation of the $CD8^+$ T cell arm of the adaptive immune system in the vaccNP clearance. Naïve B6 mice were sub-lethally irradiated and challenged with vaccNP (Fig 4.2A). Unexpectedly, virus was not detectable 5 days post virus inoculation in the ovaries of irradiated mice (Fig 4.2B).

It has been shown previously that mice exposed to total body irradiation (acute, sub-lethal dose) and challenged with wildtype vaccinia virus resulted in a time lag in the virus infection characterized by a long delay in the virus replication and

invasion (Simon & Werner 1979). Since X-irradiated B6 mice, challenged with recombinant vaccinia virus, were not able to produce a detectable vaccinia virus infection in the ovaries, therefore, mice that were chronically $CD8^+$ T cells deficient as a result of a genetic failure to generate mature T and B cells ($RAG^{-/-}$), were used to study the role of $CD8^+$ T cells in vaccNP clearance and determine the impact of L-selectin in conferring anti-virus immunity (Fig 4.2C).

The kinetics of vaccNP clearance from ovaries of B6 and $RAG^{-/-}$ mice were compared. Virus titres were similar in B6 and $RAG^{-/-}$ mice at day 5 post infection. Lymphocyte dependent clearance of virus in wildtype B6 mice was detectable at day 8 post infection when virus titers were lower than in $RAG^{-/-}$ mice which had sustained virus replication and impaired virus clearance (Fig. 4.2D).

This finding clearly illustrating that the adaptive immune system, which is including T and B cells, participates in the clearance of the recombinant vaccinia virus infection. To what extend the T cell arm of the adaptive immune system is involved in the vaccNP clearance from the infected ovaries will be dissected in the following section.

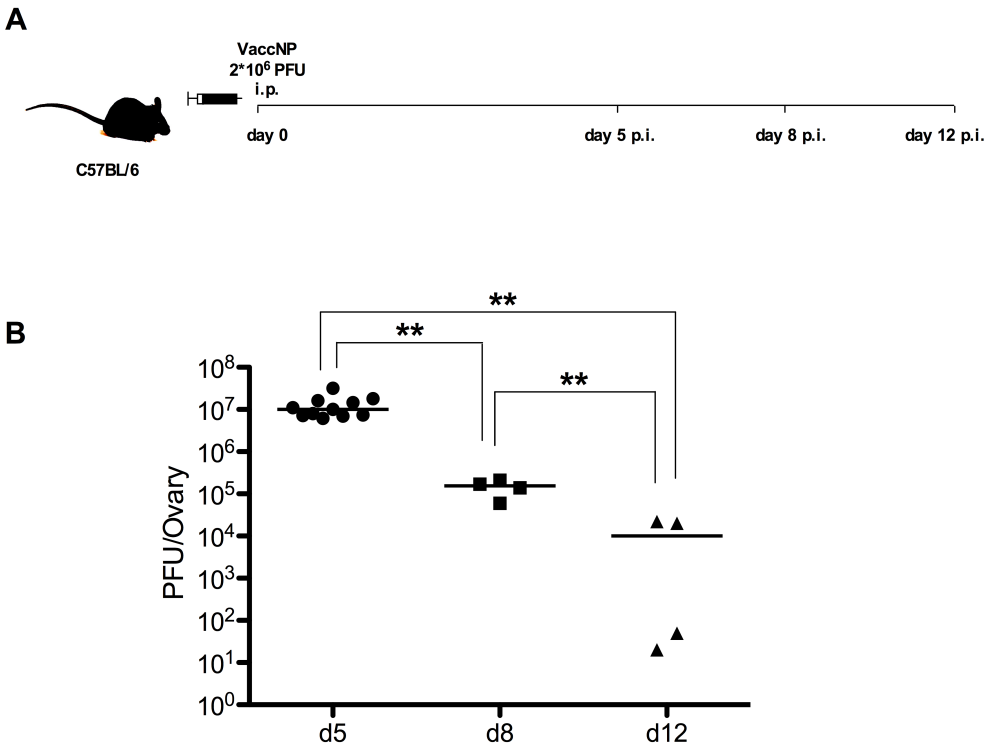


Figure 4.1: Kinetics of recombinant vaccinia virus clearance in C57BL/6 (B6) mice. (A) Naïve B6 mice were inoculated i.p. with 2 x 10^6 recombinant vaccinia virus expressing NP68 peptide (vaccNP). At days 5, 8 and 12 post infection, mice were euthanized and ovaries (site of infection) were excised for viral titer by plaque assay. **(B)** Kinetics of vaccNP clearance. Result shows titres in individual mice and median value from a single experiment (n=4-11).

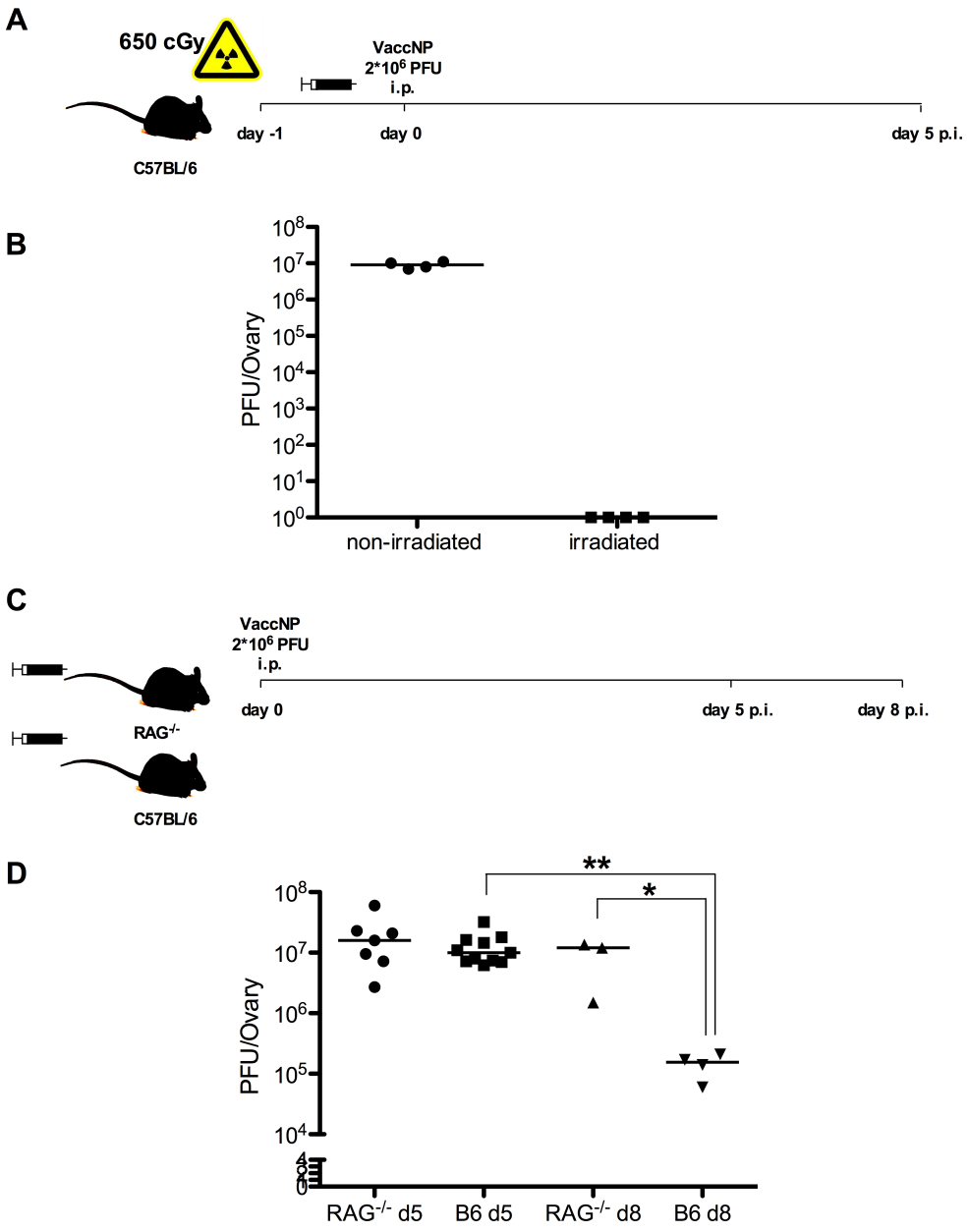


Figure 4.2: Recombinant vaccinia virus infection in immunodeficient mice. **(A)** Naïve B6 mice were sublethally irradiated with 650 cGy. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. At day 5 post infection, mice were euthanized, ovaries excised and virus titers determined in homogenized tissues by plaque assay. **(B)** Virus titres in irradiated and non-irradiated mice at day 5 post vaccNP infection. **(C)** Naïve B6 and RAG1$^{-/-}$ mice were inoculated with 2 x 10^6 pfu vaccNP. At days 5 and 8 post infection, mice were euthanized and ovaries were harvested for viral titre by plaque assay. **(D)** Virus titres in B6 and RAG1$^{-/-}$ mice at days 5 and 8 post vaccNP infection. Result shows titres in individual mice and median values from a single experiment (n=3-11)(One-Way ANOVA with post Tukey's test).

4.3.3. The role of L-selectin in $CD8^+$ T cell dependent anti-viral immunity

The significant difference in the viral load between wildtype B6 and $RAG^{-/-}$ mice at day 8 post infection led us to design an experiment to reveal the participation of $CD8^+$ T cells in the clearance of vaccinia virus infection. Also, the role of various levels of L-selectin expression on the surface of $CD8^+$ T cells in improving anti-virus immunity by enhancing the recruitment of $CD8^+$ T cells to the site of infection were investigated.

Naïve $CD8^+$ T cells co-expressing F5 TCR and wildtype L-selectin (F5/B6) or mutant L-selectin (F5/LΔP) were isolated and adoptively transferred into $RAG1^{-/-}$ recipient mice and a group of $RAG1^{-/-}$ mice did not receive T cells and were used as control. 24 hours later, mice were challenged with 2×10^6 pfu vaccNP. Then mice were treated daily from days 2 to 6 with IL-2 to support $CD8^+$ T cell survival and proliferation in the absence of CD4 helper T cells in $RAG^{-/-}$ mice (Fig 4.4A). Interestingly, adoptive transfer of F5 $CD8^+$ T cells expressing mutant L-selectin improved viral clearance. $RAG1^{-/-}$ mice that received wildtype L-selectin expressing F5$CD8^+$ T cells showed sustained virus replication and impaired viral clearance similar to control mice that did not receive T cells (Fig. 4.4B).

This finding further revealed an important role for L-selectin in supporting the enrichment of $CD8^+$ T cells at sites of viral infection and therefore enhancing anti-virus immunity.

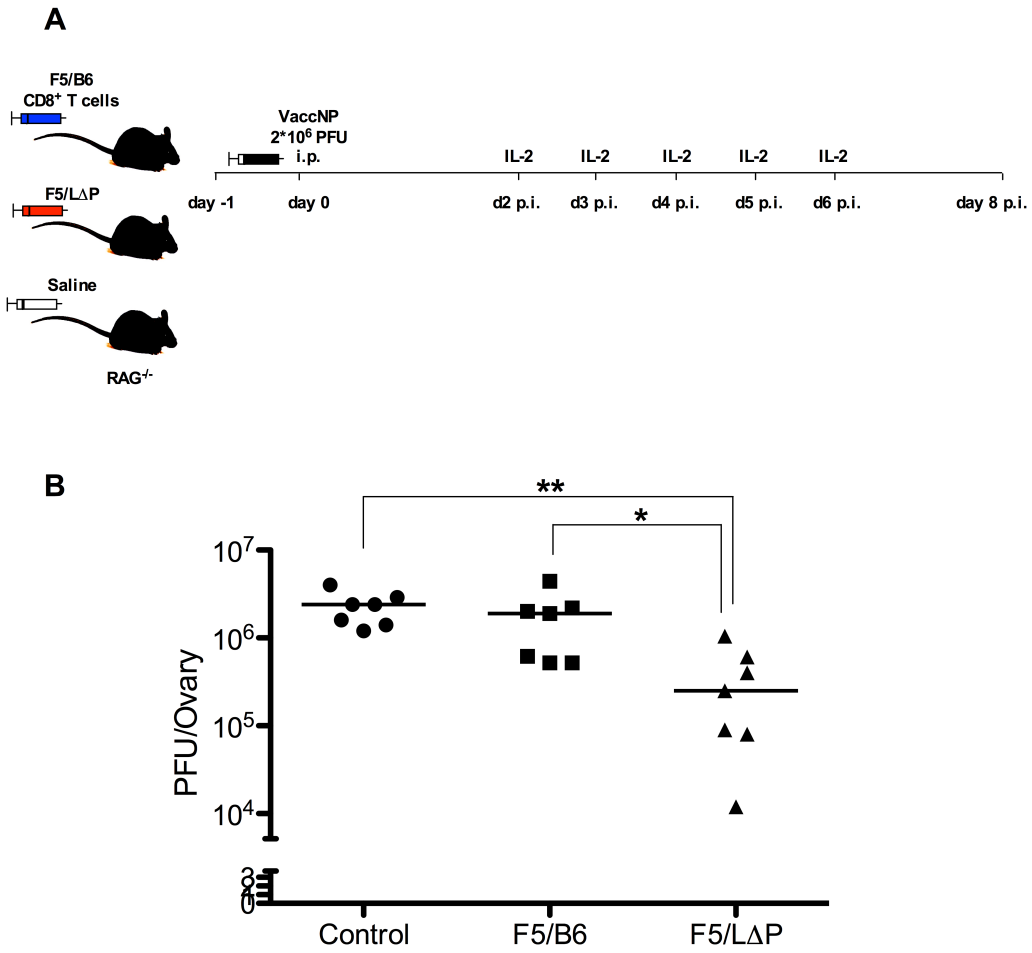


Figure 4.4: Mutant L-selectin on virus specific CD8$^+$ T cells confers protection during recombinant vaccinia virus infection. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype (F5/B6) or mutant L-selectin (F5/LΔP) were isolated and adoptively transferred into RAG1$^{-/-}$ mice. A group of RAG1$^{-/-}$ mice were also injected with saline and used as control. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. Mice were also treated with 150000 IU hrIL-2 from day 2 to day 6 post infection. At day 8 post infection, ovaries were harvested for viral titre using plaque assay. **(B)** Result shows viral titres in individual mice and median values pooled from 2 independent experiments (n=7). * P< 0.05, ** P< 0.001. One-Way ANOVA with post Tukey test.

4.3.4. L-selectin specific antibody MEL-14 blocks homing of virus specific $CD8^+$ T cells to sites of vaccNP infection in $RAG^{-/-}$ mice

The role of L-selectin in homing of virus specific $CD8^+$ T cells to the site of vaccNP infection in RAG1 mice was determined using MEL-14 blocking antibody to L-selectin and the previously employed strategy used in B6 mice. F5/LΔP $CD8^+$ T cells were transferred to naïve $RAG1^{-/-}$ mice and 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. Mice were injected with 250 µg MEL-14 or isotype control antibodies starting 52 hours following virus inoculation, and donor cell analysis was performed at day 5 post infection. MEL-14 reduced the number of donor $CD8^+$ T cells in infected ovaries (Fig. 4.5). MEL-14 treatment also reduced the number of donor $CD8^+$ T cells in ovary draining LNs but had no effect on donor $CD8^+$ T cell numbers in the spleen (Fig. 4.5B).

To exclude the possibility that MEL-14 blocked the activation and differentiation of F5/LΔP donor $CD8^+$ T cells, the activation status of F5/LΔP $CD8^+$ T cells was assessed by up-regulation of CD44. Donor $CD8^+$ T cells in both MEL-14 and isotype control antibody treated mice up-regulated CD44 in a similar manner (Fig 4.5C).

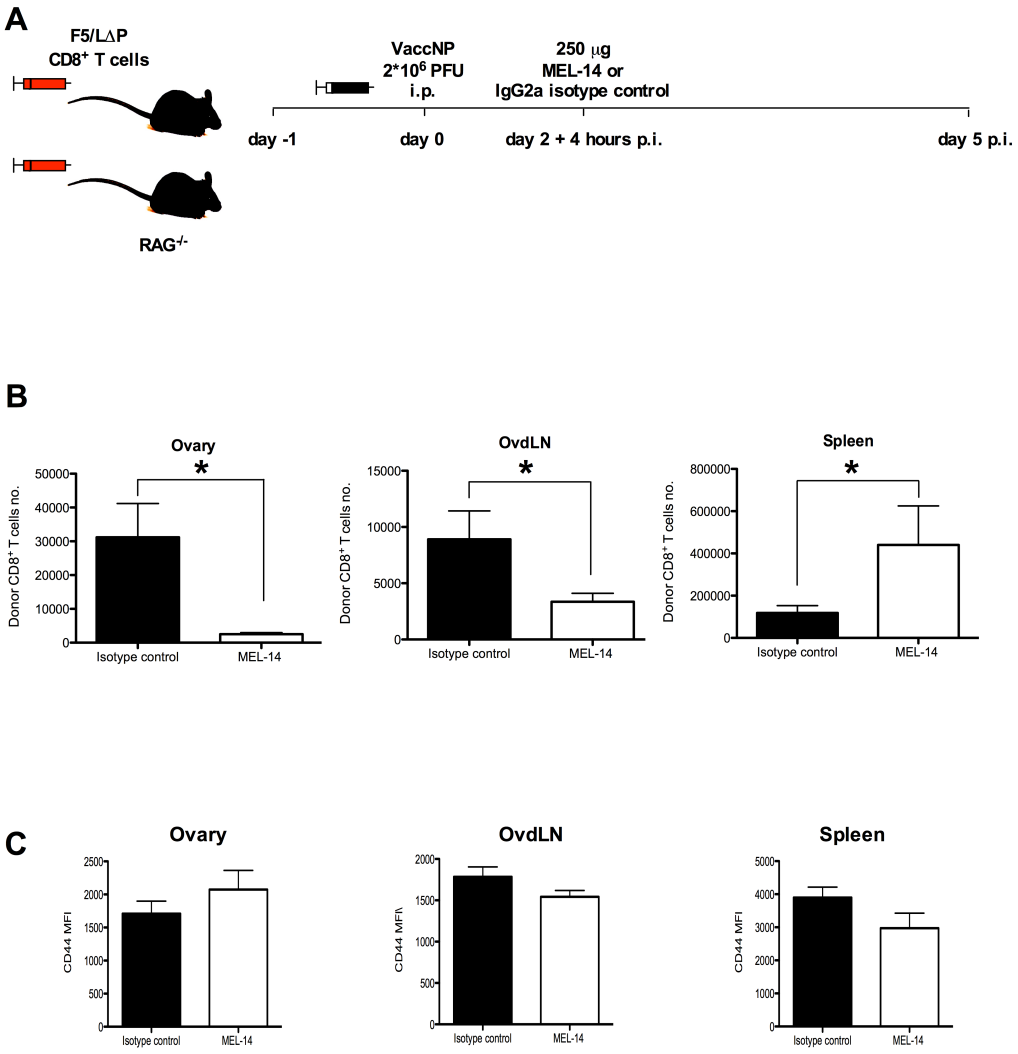


Figure 4.5: MEL-14 antibodies blocked homing of virus specific CD8$^+$ T cells to site of recombinant vaccinia virus infection in RAG1$^{-/-}$ mice. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and mutant L-selectin (F5/LΔP) were isolated and adoptively transferred into RAG1$^{-/-}$ mice. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. 52 hours following virus administration, mice were either treated with 250μg MEL-14 or IgG2a isotype control antibodies. At day 5 post infection, ovary, ovdLN and spleen were harvested for homed donor CD8$^+$ T cells analysis. **(B)** Bar charts show absolute numbers of donor CD8$^+$ T cells in ovary, ovdLN and spleen and **(C)** bar charts show the mean fluorescence intensity of CD44 expression on donor CD8$^+$ T cells in ovary, ovdLN and spleen in mice treated either with MEL-14 or isotype control antibodies at day 5 post infection (n=3). * P <0.05. Student unpaired t test.

4.3.5. Enhanced viral clearance by mutant L-selectin expressing $CD8^+$ T cells was not due to hyper-proliferation

Since the strength of the adaptive immune response against virus infection is linked to the generation of sufficient numbers of effector $CD8^+$ T cells that can home to the site of infection, proliferation analysis of donor cells at the time of protection against the virus was of great interest. Taking into consideration that $RAG^{-/-}$ mice receiving mutant L-selectin expressing $CD8^+$ T cells show improved protection against vaccNP infection, this raised the possibility that these cells might proliferate better than wildtype L-selectin expressing cells. Although mutant L-selectin expressing $CD8^+$ T cells showed a delay in entering the proliferation cycle at day 2 p.i. (Fig 3.10), the presence of mutant L-selectin on the surface of $CD8^+$ T cells could provide enhanced proliferation later during the infection. The rate of proliferation was assessed by administering the proliferation dye (EdU) at day 4 p.i., which is incorporated into the DNA of proliferating cells and analyzing donor cells in virus-infected ovaries at day 5 p.i..

There was no statistical significant difference in the percentage of F5/B6 and F5/LΔP $CD8^+$ T cells that incorporated EdU dye into their DNA, which shows that both cell types were proliferating at a similar rate. Collectively, these data show that L-selectin only provides early co-stimulatory signals for $CD8^+$ T cells to enter the proliferation cycle but has no effect at later time points and therefore enrichment of F5/LΔP $CD8^+$ T cells in virus-infected tissues was not due to enhanced proliferation (Fig 4.6).

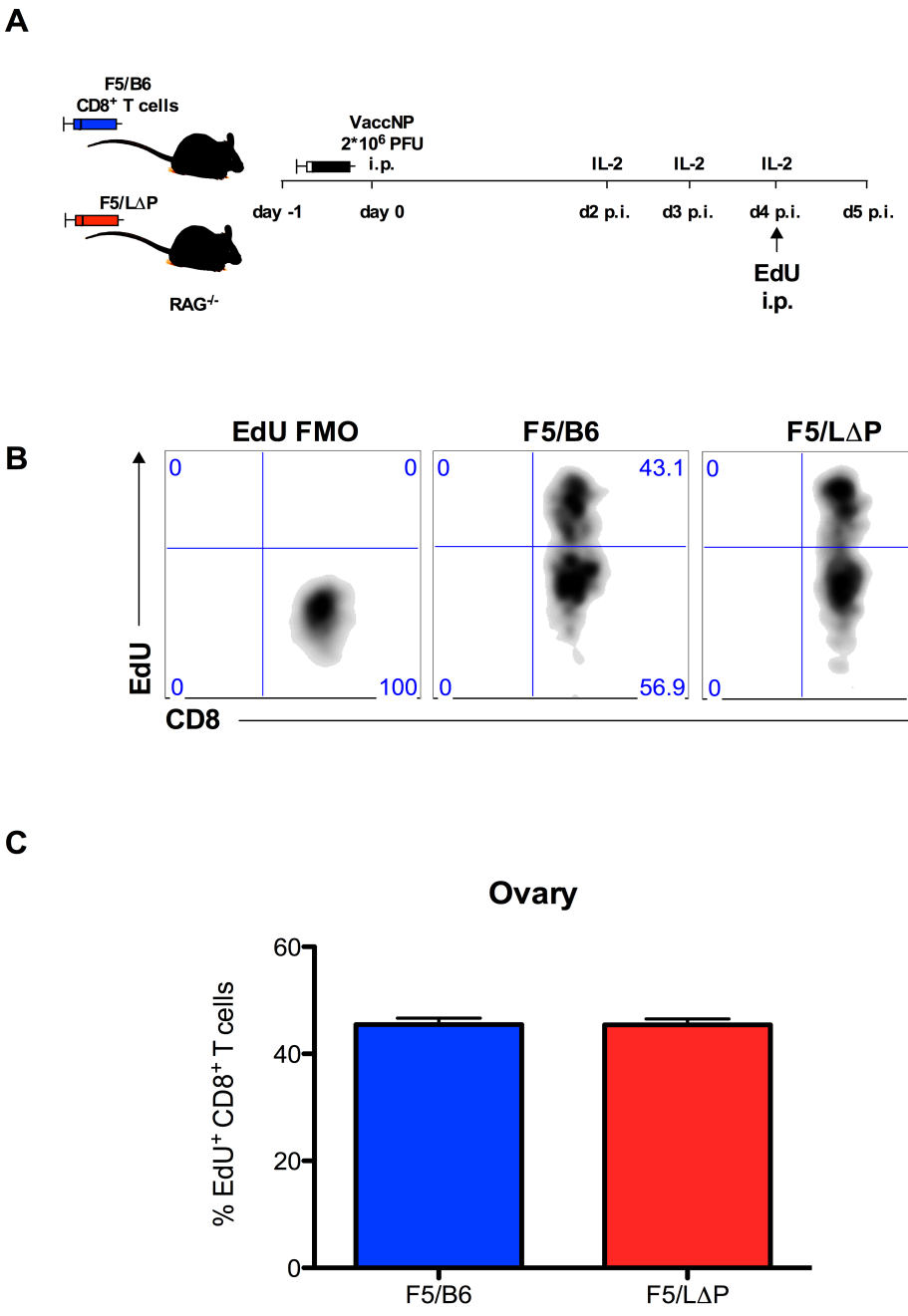


Figure 4.6: L-selectin does not co-stimulate late proliferation of virus specific CD8$^+$ T cells at the site of recombinant vaccinia virus infection. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-selectin F5/LΔP were isolated and adoptively transferred into RAG1$^{-/-}$ mice. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. Mice were also treated with 150000 IU hrIL-2 from day 2 to day 4 post infection. At day 4 post infection, 1mg EdU dye was injected i.p. and ovaries were harvested at day 5 for donor CD8$^+$ T cell analysis. **(B)** Bivariate plots show the incorporation of EdU dye into the DNA of proliferating cells as CD8 and EdU double positive cells compared with the EdU FMO. **(C)** Bar charts show the mean ± SEM (n=4).

4.3.6. Mutant and wildtype L-selectin expressing $CD8^+$ T cells show equal ability to secrete various cytotoxic chemicals

Cytokines provide both advantageous and adverse effects for the immune system. Cytotoxic $CD8^+$ T cells participate in killing virus-infected cells through secretion of chemical mediators such as Interferon-γ, granzyme B and perforin (Rodriguez et al. 2003; (Johnson et al. 2003). Whereas, regulatory T cells is secreting immunosuppressive cytokines such as interlukin-10 (IL-10) and transforming growth factor-β (TGF-β) to reduce the effectiveness of the immune system (Taylor et al. 2006). Another explanation, other than efficient homing, for the enhanced viral clearance in $RAG1^{-/-}$ mice that received mutant L-selectin expressing $CD8^+$ T cells, is that these cells secrete more cytotoxic chemicals at the site of vaccNP infection. To test the hypothesis, naïve F5/B6 or F5/LΔP $CD8^+$ T cells were adoptively transferred into $RAG^{-/-}$ mice. 24 hours later, mice were challenged with vaccNP. At day 5 post infection, donor $CD8^+$ T cells harvested from ovary and spleen were re-stimulated *ex vivo* in the presence of F5 TCR cognate peptide (NP68) to assess their abilities to secrete various cytotoxic chemicals necessary for $CD8^+$ T cells to function during primary viral infection.

F5/B6 and F5/LΔP $CD8^+$ T cells harvested from the site of vaccNP infection synthesized similar levels of, IFN-γ, TNF-α, IL-2 and granzyme B (Fig. 4.7). Donor F5/B6 and F5/LΔP $CD8^+$ T cells harvested from the spleen also synthesized similar levels of IFN-γ, TNF-α, IL-2 and granzyme B (Fig. 4.8). This finding further supports the hypothesis that L-selectin dependent enhancement of anti-virus immunity is not due to hyper-proliferation or hyper-functional $CD8^+$ T cells.

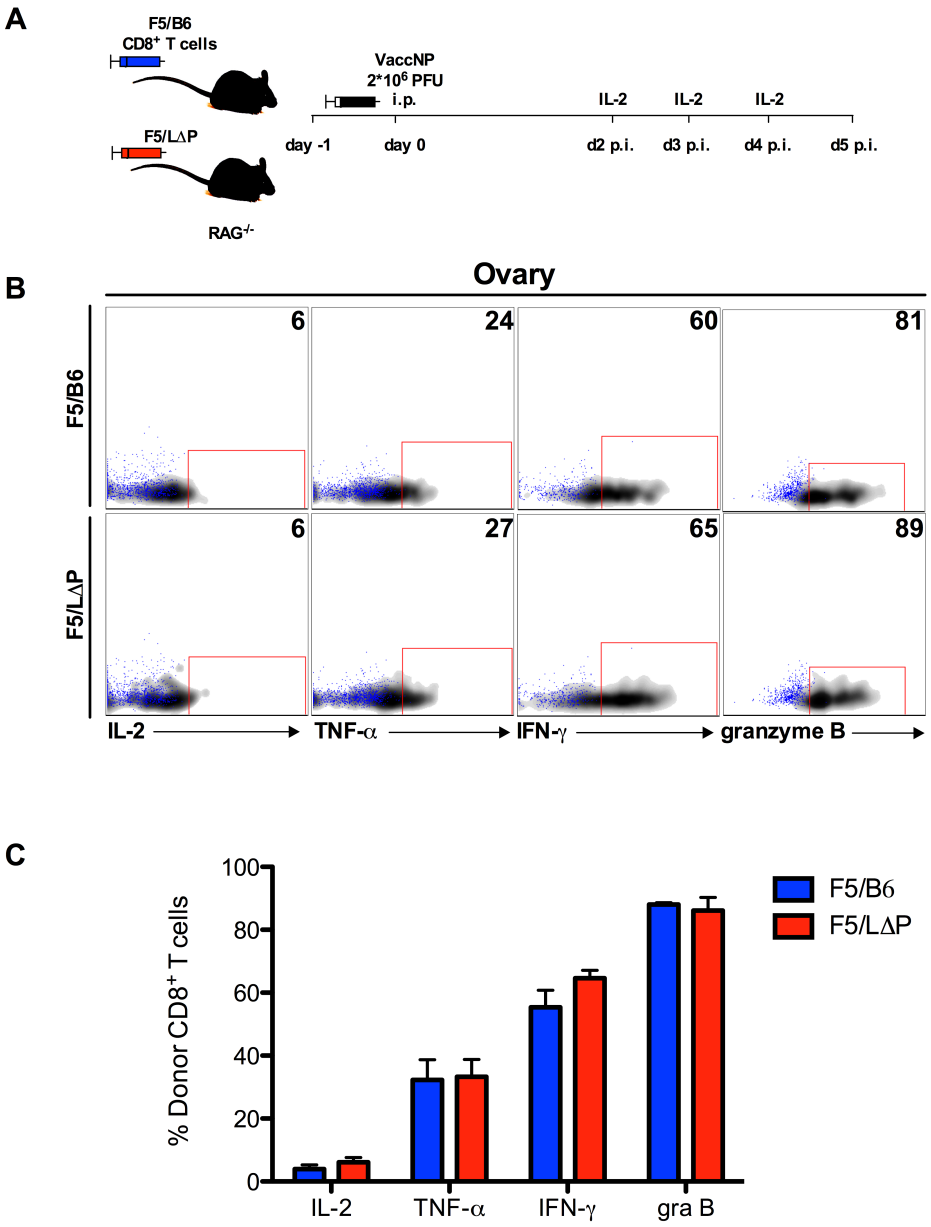


Figure 4.7: L-selectin does not affect cytokine secretion by virus specific CD8$^+$ T cells at the site of recombinant vaccinia virus infection. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-slectin F5/LΔP were isolated and adoptively transferred into RAG1$^{-/-}$ mice. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. Mice were also treated with 150000 IU hrIL-2 from day 2 to day 4 post infection. At day 5 post infection, donor CD8$^+$ T cells were harvested from ovary and re-stimulated ex vivo for 4 hours in the presence of NP68 peptide and Brefeldin A. **(B)** Representative plots show IL-2, TNF-α, IFN-γ and granzyme B secretion as density plots (black) of F5/B6 and F5/LΔP CD8$^+$ T cells overlaid on the FMO as dot plots (blue). **(C)** Bar chart shows percentage positive donor T cells as mean ± SEM (n-4).

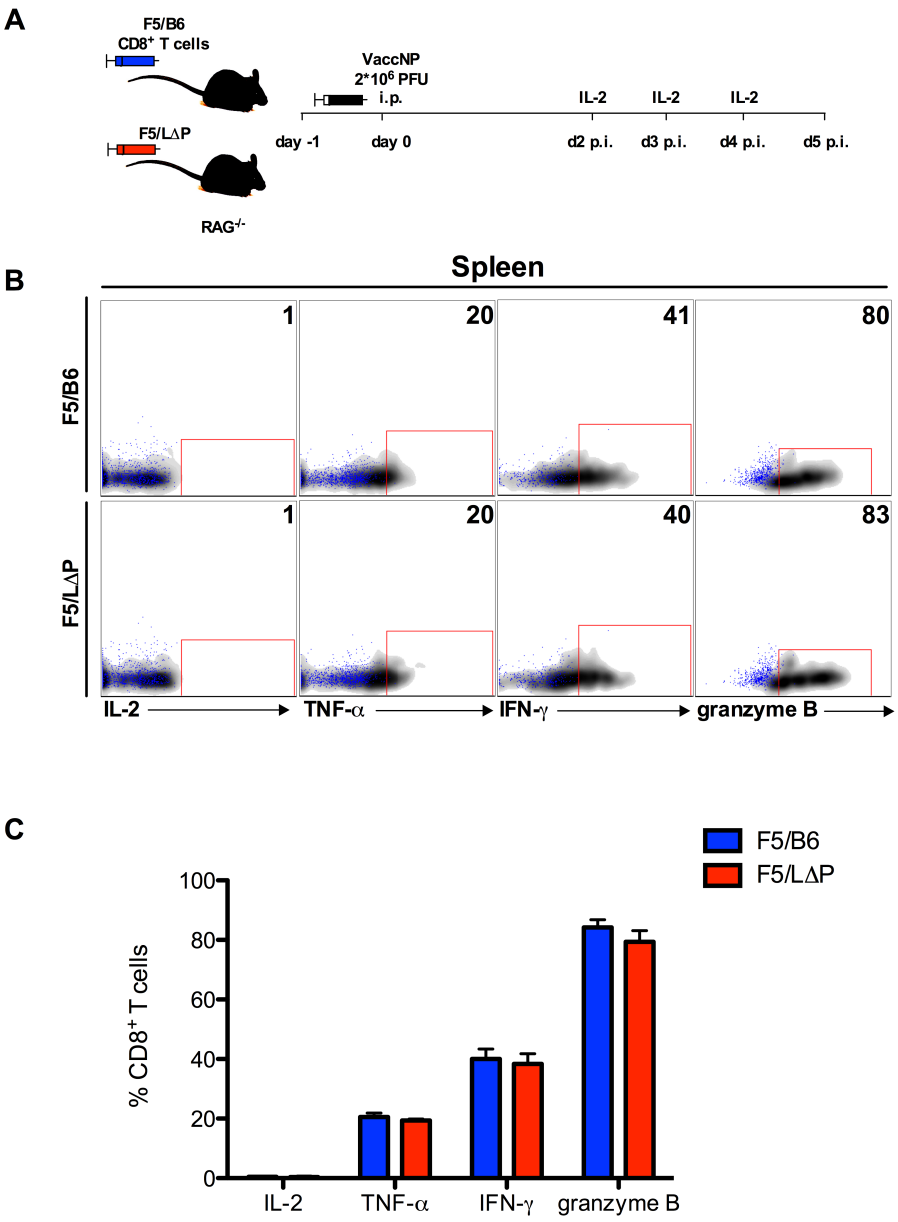


Figure 4.8: L-selectin does not affect cytokine secretion by virus specific CD8$^+$ T cells in the spleen of recombinant vaccinia virus infected mice. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-slectin F5/LΔP were isolated and adoptively transferred into RAG1$^{-/-}$ mice. 24 hours later, mice were inoculated with 2 x 10^6 pfu vaccNP. Mice were also treated with 150000 IU hrIL-2 from day 2 to day 4 post infection. At day 5 post infection, donor CD8$^+$ T cells were harvested from spleen and re-stimulated ex vivo for 4 hours in the presence of NP68 peptide and Brefeldin A. **(B)** Representative plots show the IL-2, TNF-α, IFN-γ and granzyme B secretion as density plots (black) of F5/B6 and F5/LΔP CD8$^+$ T cells overlaid on the FMO as dot plots (blue). **(C)** Bar chart shows percentage positive donor T cells as mean ± SEM (n-4).

4.3.7. Respiratory recombinant vaccinia virus infection in mice

The vaccinia (i.p.) model employed so far was very informative for cellular studies of antiviral responses, but it is of limited clinical relevance. To determine whether L-selectin expression confers protection from virus infection in other organs, recombinant vaccinia virus infection in the respiratory system was studied. It has been shown that, following intranasal inoculation of wildtype Western Reserve (WR) vaccinia virus, replicating virus was detectable in lungs and $CD8^+$ T cells exhibited a protective role, using IFN-gamma secretion mechanism to clear the virus (Goulding et al. 2014), whereas clearance of vaccinia following intraperitoneal administration is dependent on $CD4^+$ T cells and antibodies (Xu et al. 2004). Therefore, we decided to challenge the mice through an intranasal route to establish a respiratory vaccinia virus infection to test the hypothesis that L-selectin regulates $CD8^+$ T cell homing to sites of infection and confers anti-viral immunity.

Naïve B6 mice were challenged with various doses of vaccNP intranasally. Body weights were monitored daily and at day 8 post infection; lungs were harvested for viral titer by plaque assay. The mice did not show signs of infection, as they did not lose weight and there was no detectable virus in the lungs (Fig. 4.9). This finding suggests that genetic manipulation of vaccinia virus causes the virus to lose its ability (Williamson et al. 1990) to infected mouse lungs.

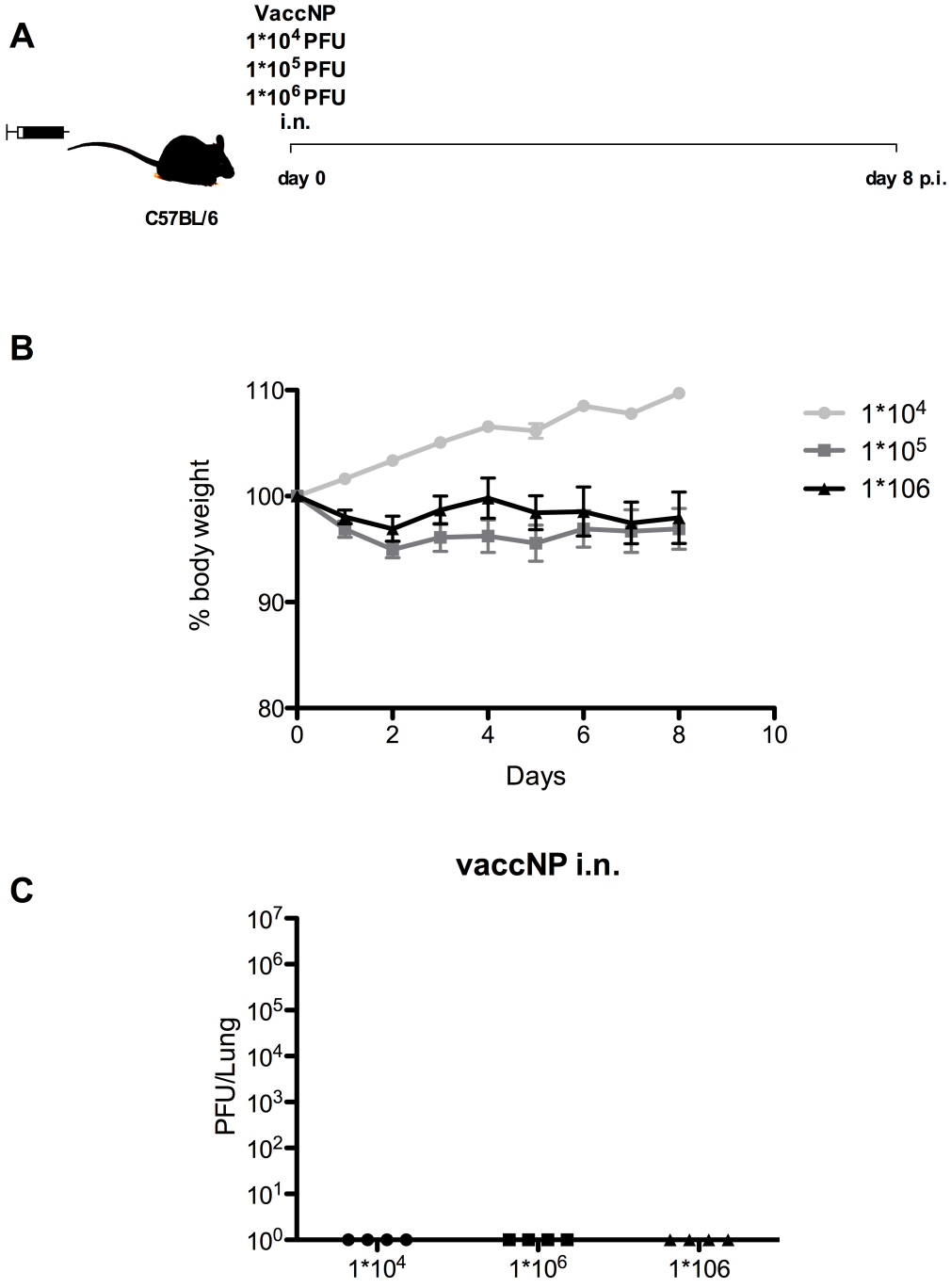


Figure 4.9: Respiratory recombinant vaccinia virus infection in C57BL/6 mice. (A) Naïve B6 mice were intranasally inoculated with 1 x 10^4, 1 x 10^5 or 1 x 10^6 pfu vaccNPunder light anaesthesia. Body weights were measured daily and at day 8 post virus administration, mice were euthanized and lungs excised for viral titre in homogenized tissue by plaque assay. (B) % Body weight loss or gain to the original body weight. (C) Viral titres in individual mice (n=4).

4.3.8. L-selectin expression promotes viral clearance during primary influenza virus infection.

Having determined that vaccNP was not able to establish a measurable respiratory infection following intranasal challenge (Fig. 4.9), a more lethal form of respiratory viral infection was established with the Influenza strain A virus. Intranasal (i.n.) infection with influenza virus in mice can be considered closest to the natural route of human influenza infections. $CD8^+$ T cells play an important role in immune response protection against influenza virus infection (Bender et al. 1992).

The impact of L-selectin expression on viral clearance from lungs following infection of various levels of L-selectin expressing mice with a pathogenic strain of influenza virus was determined. L-$sel^{-/-}$, B6 and LΔP mice were infected i.n. with influenza virus (H1N1, PR8), mice weights were monitored daily and lungs were harvested for viral titer by plaque assay. At day 8 following viral challenge, LΔP mice had the lowest virus titers which were accompanied by a striking alleviation in weight loss. In contrast, L-$sel^{-/-}$ mice demonstrated sustained viral loads and more exaggerated weight loss compared to wild type B6 mice (Fig. 4.10). These data therefore demonstrate that L-selectin is essential for the recruitment of protective, virus-specific $CD8^+$ T cells into infected tissues.

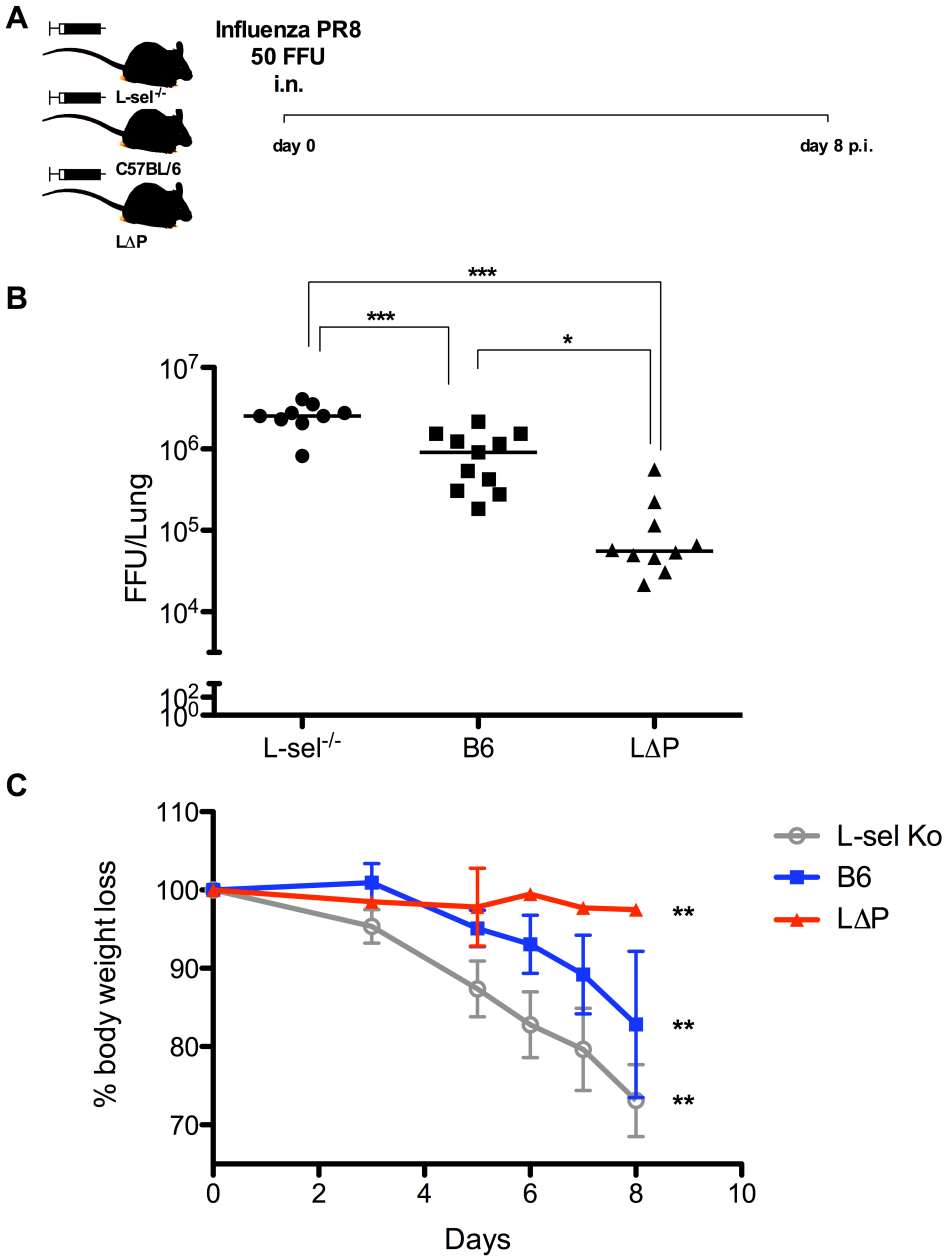


Figure 4.10: L-selectin dependent homing of virus specific CD8^{+} T cells to site of influenza virus infection confers anti-viral immunity. (A) Naïve L-sel$^{-/-}$, B6 and LΔP mice were administered with 50 ffu influenza strain A (PR8, H1N1) virus under light anaesthesia. Body weights were measured daily and at day 8 post infection, lungs were excised from the thoracic cavity and viral titres were determined in homogenized tissue by focus forming unit assay. **(B)** Result shows viral titer in individual mice and median value from two independent experiments. **(C)** Mean ± SEM % body weight loss from the original weight (n=9-11).

To further understand the role of L-selectin on the surface of activated $CD8^+$ T cells in conferring anti-viral immunity, and to dissect the strength of $CD8^+$ T cells involvement in clearing influenza A virus infection, first, L-$sel^{-/-}$, B6 and LΔP mice were challenged i.n. with a less virulent form of Influenza A virus (H3N2, H17). At day 8 post infection, lungs were harvested for viral titer. Interestingly, both B6 and LΔP mice showed enhanced viral clearance when compared with L-$sel^{-/-}$ mice. Whereas constant expression of L-selectin on the surface of activated $CD8^+$ T cells in LΔP mice did not have any impact on the viral clearance compared to the regulated expression of L-selectin in wildtype mice (Fig. 4.11).

Second, $RAG2^{-/-}$ mice, which is chronically lacking T and B cells, were replenished with purified $CD8^+$ T cells from F5-L-$sel^{-/-}$, F5B6 or F5LΔP mice. 24 hours later, mice were i.n. challenged with influenza A Virus (H3N2, H17). At day 7 post infection, lungs were harvested for viral titer. In similar consistency with previous studies, which shows that, the influenza virus virulence determines the necessity of various adaptive immune system compartments. $RAG2^{-/-}$ mice received F5LΔP showed enhanced viral clearance when compared with F5B6 and F5L-$sel^{-/-}$.

Furthermore, to confirm the L-selectin dependent homing of activated $CD8^+$ T cells and their involvement in the clearance of influenza A virus, F5L-$sel^{-/-}$, F5B6 and F5LΔP $CD8^+$ T cells were activated in vitro in the presence of NP68 pulsed irradiated APCs for 7 days. Meanwhile, $RAG2^{-/-}$ mice were challenged i.n. with influenza A virus (H3N2, H17) and 2 hours post infection, d7 CTL from various groups were instilled i.n., 5 days post T cells transfer, lungs were harvested for viral titer. Interestingly, all 3 groups with localized activated $CD8^+$ T cells in the lung were able to provide protection against influenza virus compared with the control group with no T cell transfer (Fig. 4.12).

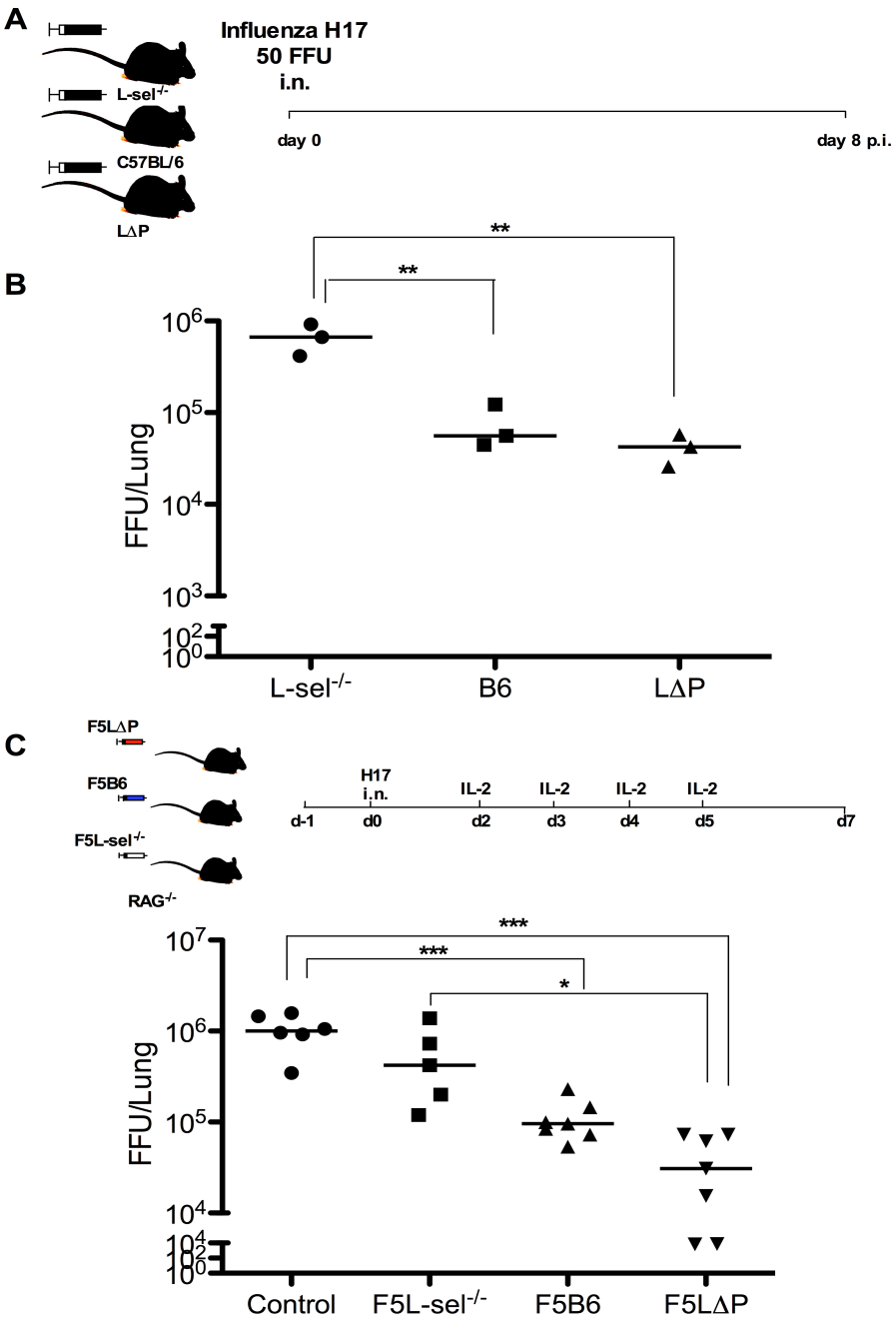


Fig 4.11: level of L-selectin on the surface of CD8$^+$ T cells determine the strength of anti-viral protection against influenza A virus infection. (A) Naïve L-sel$^{-/-}$, B6 and LΔP mice were administered with 50 ffu influenza strain A (H17, H3N2) virus under light anaesthesia. At day 8 post infection, viral titres were determined in homogenized tissue by focus forming unit assay. **(B)** RAG$^{-/-}$ mice were adoptively transferred with naïve F5LΔP, F5B6 and F5L-sel$^{-/-}$ CD8$^+$ T cells. 24 hours later, mice were administered with 50 ffu influenza strain A (H17, H3N2) virus under light anaesthesia. Mice were injected with hrIL-2 from day 2-5. Viral titers were determined at day 7 post infection. Data presented as median and each symbol represents a single mouse. (Oneway ANOVA with post Tukey's test). .

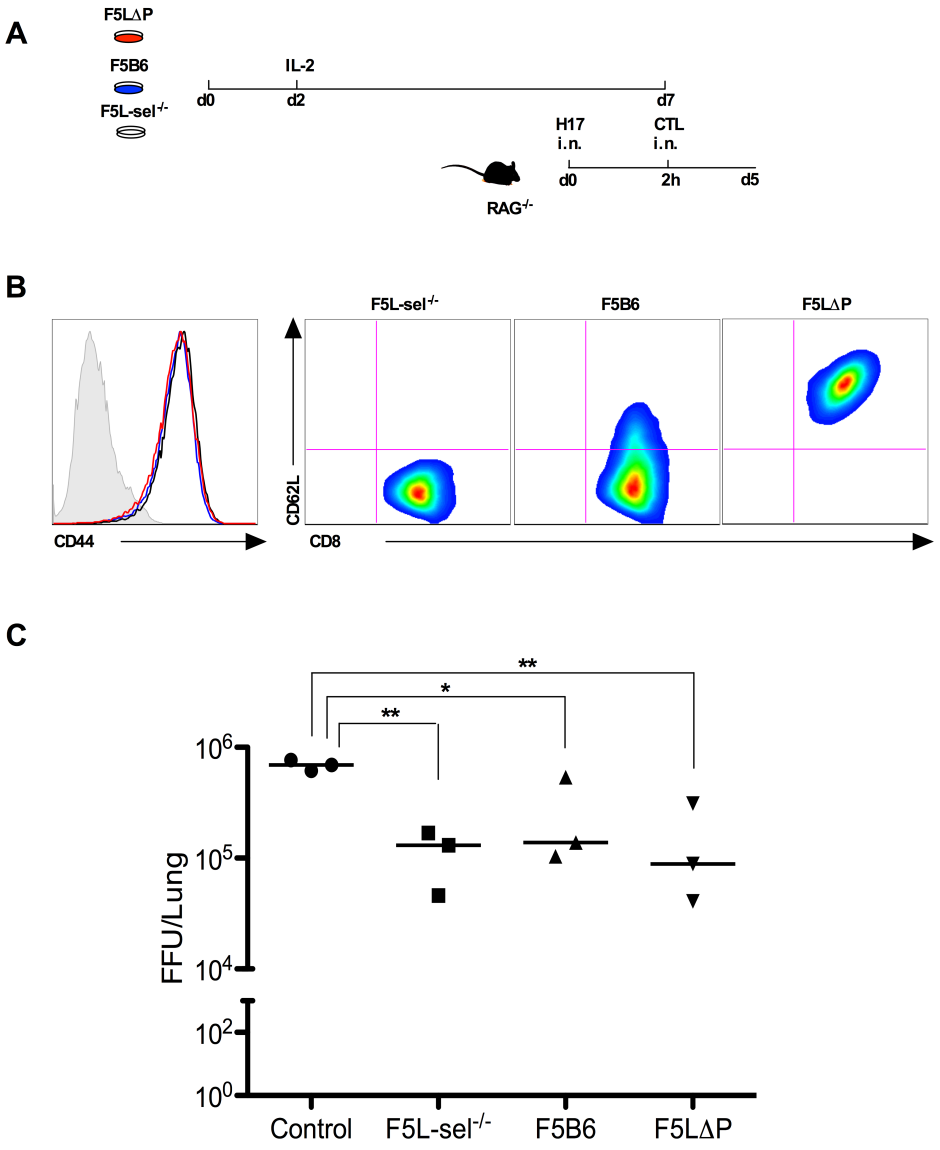


Fig 4.12: L-selectin does not affect activation and differentiation of virus specfifc CD8$^+$ T cells during influenza A virus infection. (A) naïve F5LΔP, F5B6 and F5L-sel$^{-/-}$ CD8$^+$ T cells were activated in vitro with NP68 peptide pulsed irradiated APC fro 7 days in the presence of IL-2. Meanwhile, RAG$^{-/-}$ mice were infected with 50 ffu influenza strain A virus (H17, H3N2). 2 hours post infection; mice were instilled with d7 in vitro activated F5LΔP, F5B6 and F5L-sel$^{-/-}$ CD8$^+$ T cells. Viral titers were determined at day 5 post infection by focus forming unit assay. **(B)** representative overlaid histogram shows CD44 expression and representative bivariate plots show the level of L-selectin on d7 in vitro activated F5LΔP, F5B6 and F5L-sel$^{-/-}$ CD8$^+$ T cells prior to transfer. **(C)** viral titer in RAG$^{-/-}$ mice transferred with F5LΔP, F5B6 and F5L-sel$^{-/-}$ CD8$^+$ T cells. Data presented as median and each symbol represents a single mouse. Oneway ANOVA with post Tukey's test.

Chapter Five

5. The role of L-selectin in $CD8^+$ T cell co-stimulation

5.1. Introduction

During the course of a primary viral infection, naïve $CD8^+$ T cells, which have a cognate TCR for the virus, have to differentiate and proliferate to generate enough effector cells to specifically fight the virus infection. $CD8^+$ T cells also need to undergo these steps and to generate a good pool of memory cells to protect the body against secondary challenges by the same virus. A number of molecules have been implicated in $CD8^+$ T cell co-stimulation during activation. OX40 expressed by $CD8^+$ T cells is an important molecule for co-stimulation with regards to expansion and anti-viral cytokine production during vaccinia virus infection (Salek-Ardakani et al. 2008).

Co-stimulation is necessary for $CD8^+$ T cells to be able to fight against viral infection or to sustain effector functions against antigens including those presented by tumors. The presence of Ag, antigen presenting cells and CD28 co-stimulation is critical for $CD8^+$ T cell effector function not only early during $CD8^+$ T cell priming but also during the entire effector phase (Dolfi et al. 2011). Therefore variations in L-selectin expression during $CD8^+$ T cell differentiation to effector cells could provide different levels of co-stimulation supporting anti-viral cytokine secretion and degranulation. L-selectin signaling in neutrophils is critical during adhesion and degranulation impacting on neutrophil shape change, adhesion capacity and p38 mitogen-activated protein kinase (MAPK) dependent production of granules (Smolen et al. 2000).

L-selectin expression is tightly regulated on the surface of $CD8^+$ T cells by ectdomain shedding and gene silencing (Sinclair et al. 2008). Although it has

been shown that L-selectin is an obligate lymph node homing receptor for $CD8^+$ T cells (Arbornes et al. 1994), L-selectin, engagement with its ligand, either cell-associated or soluble, in order to perform its homing function, provides a co-stimulatory signal for $CD8^+$ T cells (Nishijima et al. 2005). L-selectin binding with its ligand also provide intracellular signals for actin cytoskeleton rearrangement and enhancement of integrin-dependent binding to HEV and extracellular matrix components (Wedepohl et al. 2012). Also, L-selectin is shed by a metalloproteinase such as ADAM-17 very early during $CD8^+$ T cell activation, generating a cytoplasmic tail which could be processed further and contribute to $CD8^+$ T cell co-stimulation.

L-selectin could also provide a co-stimulus for $CD8^+$ T cell during their activation, differentiation and cytokine secretion, since engaging with a ligand, stimulates a series of signaling pathway that support $CD8^+$ T cell co-stimulation.

5.2. Hypothesis

$CD8^+$ T cell activation through TCR and L-selectin engagement by its ligand leads to a series of downstream signaling, which co-stimulates $CD8^+$ T cells. We hypothesized that if $CD8^+$ T cells express non-cleavable L-selectin, the signaling will last longer and strengthen co-stimulation.

5.3. Results

5.3.1. Constant L-selectin expression does not enhance priming and activation of $CD8^+$ T cells

To determine whether constant expression of a non-cleavable form of L-selectin strengthens or accelerates $CD8^+$ T cell priming and activation, naïve $CD8^+$ T cells co-expressing F5 TCR specific for NP68 peptide and either wildtype L-selectin (F5/B6) or mutant L-selectin (F5/LΔP) were isolated and cultured in the presence of NP68 peptide pulsed irradiated antigen presenting cells (APCs).

First, to confirm the L-selectin expression status of the F5/B6 and F5/LΔP $CD8^+$ T cells, the kinetics of L-selectin expression were analyzed at 0, 1, 4 and 24 hours post activation *in vitro*. Prior to the cells going into the culture (0h), both F5B6 and F5/LΔP $CD8^+$ T cells were fully L-selectin positive. At 1 and 4 hours post activation, F5B6 $CD8^+$ T cell down-regulated L-selectin expression. Later at 24 hours post activation, F5B6 $CD8^+$ T cells started to re-express L-selectin. This was consistent with the study by Chao et al., 1997, in which they have shown that wildtype L-selectin undergoes ectodomain proteolytic cleavage and re-expression 24-48 hours after TCR engagement. Whereas, L-selectin on the surface of F5/LΔP $CD8^+$ T cells was constantly expressed at 1, 4 and 24 hours post $CD8^+$ T cell activation (Fig. 5.1). Second, in order to test the hypothesis, F5/B6 and F5/LΔP $CD8^+$ T cells, at 0, 1, 4 and 24 hours post activation in vitro, were analyzed for activation and priming. Both F5/B6 and F5/LΔP $CD8^+$ T cells upregulated CD69 in a similar manner and with similar kinetics. This finding suggests that maintained expression of L-selectin did not affect early $CD8^+$ T cell activation (Fig. 5.2).

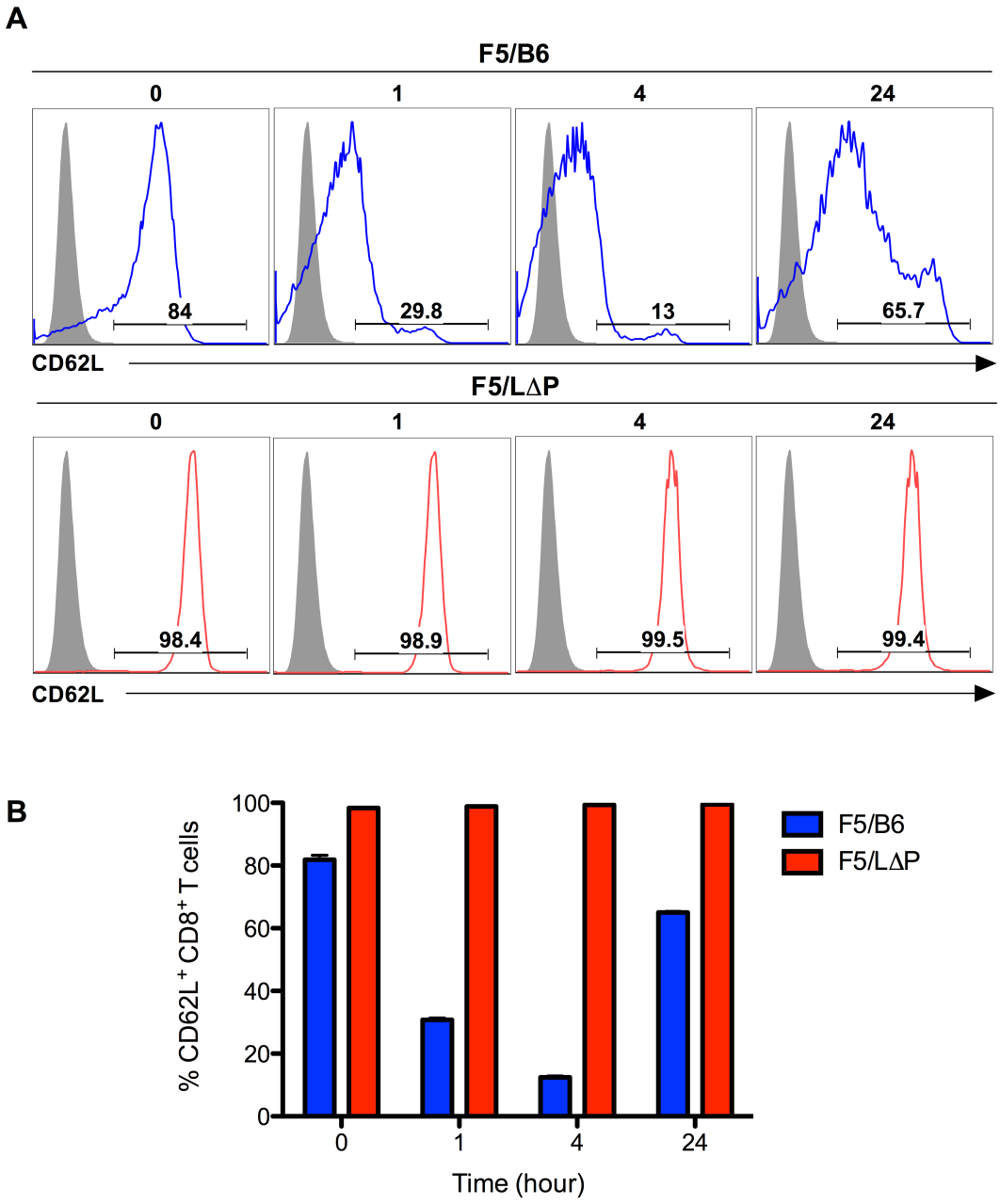


Figure 5.1: Cyclical Expression of L-selectin during CD8$^+$ T cell activation in vitro. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-selectin F5/LΔP were isolated and cultured *in vitro* in the presence of NP68 peptide pulsed irradiated APCs, and at 0, 1, 4 and 24 hours post activation, cells were harvested and stained for CD8 and CD62L. Representative histograms show % CD62L$^+$ from F5/B6 (blue) and F5/LΔP (red) CD8$^+$ T cells, the % positives were gated based on the CD62L FMO (grey) histogram. **(B)** Bar chat shows mean ± SEM (n=3).

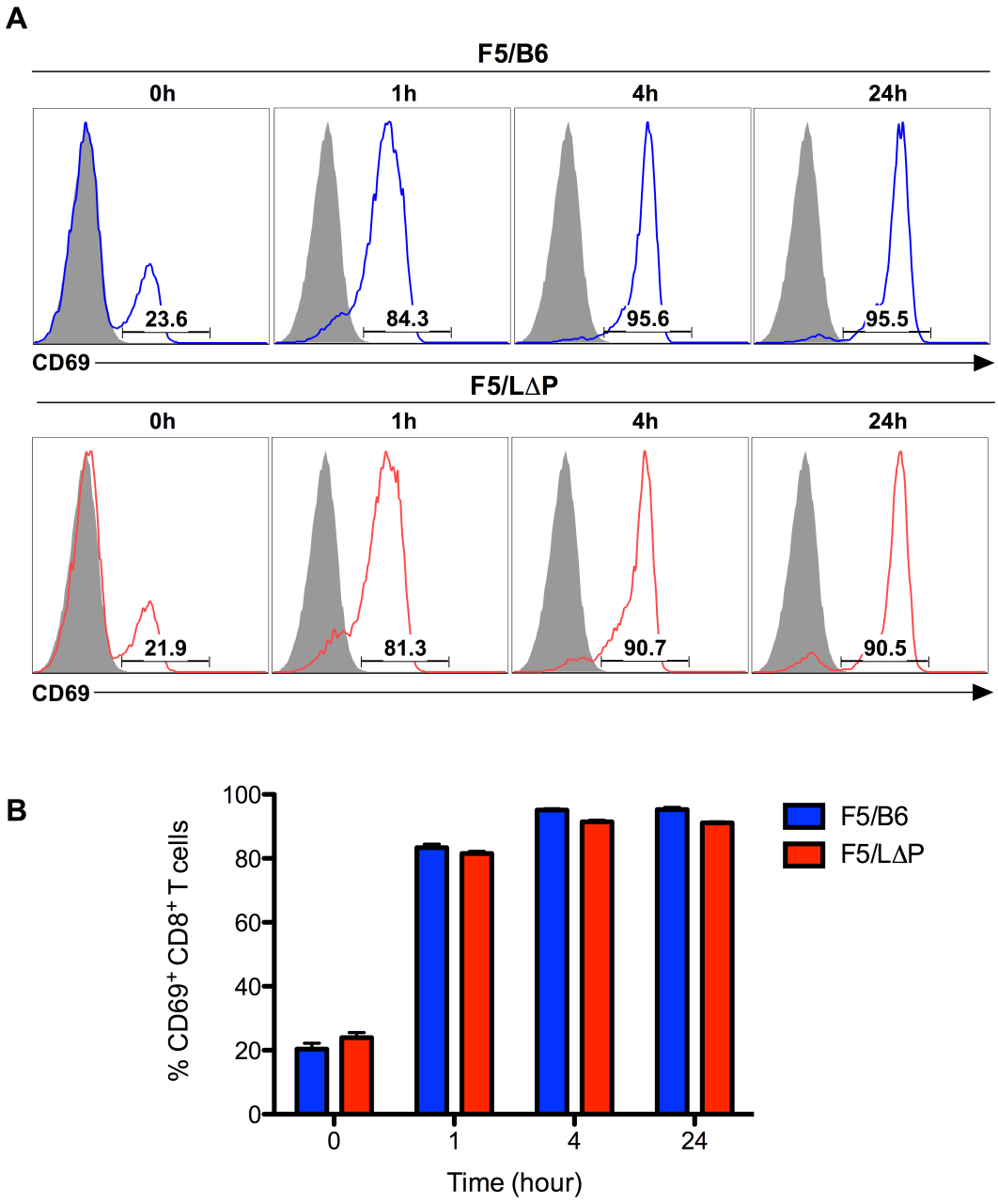


Figure 5.2: CD8$^+$ T cell activation is not affected by various level of L-selectin expression (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-selectin F5/LΔP were isolated and cultured *in vitro* in the presence of NP68 peptide pulsed irradiated antigen presenting cells (APC) at 1, 4 and 24 hours post activation, cells were harvested and stained for CD8 and CD69. Representative histograms show %CD69$^+$ of F5/B6 (blue) and F5/LΔP (red) CD8$^+$ T cells, the % positives were gated based on the CD69 FMO (grey) histogram. **(B)** Bar chart shows mean ± SEM (n=3).

5.3.2. L-selectin co-stimulates early proliferation of $CD8^+$ T cells

To determine whether mutant L-selectin enhances co-stimulation, naïve F5/B6 and F5/LΔP $CD8^+$ T cells were isolated, labeled with the dilution sensitive dye (CFSE) to assess their proliferation ability. After incubation with CFSE, $CD8^+$ T cells were cultured in the presence of peptide pulsed APCs and IL-2 for 6 days (Fig 5.3). Similar to the findings *in vivo*, $CD8^+$ T cells expressing wildtype L-selectin initiated the proliferation earlier than mutant L-selectin expressing $CD8^+$ T cells. Earlier entry into the proliferation cycle was indicated by the significant difference in division indices between F5/B6 and F5/LΔP $CD8^+$ T cells at day 4 post activation, however, the effect was transient since both F5/B6 and F5/LΔP $CD8^+$ T cells had similar division indices at day 5 post activation, illustrating that both cell types were proliferating at a similar rate (Fig. 5.3).

Cytokines such as IL-2 are pivotal for the clonal expansion of $CD8^+$ T cells. $CD8^+$ T cells receive signals from the IL-2 receptor (IL-2R) upon engagement with IL-2 in order to induce further cytokine secretion and differentiation into cytotoxic effector cells (Cheng et al. 2002). IL-2R is a heterotrimer composed of a 3 unique chains, α-chain (CD25), β-chain (CD122) and γ-chain (CD132). IL-2 and IL-15 share the β-chain, and IL-2, IL-4, IL-7 and IL-15 share the γ-chain (Nelson & Willerford 1998). Since IL-2R α-chain is unique among the IL-2R binding to IL-2 cytokine, we tested the expression of the IL-2R α-chain (CD25) by F5/B6 and F5/LΔP $CD8^+$ T cells following activation *in vitro*. Earlier entry into proliferation cycle of wildtype L-selectin expressing $CD8^+$ T cells was accompanied by an increased up-regulation of CD25 (IL2Rα), which is an important factor in the initiation of proliferation of $CD8^+$ T cells (Fig 5.4).

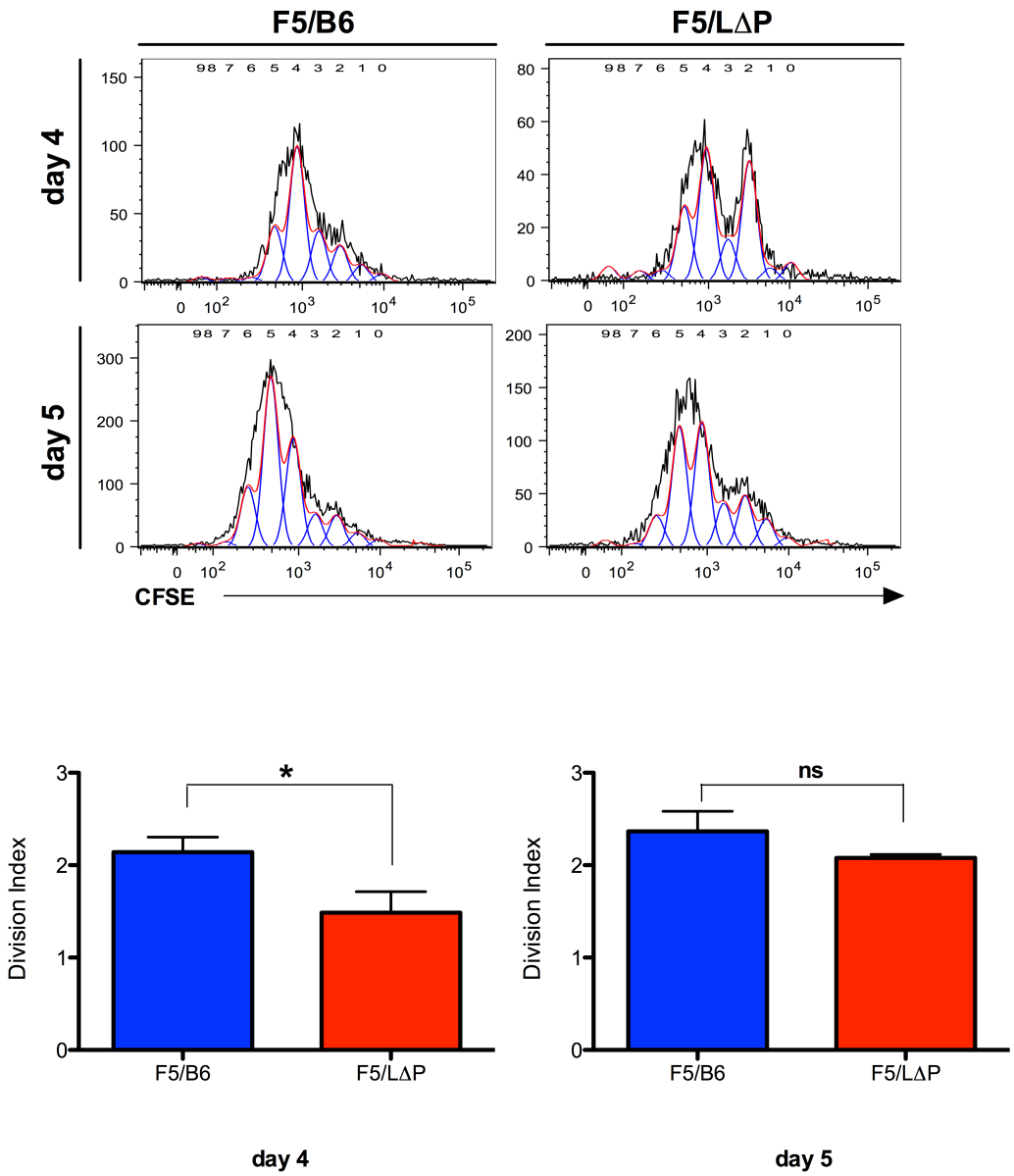


Figure 5.3: Upon TCR activation, wildtype L-selectin expressing CD8$^+$ T cells initiate proliferation earlier than mutant L-selectin expressing cell. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-selectin F5/LΔP were isolated and stained with 2μM CFSE. Later, cells were cultured *in vitro* in the presence of NP68 peptide pulsed irradiated APCs and IL-2. Representative histograms show the CFSE dilution of F5/B6 and F5/LΔP CD8$^+$ T cells at day 4 and 5 post activation. **(B)** Bar charts show the division index of F5/B6 and F5/LΔP CD8$^+$ T cells at day 4 and 5 during *in vitro* activation, division index analyzed by flow jo software proliferation analysis tab. Result is from two independent experiments (n=3-6). * P <0.05, unpaired student t-test.

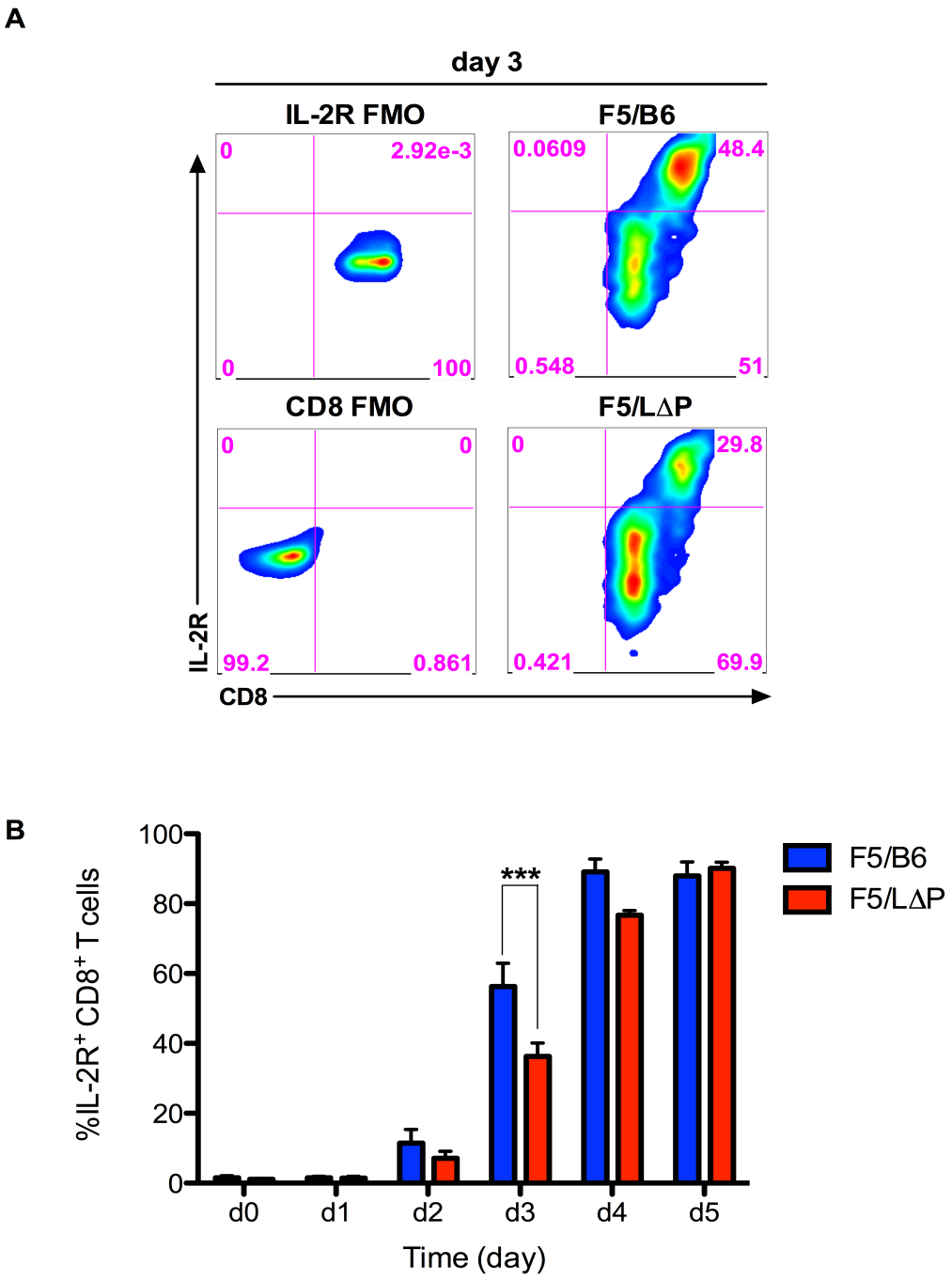


Figure 5.4: Wildtype L-selectin supports increased IL-2R up-regulation, which enhances entry into proliferation cycle. Naïve $CD8^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-selectin F5/LΔP were isolated and cultured *in vitro* in the presence of NP68 peptide pulsed irradiated APCs and IL-2. Representative bivariate flow cytometry plots show the % $IL\text{-}2R^+$ (CD25) by F5/B6 and F5/LΔP $CD8^+$ T cells at day 3 post activation in the culture compared to their FMO (CD8 FMO and CD25 FMO). **(B)** Bar chart shows the % $IL\text{-}2R^+$ by F5/B6 and F5/LΔP $CD8^+$ T cells during 5 day culture. Results are means ± SEM (n=3)(One-Way ANOVA with post Tukey's test).

5.3.3. Mutant and wildtype L-selectin expressing CD8$^+$ T cells differentiate similarly into effector cells.

Classically, L-selectin is used as a marker of activation. L-selectin, in combination with other activation antigens such as CD44, is used to distinguish naïve CD8$^+$ T cells and their differentiated forms of effector and memory populations. In our study, because the role of L-selectin was interested and wildtype L-selectin has been compared with a mutant form of L-selectin, in which L-selectin does not undergo shedding and gene silencing, an alternative means to identify effector and memory subsets of F5/B6 and F5/LΔP CD8$^+$ T cells was required.

To study the impact of mutant L-selectin on the differentiation of CD8$^+$ T cells into effector cells, naïve F5/B6 and F5/LΔP CD8$^+$ T cells were purified and cultured in the presence of NP68 peptide pulsed irradiated APCs and IL-2 for 7 days. Up-regulations of late activation antigen CD44 in combination with expression of chemokine receptor 7 (CCR7) was used. Both naïve F5/B6 and F5/LΔP CD8$^+$ T cells expressed low level of CD44 and high level of CCR7. F5/B6 and F5/LΔP CD8$^+$ T cells activation status was measured by the up-regulation of CD44 and down-regulation of CCR7. At days 6 and 8 post activation, there were no statistical significant difference in the percentage of CD44^{hi}CCR7^{low} effector subset between F5/B6 and F5/LΔP CD8$^+$ T cells (Fig. 5.6). This finding indicates that the presence of mutant L-selectin on the surface of CD8$^+$ T cells does not affect the generation of effector and memory subsets of CD8$^+$ T cells upon TCR activation. In order to determine the role of L-selectin in activation and differentiation of CD8$^+$ T cells, wildtype L-selectin can be compared with an L-selectin knock out CD8$^+$ T cells to precisely determine the effect of L-selectin.

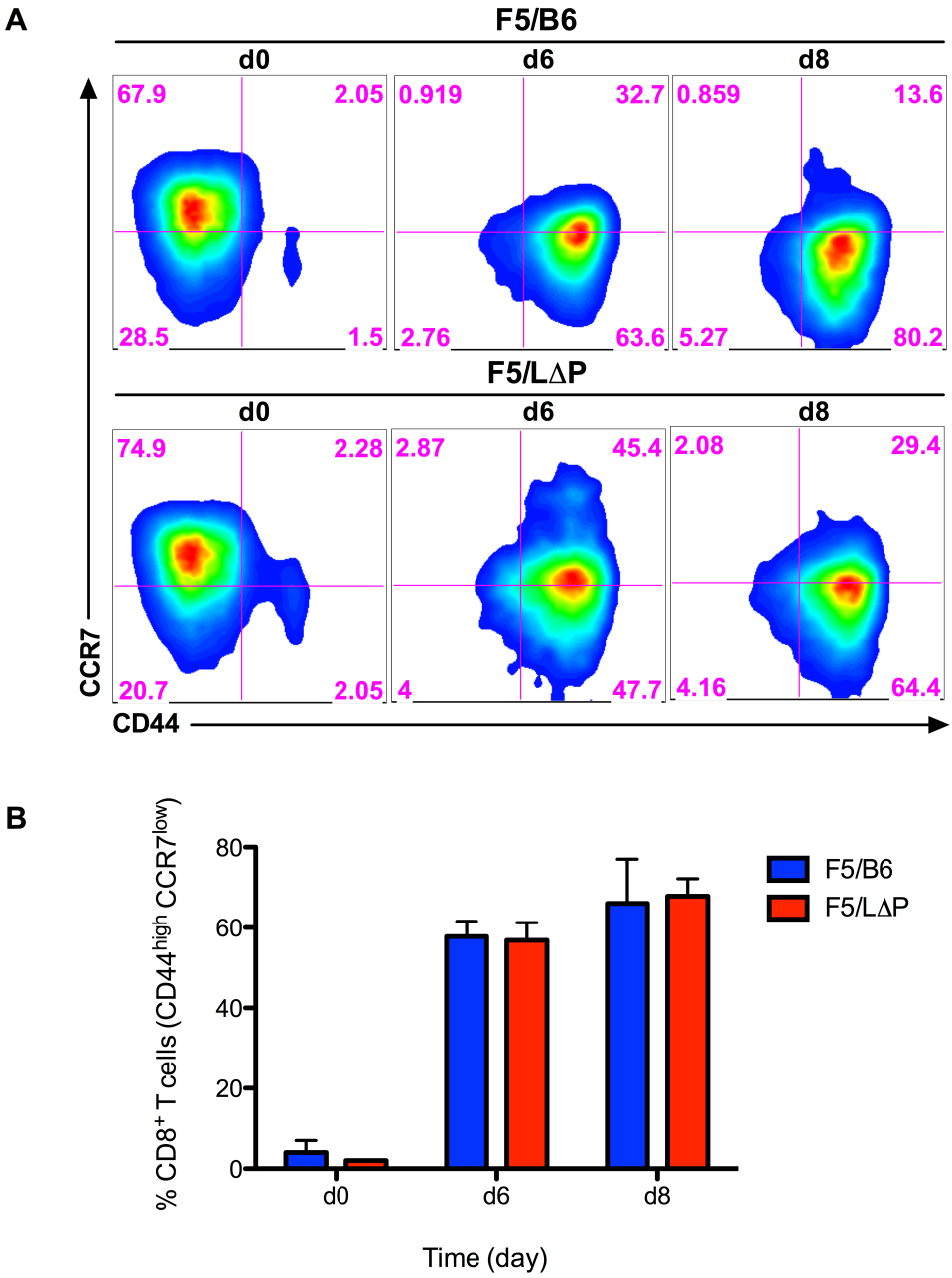


Figure 5.6: Mutant L-selectin does not impact on effector CD8$^+$ T cell differentiation. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-selectin F5/LΔP were isolated and cultured *in vitro* in the presence of NP68 peptide pulsed irradiated APCs and IL-2. Representative bivariate flow cytometry plots show naïve F5/B6 and F5/LΔP CD8$^+$ T cells at day 0, which express low level of CD44 and high CCR7, and differentiated effector CD8$^+$ T cells at day 6 and 7, which express high CD44 and low CCR7. **(C)** Bar char shows % CD44 high CCR7 low for F5/B6 and F5/LΔP CD8$^+$ T cells at various time points during in vitro activation. Results are means ± SEM from two independent experiment (n=4).

5.3.4. Mutant L-selectin does not affects $CD8^+$ T cell's ability to secrete various cytokines.

Various factors impact on $CD8^+$ T cells differentiation into effector cytotoxic cells. L-selectin can co-stimulate $CD8^+$ T cells and could impact on cytokine secretion by activated $CD8^+$ T cells. To determine whether mutant L-selectin impacts $CD8^+$ T cell differentiation, naïve F5/B6 and F5/LΔP $CD8^+$ T cells were activated *in vitro* for 8 days. Activated $CD8^+$ T cells at the indicated times (days 6 and 8) were re-stimulated with cognate peptide for 4 hours in the presence of brefeldin A. Re-stimulated F5/B6 and F5/LΔP activated $CD8^+$ T cells at day 6 post activation secreted similar levels of IL-2, TNF-α and IFN-γ (Fig. 5.7). F5/B6 and F5/LΔP activated $CD8^+$ T cells also secreted similar levels of the above cytokines at day 8 post activation (Fig. 5.8).

Immune cells secrete cytokines in order to communicate with each other, for their survival and also to lyse pathogen infected cells. For example, effector $CD4^+$ T helper 1 cells secrete interferon-γ to activate macrophages (Sher & Coffman 1992). Effector $CD4^+$ and $CD8^+$ T cells secrete IL-2 for their expansion and survival (Cheng et al. 2002) and effector $CD8^+$ T cells mainly secrete cytokines such as tumor necrosis factor-α and IFN-γ to directly eliminate invading pathogens. This finding indicates that mutant L-selectin did not affect the secretion of various cytotoxic chemical mediators necessary for activated $CD8^+$ T cells to function during an immune response.

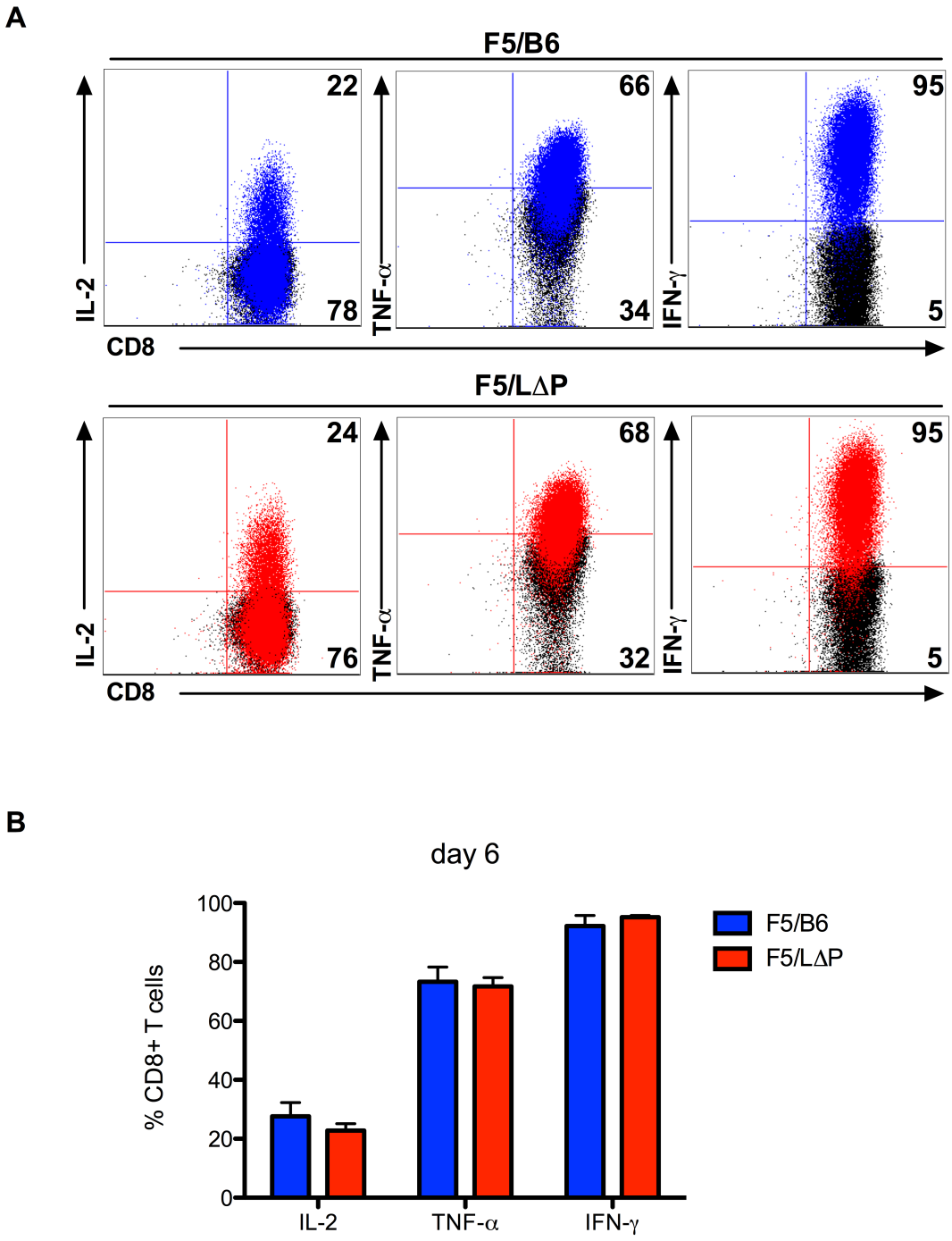
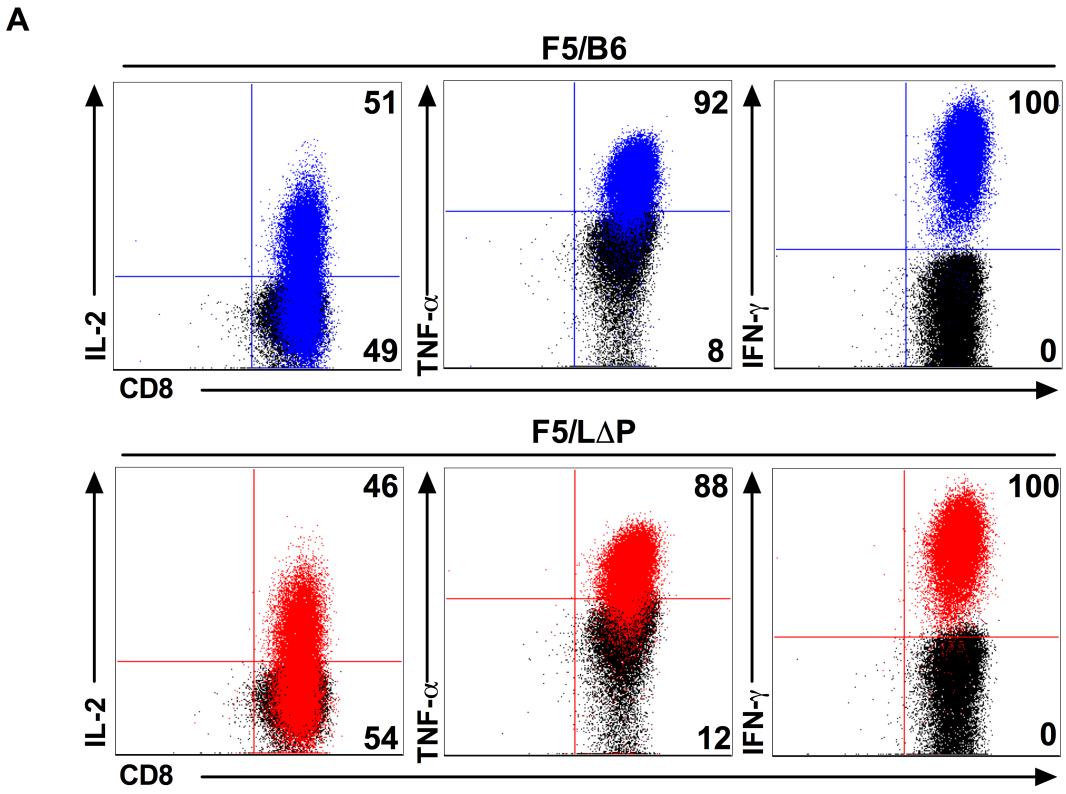


Figure 5.7: L-selectin does not enhance cytokine secretion by early effector CD8$^+$ T cells. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 (blue) or mutant L-selectin F5/LΔP (red) were isolated and cultured *in vitro* in the presence of NP68 peptide pulsed irradiated APCs and IL-2. At day 6 post activation, cells were re-stimulated with NP68 peptide in the presence of Brefeldin A for 4 hours, and then stained for CD8 and intracellularly for IL-2, TNF-α and IFN-γ. Representative bivariate flow cytometry plots show a similar level of cytokine secretion by F5/B6 and F5/LΔP CD8$^+$ T cells at day 6 post activation upon re-stimulation. **(C)** Bar chart show means± SEM (n=3).



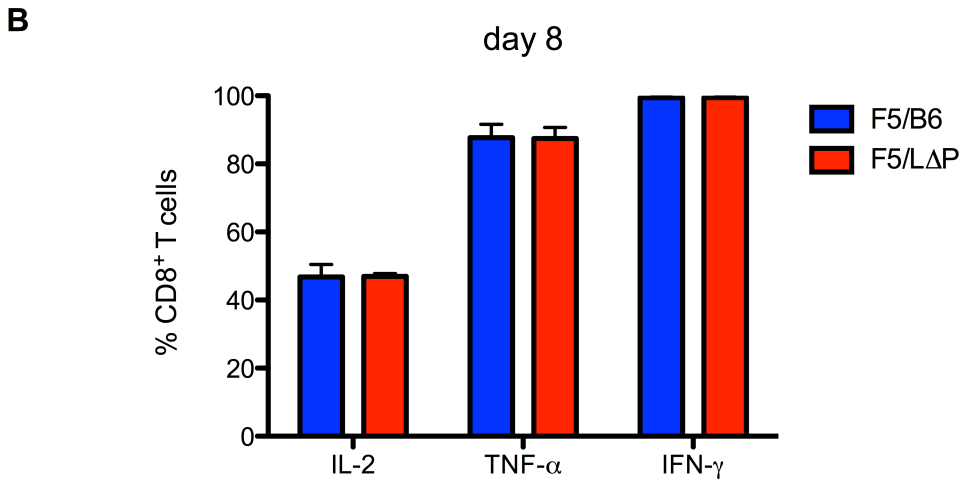


Figure 5.8: L-selectin does not enhance cytokine secretion by late effector CD8$^+$ T cells. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 (blue) or mutant L-selectin F5/LΔP (red) were isolated and cultured *in vitro* in the presence of NP68 peptide pulsed irradiated APCs and IL-2. At day 8 post activation, cells were re-stimulated with NP68 peptide in the presence of Brefeldin A for 4 hours, and then stained for CD8 and intracellularly for IL-2, TNF-α and IFN-γ. Representative bivariate flow cytometry plots show a similar level of cytokine secretion by F5/B6 and F5/LΔP CD8$^+$ T cells at day 8 post activation upon re-stimulation. **(C)** Bar char shows mean ± SEM (n=3).

Although F5/B6 and F5/LΔP $CD8^+$ T cells secreted similar levels of individual cytokines at different time points during activation *in vitro,* the ability of a single cell to secrete all three cytokines and adopt a polyfunctional status, which has been correlated with protective immunity to invading pathogens (Precopio et al. 2007), was acquired during prolonged culture. An increase in the percentage of F5/B6 cells that secrete all the three cytokines (+++) (IFN-γ, TNF-α and IL-2) was seen between days 6 and 8 with concomitant reductions in the proportions of cells that secrete two (++)(IFN-γ and TNF-α) or only one cytokine (+)(IFN-γ) (Fig. 5.9). Also, increase in the proportion of (+++) and reduction of (++) and (+) cytokine secreting F5/LΔP $CD8^+$ T cells were similar to F5/B6 cells. Time is a critical requirement for the cellular arm of the adaptive immune system to develop and generate a strength response against invading pathogens.

5.3.5. L-selectin does not impact the degranulation of $CD8^+$ T cells upon activation

Granules are key characteristics of cytotoxic T cell. To examine whether mutant L-selectin has any effect on the level of degranulation, F5/B6 and F5/LΔP $CD8^+$ T cells were cultured for either 6 or 8 days as previously described. They were then analyzed for cell surface expression of LAMP-1 (CD107a), which is a lysosomal associated glycoprotein within the cytotoxic granule membrane and can be used as an indicator of the ability of $CD8^+$ T cells to degranulate upon activation. F5/B6 and F5/LΔP $CD8^+$ T cells de-granulated at similar levels upon re-stimulation on day 6 and day 8 post culture (Fig. 5.10).

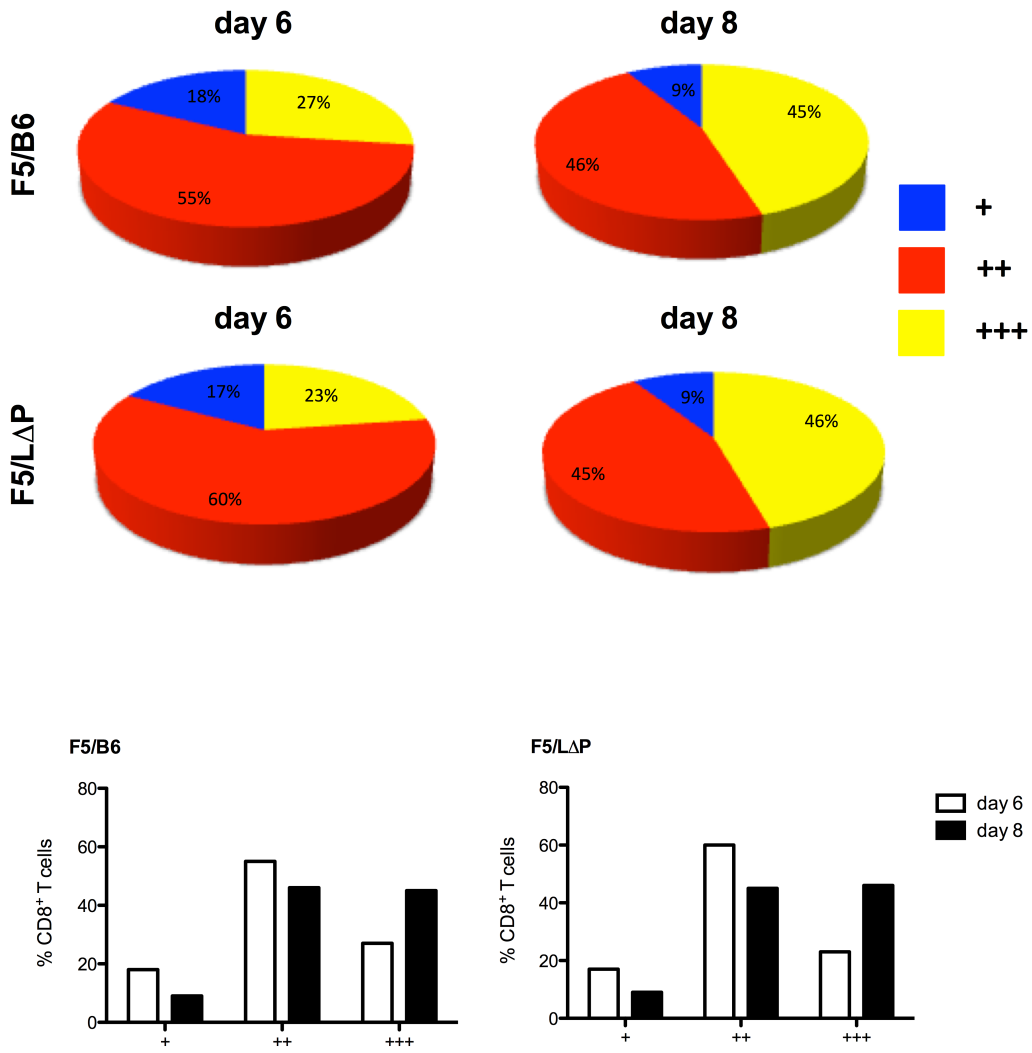


Figure 5.9: Mutant L-selectin does not affect the development of cytotoxic CD8$^+$ T cells. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-selectin F5/LΔP were isolated and cultured *in vitro* in the presence of NP68 peptide pulsed irradiated APCs and IL-2. At days 6 and 8 post activation, cells were re-stimulated with NP68 peptide in the presence of Brefeldin A for 4 hours, and then stained for CD8 and intracellularly for IL-2, TNF-α and IFN-γ. Pie charts show the % triple positive (+++; IFN-γ, TNF-α, IL-2), double positive (++; IFN-γ, TNF-α) from F5/B6 and F5/LΔP CD8$^+$ T cells at days 6 and 8 post activation in vitro and re-stimulation. **(B)** Bar charts show the difference between day 6 and day 8 activated and re-stimulated CD8$^+$ T cells from either F5/B6 or F5/LΔP CD8$^+$ T cells in the % of single, double and triple positive. Results are mean ± SEM (n=3).

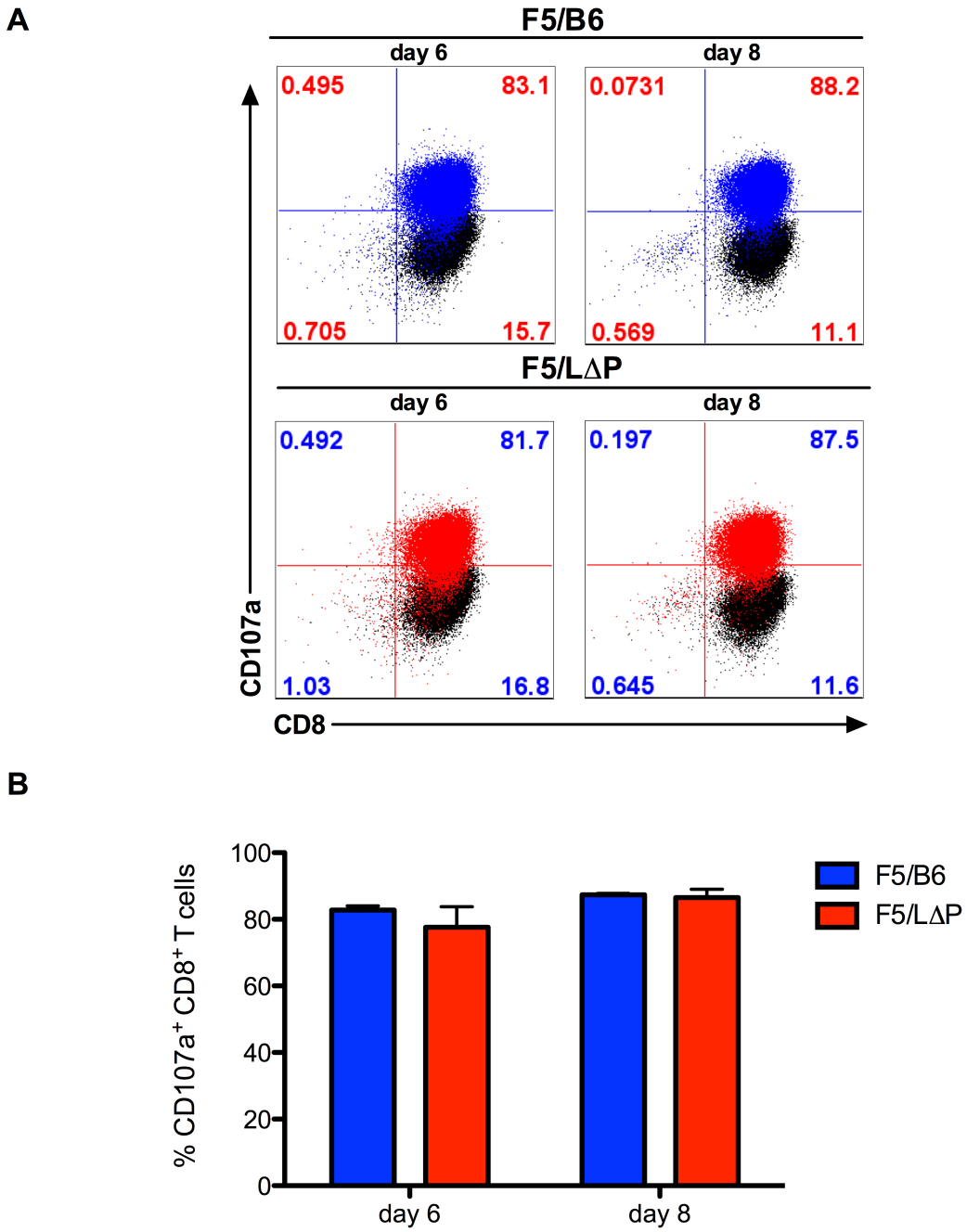


Figure 5.10: Mutant L-selectin does not affect effector CD8$^+$ T cell degranulation. (A) Naïve CD8$^+$ T cells co-expressing transgenic F5 TCR and either wildtype F5/B6 or mutant L-selectin F5/LΔP were isolated and cultured *in vitro* in the presence of NP68 peptide pulsed irradiated APCs and IL-2. At days 6 and 8 post activation, cells were re-stimulated with NP68 peptide and added with CD107a staining antibody. After 1 hour, cells were incubated with monensin and further re-stimulated for 4 hours. Cells then stained for CD8 as well. Representative bivariate flow cytometry plots show intracellular staining of F5/B6 and F5/LΔP CD8$^+$ T cells for CD107a at days 6 and 8 post activation in vitro and re-stimulation. (B) Bar chart show means ± SEM (n=3).

Chapter Six

6. Discussion

6.1. The role of L-selectin in homing of activated $CD8^+$ T cells to virus infected organs.

The human body can naturally defend itself against pathogens through its potent immune system. The functional diversity of the immune system originates from the specificity of the adaptive arm of the system. Upon entering the body, the virus, airborne, consumed or via skin, will be dealt with through the specialized antigen presenting cells (APCs) and particle of the virus will be presented on the surface in the context of either MHC class I or II molecules. Meanwhile, naïve $CD8^+$ T cell's continuous circulation through bloodstream and lymphatic in and out of secondary lymphoid tissues allow encounter between the APCs and the T cell in order for the T cell with cognate T cell receptor to activate and differentiate into cytotoxic $CD8^+$ T cells (CTL)(Goulding et al. 2014). At this stage, the CTL is required to migrate to infected organs in order to provide protective immunity via a direct contact with the infected cells.

The process of $CD8^+$ T cell migration into lymphoid organs for immune T cell surveillance and priming, in the presence of an antigen, and further migration into sites of infection involve a number of adhesion molecules, chemokine receptors and their ligands (Ager & May 2015).

Using recombinant vaccinia virus (vaccNP) expressing NP68 peptide (Townsend et al. 1986), which is recognized by F5 TCR, and influenza A virus allowed us to study the role of adhesion molecules L-selectin in the migration of virus specific $CD8^+$ T cells to sites of infection. It has been shown that parental Vaccinia virus

Western Reserve strain infects a range of visceral organs when the virus is inoculated intraperitoneally (Zhao et al. 2011), therefore, we determined the site of vaccNP infection first. Upon vaccNP infection i.p., the virus replicated only in the ovaries whereas, the virus in the heart, liver and lungs were below the level of detection when determined by vaccinia virus plaque assay. The difference between the parental and the recombinant form of the virus may result from the recombination process, which has been shown to reduce the pathogenicity and infectivity (Williamson et al. 1990). The preferential replication and infectivity of vaccNP in the ovaries of mice were consistent with a previous finding, which reported that vaccinia virus replicates in ovaries following intraperitoneal administration (Zhao et al. 2011).

The site of $CD8^+$ T cell priming during vaccNP infection was identified. As it has been shown previously in a research study, in which mediastinal LN serve as a primary site of $CD8^+$ T cells priming during lymphcytic choriomeningitis virus (LCMV) infection when the virus inoculated i.p. (Olson et al. 2012), the main site of antigen presentation to $CD8^+$ T cells during vaccNP infection was mostly restricted to Med LN. Med LN drains the site of influenza virus infection and influenza specific $CD8^+$ T cell activation and priming occurs in the Med LN (Flynn et al. 1998) allowed us to study the kinetics L-selectin expression and its contribution in virus specific $CD8^+$ T cell homing during two different virus infection with two distinct sites of replication and a site of priming to be investigated.

It is clear that lymphocyte migration involves a complex and well-orchestrated process. Manipulation of these pathways could lead to enhancement of T cell infiltration into sites of virus infection in which the virus clearance is $CD8^+$ T cell dependent. Various adhesion molecules and chemokine receptors have been

reported to play a role in homing of activated $CD8^+$ T cells into peripheral non-lymphoid tissues. The contribution of these molecules depends on the type and site of infection. For example, P and E selectins have been shown to play a role in homing of activated $CD8^+$ T cells into the skin during hypersensitivity reaction (Hirata et al. 2002). Whereas, P and E selectin double knockout mice showed equal viral clearance in internal organs such as liver, lung and spleen during LCMV infection, which suggests that P and E selectins are not required for migration of activated $CD8^+$ T cells to sites of virus infection (Bartholdy et al. 2000). Chemokines such as CXCL12 secreted by newly infiltrated neutrophils play a prominent role in the migration of influenza specific $CD8^+$ T cells into the trachea (Lim et al. 2015).

The current dogma in immunology suggests that $CD8^+$ T cells only utilize L-selectin molecules on their surface to enter the peripheral lymph nodes for priming. L-selectin will be shed on the surface soon after $CD8^+$ T cell activation via ectodomain proteolytic shedding and later, L-selectin is not thought to require for homing into sites of infection such as viruses, since virus specific $CD8^+$ T cells, harvested from sites of infection late during the primary course of infection, expressed low level of L-selectin.

In this study, we were able to map the cyclical expression of L-selectin during virus specific $CD8^+$ T cell activation and differentiation *in vivo* during infection with two evolutionary distinct viruses, vaccinia and influenza virus which infect visceral and mucosal organs, respectively. L-selectin reversibly cycles between high and low expression during the course of a primary virus infection. L-selectin cyclical expression occurred when the virus specific $CD8^+$ T cells are in different locations of the body. L-selectin expression was reduced when the $CD8^+$ T cells were resident in the draining lymph node for priming during vaccNP and influenza A

virus infection. Re-expression of L-selectin occurred when the cells were still in the LN, virus specific $CD8^+$ T cells left the LN entered peripheral blood circulation started to lose their L-selectin again. The first population of virus specific $CD8^+$ T cells enriched the sites of virus infection, expressed a significant amount of L-selectin on their surface and later, L-selectin was fully downregulated. The timing of cyclical expression of L-selectin during virus specific $CD8^+$ T cell activation and differentiation *in vivo* in this study were almost comparable with what have been reported *in vitro* which mirrors the L-selectin early ectodomain shedding, re-expression and later gene silencing (Chao et al. 1997).

Since virus specific $CD8^+$ T cells expressed a significant amount of L-selectin when they enter the sites of virus infection, mechanisms to maintain L-selectin on the surface of virus specific $CD8^+$ T cells might play a significant role in the enhancement of T cell migration to sites of infection. A number of interventions are possible to maintain L-selectin on the surface, such as blocking A metalloproteinase and Disintigrins (ADAMs) responsible for L-selectin ectodomain shedding and proteolysis.

Other mechanisms that maintain L-selectin expression on the surface is through manipulating L-selectin gene silencing through mTOR and PI3K inhibiting the action of KLF2 responsible for transcription of L-selectin gene (Sinclair et al. 2008) intervention or using a mutant L-selectin transgene in which the ectodomain cleavage site was replaced with the one from P-selectin which is no longer vulnerable to shedding and expression controlled by human CD2 (hcd2) promoter which resists gene silencing (Galkina et al. 2007; Richards et al. 2008).

When the hypothesis was tested maintained expression of L-selectin (LΔP) on the surface of virus specific $CD8^+$ T cells showed enhanced homing ability into

vaccNP-infected ovaries at days 5 (5-fold) and 8 (10-fold) post infection when compared to its wildtype counterpart. The enhancement in homing was not due to retention of the virus specific $CD8^+$ T cells expressing LΔP L-selectin since their numbers contracted at day 12 post infection. The role of re-expressed wildtype L-selectin in homing of virus specific $CD8^+$ T cells into site of infection was confirmed by comparing wildtype L-selectin expressing $CD8^+$ T cells with L-selectin knockout (L-$sel^{-/-}$) cells. During a 5-day primary course of vaccinia virus infection, L-$sel^{-/-}$ cells showed impaired homing into the site of vaccNP infection (ovary) at day 5 post infection but not into spleen, since L-selectin is not a dominant homing molecule for leukocyte homing into the spleen (Arbornes et al. 1994).

Furthermore, when LΔP mice were challenged with influenza A virus, the number of endogenously generated virus specific $CD8^+$ T cells were 2-fold higher than wildtype mice in the lungs 8 days post infection but not in the spleen. This finding was also consistent with the finding reported for LΔP mice during vaccNP infection that there was an increased number of endogenously generated vaccNP specific $CD8^+$ T cells in the ovaries at day 8 post infection (Richards et al. 2008).

Cell surface molecules can impact on the activation and differentiation of $CD8^+$ T cells upon activation through its T cell receptor. For example, the signalling receptor, Notch, play a fundamental role in T cell differentiation (Backer et al. 2014). Therefore, we tested whether constant expression of L-selectin on the surface of mutant L-selectin expressing $CD8^+$ T cells affects the way these cells activate and differentiate into effector cells and, therefore, impact on the bulk of the cells generated. Having an increased number of cells generated during activation and differentiation stage could account for an increased end number of $CD8^+$ T cells entering the sites of infection.

The early activation antigen, CD69, has been shown to be upregulated upon $CD8^+$ T cell priming by antigen presenting cells (Alari-pahissa et al. 2012). CD69 play an important role in holding the $CD8^+$ T cells in the lymph node in order to receive the necessary signals and undergo activation and proliferation (Schenkel 2014). The kinetics of $CD8^+$ T cell activation expressing mutant, wildtype or deficient in L-selectin during an infection with vaccNP or influenza virus infection were tested by the kinetics of CD69 up-regulation. F5/LΔP $CD8^+$ T cells up-regulated CD69 at day 1 post vaccNP infection in the mediastinal LN, which drains the site of virus entry, in a similar manner as the wildtype L-selectin expressing cells F5/B6. Also, L-selectin deficient $CD8^+$ T cell's, F5L-$sel^{-/-}$, CD69 up-regulation at day 2 post infection with influenza virus were identical with the wildtype L-selectin cells. This finding is the first indicator that the increased number of mutant L-selectin expressing cells at the site of virus infection is due to the enhanced homing ability of the cells rather than the initial preferential activation in the draining lymph node.

Next, the differentiation status of the cells was investigated. Cell surface receptor such as CD44, which is a well-characterized differentiation marker, involve in the migration of activated $CD8^+$ T cells into peripheral tissues. CD44 expression increased with the stage of the $CD8^+$ T cell differentiation during a primary virus infection or $CD8^+$ T cell activation in vitro the cell activate through its T cell receptor (Wherry et al. 2007). The CD44 expression and up-regulation of F5/LΔP $CD8^+$ T cells during 5, 8 and 12 days post infection with vaccNP were identical with F5/B6 $CD8^+$ T cells in the ovary, ovdLN and spleen. CD44 up-regulation of L-selectin deficient $CD8^+$ T cells, F5L-$sel^{-/-}$, was also identical with F5B6 $CD8^+$ T cells at day 5 post infection with vaccNP. Furthermore, endogenously generated virus specific $CD8^+$ T cells from the mutant L-selectin mice, LΔP, was up-

regulated the late activation antigen, CD44, in a similar manner with the cells from the L-selectin wildtype, B6, at day 8 post infection influenza virus in the lungs and spleen. These findings collectively indicate that L-selectin on the surface of $CD8^+$ T cells does not affect the conjugate formation with the antigen presenting cells initially for the T cell activation and also the T cell differentiation during a primary course of the virus infection.

The immune system is a diverse system. $CD8^+$ T cells have T cell receptor with specificity for a foreign antigen from pathogens. Since naïve $CD8^+$ T cells with specificity for a peptide are rare, massive proliferation capacity of the $CD8^+$ T cells with the cytokine storm help from other cells is critical in purging the acute viral infection, limiting persistence infection and also, in the production of life-long protective immunity (Cox et al. 2013). Innate immune cells such as plasmacytoid dendritic cells (pDCs) play a role in driving $CD8^+$ T cells proliferation during primary virus infection by secreting proinflammatory chemokines (Gilliet et al. 2003). The molecule, such as 2B4 (CD244) is also important for $CD8^+$ T cells proliferation (Kambayashi et al. 2001). Therefore, we tested the contribution of L-selectin in enhancing the $CD8^+$ T cell proliferation during primary virus infection with vaccNP. Interestingly, wildtype L-selectin expressing $CD8^+$ T cells, in which L-selectin expression is controlled by separate mechanisms of shedding and gene silencing, started to proliferate earlier than the mutant L-selectin expressing cells in the mediastinal LN at day 2 post vaccNP infection. Constant expression of L-selectin does not provide enhanced proliferation. Therefore, the increased number of virus specific $CD8^+$ T cells in the site of infection was not due to the enhanced proliferation but rather due to homing.

L-selectin engaging with its ligand or antibody to initiate the signalling cascade has been shown to play a role in oxygen synthesis (Brenner et al. 1996). L-

selectin shedding, re-expression and later gene silencing could play an important role in the $CD8^+$ T cell proliferation and it might be manipulated to enhance the generation of effector $CD8^+$ T cells to generate enough number of cells in a short period of time to specifically eliminate a virus from the body.

Virus specific $CD8^+$ T cells leaving the draining lymph nodes, entering the blood stream and infiltrating the site of infection expressed a significant amount of L-selectin. We exploited a different method to determine whether L-selectin is involved in homing of virus specific $CD8^+$ T cells into virus infected organs. The antibody blockade system to prevent the receptor for its known function allows demonstrating the involvement of L-selectin in migration pattern of $CD8^+$ T cells. MEL-14, an antibody that was identified to bind to the NH2-terminal 53 amino acids of L-selectin's lectin domain in the presence of its epidermal factor domain, binds to L-selectin and prevents it from all its known function (Bowen et al. 1990). MEL-14 has been shown to block homing of lymphocytes into peripheral lymph nodes (Gallatin et al. 1987), together with the L-selectin knockout finding, allowed the characterization of L-selectin as peripheral lymph node homing receptor.

Since virus specific $CD8^+$ T cells expressing either mutant or wildtype L-selectin were activated in the mediastinal LN at day 1 and 2 post infection with vaccNP and influenza virus, respectively, MEL-14 administration was started at 52 hours hours post virus administration in order to allow the cells to be primed in the draining lymph node, enter the bloodstream and start to infiltrate the site of virus infection. MEL-14 treatment significantly blocked homing of the donor virus specific $CD8^+$ T cells in the ovaries and ovdLN compared to the IgG2a isotype control treated mice when the organs harvested at day 5 post infection. MEL-14 treatment did not affect the number of donor cells that homed to the spleen

because lymphocyte migration into spleen is not L-selectin dependent (Arbornes et al. 1994).

Furthermore, when wildtype B6 mice were infected with influenza A virus and then treated with MEL-14 daily started at 52 hours post infection. Because the endogenously generated virus specific $CD8^+$ T cells just started to infiltrate the site of infection, at day 4 post infection, there was only a slight difference between the number of virus specific $CD8^+$ T cells that infiltrated into the infected lungs in the MEL-14 treated mice and the IgG2a isotype control group. Whereas, interestingly, there was a significant blockade by MEL-14 treatment compared with the control group at day 8 post infection, which is a time, where the adaptive immune system $CD8^+$ T cells play a pivotal role in the influenza virus elimination.

Similar to the previous model, MEL-14 did not have any effect on the numbers of endogenously generated virus specific $CD8^+$ T cells homing into the spleen neither at day 4 or day 8 post infection with influenza virus infection. Also, the number of virus specific $CD8^+$ T cells was not affected by the MEL-14 treatment in the mediastinal LN. This finding was consistent with the previous study by (Caucheteux et al. 2013), showing that lung $CD4^+$ T cells use other mechanisms to enter the mediastinal LN rather than L-selectin pathway. Similar to mesenteric lymph node, in which $CD8^+$ T cells utilize α4β7 binding to MAdCAM-1 in order to enter (Shyjan et al. 1996), suggest that homing of $CD8^+$ T cells to these particular mucosal lymph nodes are not L-selectin dependent.

Further to test the hypothesis that L-selectin is directly involved in homing of virus specific $CD8^+$ T cells into the site of virus infection. A short term homing assay to investigate the role of L-selectin in the migration of virus specific $CD8^+$ T cells directly from the blood vessel into the site of infection was used.

Short term homing assays have been used widely to investigate the role of various adhesion molecules. For example, Using cell membrane dyes, CFSE and CMTMR, to track donor cells in a short term homing assay, naive mutant L-selectin (LΔP) expressing $CD8^+$ T cells can home equally as the wildtype L-selectin expressing cells into peripheral lymph nodes, in which the homing is L-selectin dependent (Galkina et al. 2003). The role of L-selectin in homing of virus specific $CD8^+$ T cells into sites of infection was investigated using a similar strategy. Instead of using fluorescent dyes, the congenic CD90.1/CD90.2 markers were used to identify the donor cells within the host and exploited the non-shedding mutant L-selectin expression to differentiate them from the L-selectin knockout cells.

L-selectin sufficient and deficient $CD8^+$ T cells were either activated *in vitro*, using cognate peptide pulsed irradiated APCs for 7 days, or activated *in vivo* using recombinant virus expressing cognate peptide and harvested from spleen 5 days post infection. After that, both activated mutant L-selectin and L-selectin knockout $CD8^+$ T cells were mixed 1:1 and intravenously transferred into vaccNP infected mice (day 3 post infection) and then very shortly, after 3 hours, site of vaccNP infection (ovary), a range of L-selectin dependent and independent lymphoid organs such as ovdLN, Axillary, inguinal lymph nodes and spleen were harvested for homed donor $CD8^+$ T cells analysis. Compared to the circulating L-selectin sufficient and deficient $CD8^+$ T cells in the peripheral blood at the time of harvest (50% F5LΔP/ 50% F5L-$sel^{-/-}$), there was a significant enrichment of L-selectin sufficient $CD8^+$ T cells in the ovaries of vaccNP infected mice compared with the knockout cells when the invitro activated cells were used (3-fold; 70% F5LΔP/ 30% F5L-$sel^{-/-}$). The L-selectin dependent sites such as inguinal and axillary lymph nodes showed a full L-selectin dependency (100% F5LΔP) and L-selectin

independent sites such as spleen and mediastinal lymph node showed L-selectin independent migration (50% F5LΔP/ 50% F5L-sel$^{-/-}$).

Interestingly, when the *in vivo* activated CD8^{+} T cells were used, there was a much stronger L-selectin dependency in homing of the cells into the site of infection (6-fold; 86% F5LΔP/ 14% F5L-sel$^{-/-}$). This finding reveals that it is important for lymphocyte migration assays that the cells are activated *in vivo* rather than *in vitro*. All the necessary signals may not exist *in vitro* and CD8^{+} T cells activation *in vitro* could drive through a different differentiation pathway.

The diversity and the strength of the immune system originates from the adaptive arm of the system. T cells, in particular, CD8^{+} T cells, play a pivotal role in the elimination of viruses from the body (Thimme et al. 2003). Although virus specific CD8^{+} T cells, upon direct contact with the infected cells, use specific mechanisms to eliminate the virus from the infected tissues such as IFNγ secretion (Goulding et al. 2014) and granzyme B (Zajac et al. 2003), the virus specific CD8^{+} T cells must migrate to the site of infection first.

Cells of the immune system orchestrate each other to provide an efficient protection during a virus infection. It has been shown recently that neutrophils, cells from the innate immune system that participate in early events during a virus infection, leave trails containing chemokine CXCL12, which guide the virus specific CD8^{+} T cells to the trachea during influenza virus infection to participate in the virus elimination (Lim et al. 2015). Other cells such as CD4^{+} T cells, through the secretion of cytokines such as IFN-γ, help the virus specific CD8^{+} T cells to mobilize to the site of infection in order to provide antiviral protection (Nakanishi et al. 2010).

6.2. The impact of various level of L-selectin on the surface of $CD8^+$ T cells in providing anti-viral immunity.

It is clear that lymphocyte homeostasis, during normal circumstances, and lymphocyte migrations to the site of infection, during a virus infection, are complex processes. Adhesion molecules and chemokine receptors play an important role in the lymphocyte migration process. Since the virus specific $CD8^+$ T cells enriched at the site of virus infection were expressing L-selectin, therefore, the role of various level of L-selectin on the surface of virus specific $CD8^+$ T cells were tested in providing antiviral immunity against two evolutionary distinct viruses, vaccinia and influenza, which infect mucosal and visceral organs, respectively.

It has been shown that inserting genes from different viruses into other, in order to express the peptide of interest, can affect the pathogenicity of the virus. First, we determined the kinetics of vaccNP clearance in a normal C57BL/6 mouse. Based on our finding that vaccNP mainly replicates in and infects ovaries, B6 mice infected with vaccNP i.p. were euthanized at day 5, 8 and 12 post infection and ovaries were harvested for vaccNP titer by plaque assay. At the time point analyzed, the B6 immune system appear to significantly clear the virus from the infected ovaries as there was 2 log difference between day 5 and 8 post infection in the virus titer. Although the virus was not completely cleared, there were further signs of viral clearance at day 12 post infection. This finding was comparable to the previous finding with the parental Western Reserve vaccinia virus infection in ovaries in which the titer fell between day 7 and 15 post infection (Zhao et al. 2011).

The various immune system compartments in clearing vaccinia virus have been investigated in details. Based on the site of virus entry and the strain of the virus, antibody and $CD4^+$ T cell mediated viral clearance versus $CD8^+$ T cells can vary. When the Western Reserve vaccinia virus was inoculated intraperitoneally, $CD4^+$ T and antibody mediated viral clearance dominate the $CD8^+$ T cell antiviral immunity (Xu et al. 2004). Whereas, intranasal inoculation of the virus leads to a robust $CD8^+$ T cell response that clears the virus through IFN-γ secretion by the CTLs (Goulding et al. 2014).

In order to investigate to what extent $CD8^+$ T cells contribute to the clearance of vaccinia virus infection, mice were either acutely depleted of circulating lymphocytes, by using a sublethal dose of irradiation, or chronically depleted, by using mice that are knockout for the recombination activating gene (RAG). Then, adoptive transfer of purified $CD8^+$ T cells only into these lymphocyte-depleted mice was performed to investigate the role of $CD8^+$ T cells in controlling virus infection and, whether L-selectin on the surface of $CD8^+$ T cells could enhance virus clearance.

Mice acutely depleted of circulating lymphocytes by sublethal irradiation were no longer susceptible to vaccNP infection. There was no detectable virus in ovaries at day 5 post infection. The irradiation could have affected the ovarian tissues and made them no longer susceptible to virus entry into the ovarian cells and subsequent infection. Therefore, $RAG^{-/-}$ mice were used for further investigation of the role of $CD8^+$ T cells and impact of L-selectin in protection against the virus.

First, the ability of the complete immune system in B6 mice was compared with that in $RAG^{-/-}$ mice that lack T and B lymphocytes. When both groups were infected with the vaccNP i.p., there was no difference between B6 and $RAG^{-/-}$

mice in the viral titer at day 5 post infection, which suggests that the lymphocyte dependent viral clearance was not yet involved. Whereas by day 8 p.i., there was a significant difference in the viral titers between the B6 and $RAG^{-/-}$ mice in which the B6 mice started to clear virus by 2 logs compared to the $RAG^{-/-}$ mice that had a similar virus titer as day 5 p.i.. This finding suggests that there is a window for lymphocyte dependent antiviral activity that can be used to study the impact of L-selectin in homing of virus specific $CD8^+$ T cells and therefore on the antiviral protection.

The involvement of $CD8^+$ T cells was then investigated by adoptive transfer of purified naïve $CD8^+$ T cells expressing either wildtype or mutant L-selectin into $RAG^{-/-}$ recipient, and a group was left with no T cell transfer and used as a control. Then mice were challenged with vaccNP and 8 days later, ovaries were harvested for viral titer by plaque assay. We have shown that upon vaccNP inoculation intraperitoneally, wildtype L-selectin expressing $CD8^+$ T cells did not provide protection against the vaccNP infection in the ovaries when compared with the control no T cell transfer group. This result is consistent with the previous finding that vaccinia virus clearance in the ovaries depends on $CD4^+$ T cells and antibody protection from B cells (Xu et al. 2004). Whereas, mutant L-selectin expressing $CD8^+$ T cells provided a significantly enhanced level of protection against vaccNP as the F5LΔP $CD8^+$ T cell transfer group showed lower viral titers in the ovaries when compared wildtype L-selectin $CD8^+$ T cell transfer and control groups.

Although we have shown that wildtype L-selectin expressing $CD8^+$ T cells homed to the site of vaccNP infection, their inability to provide protection against vaccNP infection might be due to the limited numbers of cells that are homed to the site of

virus infection which in turn due to the limited amount of L-selectin expressed on their surface by the time that is started to enrich at the site of infection.

Since mutant L-selectin expressing $CD8^+$ T cells provided protection against vaccNP infection in the ovaries of $RAG^{-/-}$ mice, L-selectin dependent homing of these cells into the site of the vaccNP infection was confirmed using L-selectin specific antibody blockade strategy. $RAG^{-/-}$ mice were adoptively transferred with mutant L-selectin expressing $CD8^+$ T cells and 24 hours later, after the T cells equilibrated in the body environment, vaccNP inoculated i.p. then, mice were injected with MEL-14 antibodies at 52 hours p.i.. At day 5 p.i., ovaries, ovdLN and spleen were harvested for homed $CD8^+$ T cells enumeration by flow cytometry. Interestingly, similar to the wildtype B6 host, MEL-14 blocked homing of the virus specific $CD8^+$ T cells expressing mutant L-selectin into the ovaries of $RAG^{-/-}$ mice as well as into the ovdLN. Whereas the number of the cells was identical in the spleen between the MEL-14 treated mice and the IgG2a isotype control group. Using MEL-14 blockade strategy, together with the short-term homing experiments further revealed that homing of $CD8^+$ T cells into the ovdLN are L-selectin dependent. Although ovdLN is not a peripheral or cutaneous LN, it is interesting that lymph node which is located in the peritoneal cavity and lymohocyte migration to this particular lymph node is L-selectin dependent. This is further revealing the diversity of L-selectin function in enabling $CD8^+$ T cells to specifically migrate to certain parts of the body at various times such as normal homeostasis or during a primary or secondary viral infection.

Various molecules have been reported to play an important role in driving the $CD8^+$ T cell expansion during vaccinia virus infection. TNF receptor family member OX40 plays a pivotal role in driving the primary $CD8^+$ T cell response and cytokine production (Salek-Ardakani et al. 2008). Therefore, the impact of L-

selectin on the $CD8^+$ T cell proliferation during primary vaccNP infection was tested to exclude the possibility that the enhanced antiviral protection by the mutant L-selectin expressing $CD8^+$ T cells was due to enhanced proliferation rather than their enhanced ability to home to the site of infection. The experiment was conducted by adoptive transfer of purified $CD8^+$ T cells expressing either wildtype or mutant L-selectin into $RAG^{-/-}$ recipient mice. Mice were inoculated with vaccNP and then they were treated with IL-2 to drive $CD8^+$ T cell expansion in the absence of $CD4^+$ helper T cells. At day 4 p.i., 24 hours prior to harvest, mice were injected with EdU (5-Ethynyl 2-deoxyuridine), which is incorporated into the DNA of proliferating cells, to investigate the proliferative state of the cells *in vivo*. In support of the hypothesis, both wildtype and mutant L-selectin expressing $CD8^+$ T cells harvested from the ovaries of vaccNP infected mice incorporated a similar level of EdU into their DNA, which suggests that both cell types expanded equally in response to the vaccNP infection.

Upon vaccinia virus infection, $CD8^+$ T cell shows site dependent heterogeneity of functional responses. It has been shown that OT-I T cells, which recognize the class I epitope, can expand robustly against i.p. infection with recombinant vaccinia virus expressing OVA-peptide. OT-I T cells start to secrete IFN-γ as they proliferate and differentiate in the lymph node and spleen. They secrete TNF-α upon recognition of the virus (Xiao et al. 2007). Even if $CD8^+$ T cells do not participate in direct protection against the virus (Xu et al. 2004), this cytokine storm is necessary to initiate various responses against the virus. Therefore, it was necessary to understand that whether a various level of L-selectin on the surface of $CD8^+$ T cells have an impact on the secretion of various cytokines and chemical mediators during the primary course of vaccNP infection. $RAG^{-/-}$ mice were adoptively transferred with $CD8^+$ T cells expressing either wildtype or mutant L-selectin and then challenged with vaccNP, both cell types responded to

the virus in a similar way with regards to cytokine secretion such as IL-2, TNF-α, IFN-γ and granzyme B at day 5 p.i. the ovaries. Also, adoptively transferred virus specific $CD8^+$ T cells harvested from the spleen are secreted similar level of IL-2, TNF-α, IFN-γ and granzyme B as in the ovaries.

More recently, research has shown that $CD8^+$ T cells play a dominant role in the clearance of highly virulent vaccinia virus infection when the virus is administered intranasally (Goulding et al. 2014). Other studies also concluded that there was a robust intrapulmonary $CD8^+$ T cell response upon an intranasal infection with vaccinia virus (Mathew et al. 2008). Therefore, the role of $CD8^+$ T cells in clearing vaccNP was tested in the context of intranasal infection. B6 mice were challenged with vaccNP i.n., lungs were harvested at day 8 post infection for viral titer by plaque assay. However, there was no detectable vaccNP in the lung, even high inoculating dose of 10^6 pfu, whereas the parental wildtype Western Reserve vaccinia virus is reported to cause severe lung infection using 10^4 pfu (Goulding et al. 2014). Also, in order to confirm the inability of vaccNP to produce a lung pathology when administered i.n., body weights were measured during the course of the primary vaccNP infection. There were some signs of initial distress and infection with the high doses of 10^5 and 10^6 pfu during the first 2 days, but the mice gained weight again from day 3 onward. Using 10^4 pfu, there was no weight loss during 8 days following intranasal administration of the virus.

Since vaccNP did not establish a lung infection, which is consistent with the finding that genetic manipulation of viruses leads to changes in pathogenicity (Williamson et al. 1990), a more virulent lung virus was used to study the impact of L-selectin on homing of these virus specific $CD8^+$ T cells into the site of infection for protective antiviral immunity.

The more virulent viruses used were influenza A viruses including serotypes (H1N1, PR8) and (H3N2, H17). It has been reported that $CD8^+$ T cells play a pivotal role in clearing influenza virus-induced pneumonia (W Allan et al. 1990)(Graham & Braciale 1997). Therefore, the endogenous immune response against influenza A virus was studied in the mutant, wildtype and L-selectin knockout mice. Influenza A virus (PR8, H1N1) was administered i.n. into mutant, wildtype and L-selectin knockout mice, body weights were measured daily and lungs were harvested at day 8 p.i. for viral titer by focus forming unit assay. Interestingly, there was a hierarchy in virus clearance based on the level of L-selectin expression. The mutant L-selectin mice, in which the L-selectin is selectively expressed on T lymphocytes and expression is maintained following T cell activation (Galkina et al. 2003)(Richards et al. 2008), provided highest level of protection with the lower level of virus in the lungs compared with the wildtype and L-selectin knockout mice. Wildtype L-selectin expressing mice showed a moderate level of protection compared with the other groups, whereas L-selectin knockout mice provided the least level of protection. The virulence of the PR8 strain and the impact of level of L-selectin were further confirmed by the differences in the body weight loss which accompanied the primary infection. The mutant L-selectin mouse had the lowest level of weight loss across the 8 days of primary influenza A virus PR8 infection compared with the wildtype and L-selectin knockout mice.

Mutant and wildtype L-selectin mice and the L-selectin knockout mice were also challenged with a less virulent strain of the influenza A virus (H17, H3N2) and viral titers were measured in the lungs at day 8 p.i.. Wildtype L-selectin expressing mice showed enhanced virus clearance when compared with L-selectin knockout mice which clearly shows a role for L-selectin in controlling the virus replication. However, mutant L-selectin expressing mice did not show

enhanced protection compared with wildtype mice. One possibility is that the necessity of L-selectin on the surface of $CD8^+$ T cells and its impact on virus clearance may rely on the virulence of the virus. When the mouse has its complete immune system, other cells can participate in clearance of the virus and reduce the dependency on $CD8^+$ T cells. It has been shown that $CD8^+$ T cells play an important role in influenza virus infection, but the involvement of other immune cells such as $CD4^+$ T cells and B cells in virus clearance depends on the virulence of the virus (Mozdzanowska et al. 2000).

To further understand the role of L-selectin on the surface of $CD8^+$ T cells in clearing influenza virus infection, immunodeficient $RAG^{-/-}$ mice, which chronically lack T and B cells, were adoptively replete with purified naïve $CD8^+$ T cells co-expressing F5 transgenic TCR and either mutant or wildtype L-selectin or knockout for L-selectin. Then mice were challenged with influenza A virus (H17, H3N2) and virus clearance was measured by focus forming unit assay 7 days p.i.. Similar to the highly virulent influenza A virus, PR8, $RAG^{-/-}$ mice that were complemented with mutant L-selectin expressing $CD8^+$ T cells provided enhanced H17 influenza virus clearance when compared with the $RAG^{-/-}$ mice transferred with wildtype L-selectin expressing or L-selectin knockout $CD8^+$ T cells. L-selectin knockout $CD8^+$ T cell's complemented $RAG^{-/-}$ mice showed no signs of viral clearance when compared with the no T cell transfer control group.

Although it was clear from the vaccinia model that L-selectin did not impact on the activation and differentiation of the virus specific $CD8^+$ T cells during the primary vaccNP infection, $CD8^+$ T cells co-expressing F5 TCR and various levels of L-selectin were activated *in vitro* for 7 days and instilled directly into the lungs of influenza A virus infected $RAG^{-/-}$ mice. T cells delivered intratracheally were able to clear virus when compared with no T cell transferred group and the level

of protection was not dependent on L-selectin expression. Therefore, the inability of *in vivo* primed L-selectin knockout T cells to clear virus was not due to their functional inability rather due to their inability to home to the site of infection.

6.3. L-selectin impacts on $CD8^+$ T cell co-stimulation

L-selectin expression is tightly regulated on the surface of $CD8^+$ T cells. Shedding and gene silencing are two mechanisms that account for the level of L-selectin on the surface of naïve and activated $CD8^+$ T cells (Chao et al. 1997)(Sinclair et al. 2008). L-selectin has been implicated in a number of signalling processes during $CD8^+$ T cells undergoing its developmental changes upon activation through a peptide MHC class I on the surface of an APC. (Brenner et al. 1996), have shown that cross-linking L-selectin with the antibody (Dreg56) leads to shedding of the L-selectin on the surface, this process has been reported to induce signalling which is important for a number of functional activity including Ceramide and kinases function such as the $p56^{LCK}$ kinase. Ceramide is involved in cellular function, development and differentiation and apoptosis. Whether L-selectin shedding on the surface leads to the initiation of the signalling cascade or further processing of the tail of L-selectin by other enzymes results in the initiation of the signalling cascade is not known.

Although we have investigated the impact of various levels of L-selectin such as higher than normal level in mutant L-selectin (LΔP) and normal level of L-selectin as in wildtype on the biology of $CD8^+$ T cells activated *in vivo* through virus and looking at the endogenous response or T cells co-expressing various levels of L-selectin and F5 transgenic TCR through the recombinant virus expressing peptide of interest, mutant L-selectin enhances on the $CD8^+$ T cell's proliferation,

activation and differentiation upon *in vitro* activation with a cognate peptide MHC class I complex with an APC was investigated. Furthermore, we have also evaluated the amount and types of cytokines secreted by $CD8^+$ T cells expressing either wildtype or mutant L-selectin.

$CD8^+$ T cells expressing the mutant form of L-selectin (F5/LΔP) show no significant difference from the wild type (F5/B6) with regards to very early activation (first 24 hours) in the culture system upon stimulation with a cognate peptide NP68. As both $CD8^+$ T cells showed a similar rate of CD69 up-regulation.

Another aspect of our investigation was a detailed proliferation analysis. L-selectin has been shown to play an important role during $CD8^+$ T cell proliferation and cytokine secretion. A study has shown that mice lack L-selectin (L-selectin $^{-/-}$) are impaired in the level of IL-2, IL-4 and IFN-γ secretion and also they have shown that $CD8^+$ T cells proliferate more slowly than wild type cells (Xu et al. 1996).

Although we have shown that wildtype L-selectin expressing $CD8^+$ T cells entered the proliferation cycle earlier upon *in vivo* activation, $CD8^+$ T cells expressing various levels of L-selectin were activated *in vitro* and the proliferation cycle were investigated in detail. Using proliferation sensitive dye, CFSE, both cell types, wildtype and mutant L-selectin expressing $CD8^+$ T cells, started to proliferate equally at day 1 post activation indicated by equal dilution of the CFSE dye. Whereas, the regulated expression of wildtype L-selectin, including initial ectodomain proteolytic cleavage and shedding and later gene silencing, enhanced the $CD8^+$ T cell's proliferation cycle same as $CD8^+$ T cells started to proliferate during *in vivo* activation. F5/B6 $CD8^+$ T cells showed higher division index and more CFSE dilution at day 4 post activation when compared with

F5/LΔP cells, whereas at day 5 post activation, there were no significant difference between their division indices and CFSE dilution ability, indicated that both cells were became proliferating in a similar manner. It has been reported that $CD8^+$ T cells enter the proliferation cycle independent on interleukin-2 (IL-2) for the first proliferation cycle (D'Souza & Lefrançois 2003), which is consistent with our finding as both cell types started to proliferate in a similar manner at day 1 post activation. During the wildtype L-selectin expressing $CD8^+$ T cells showed enhanced ability to proliferate ad day 4 post activation, we also showed that these cells upregulated the IL-2 receptor (IL-2R) earlier at day 3 and 4 post activation, whereas at day 5 post activation, the level of IL-2R became identical between the two cell types. Regulated expression of L-selectin in the wildtype may lead to an intracellular signalling event, which may be important for IL-2R upregulation, which can be investigated in detail in the future. Mutant L-selectin (LΔP) expressing $CD8^+$ T cells was delayed in entering the IL-2 dependent proliferation cycle only. Similar to the previous research study by (Xu et al. 1996), $CD8^+$ T cells will find an alternative mechanism to compensate for the missing signals and co-stimulation that suppose to be provided by the regulated expression of L-selectin.

Upon naïve $CD8^+$ T cells activation through peptide MHC class I on the surface of an APC, $CD8^+$ T cells undergo developmental changes that lead to the rise of the distinct subset of $CD8^+$ T cells classified as effector and memory CD8+ T cells (Brinkman et al. 2013). Effector and memory $CD8^+$ T cells have different functions and migratory pattern during an on going immune response (Brinkman et al. 2013)(Laouar et al. 2008). Therefore, it was necessary to study the impact of mutant L-selectin expression on the concurrent generation of effector and memory $CD8^+$ T cells. We have shown, upon activation in vitro, that the $CD8^+$ T cells on which L-selectin expression is maintained during T cell activation and

differentiation (F5/LΔP) can be classified into effector and memory subsets based on the cell surface markers such as CD44 and CCR7, as the expression of those markers changes upon activation with a cognate peptide MHC class I with an APC. There was not any significant difference in the generation of effector $CD8^+$ T cells between the F5/LΔP and F5/B6 $CD8^+$ T cells.

Cytokine secretion by various subsets of $CD8^+$ T cells is considered pivotal for various stages of the adaptive immune response. Effector and memory $CD8^+$ T cells secrete almost the same cytokines but at a different rate according to their necessity. Although $CD4^+$ T cells are the main T cell responsible for cytokine secretion and providing co-stimulation and help, $CD8^+$ T cells can secrete various types of cytokines and show their cytotoxic abilities (Kristensin et al. 2004). Furthermore, the types and the amount of cytokines secreted by $CD8^+$ T cells expressing mutant or wild type L-selectin upon *in vitro* activation were investigated. The presence of mutant L-selectin did not show any impact on the rate of cytokine secretion and there was no significant difference between mutant (F5/LΔP) and wild type L-selectin (F5/B6) $CD8^+$ T cells with regards to cytokine secretion upon re-stimulation.

Furthermore, the degranulation ability of $CD8^+$ T cells expressing either mutant or wild type L-selectin molecules were also investigated. The result showed that there was no significant difference between the two types of cells with regards to intracellular CD107a staining. Degranulation is a characteristic ability of activated $CD8^+$ T cells. After activation and differentiation of naïve $CD8^+$ T cells, both effector and memory $CD8^+$ T cells can degranulate but the protein content of their granules (most commonly perforins and granzymes) can vary and perform a lytic activity in the case of effector population (Wolint et al. 2004).

The presence of mutant L-selectin (LΔP) on the surface of $CD8^+$ T cells did not show any significant impact on the way these cells activate, differentiate and proliferate upon activation through a specific peptide transgenic TCR recognition *in vitro* apart from a short delay (24 hours) in the onset of IL-2 dependent proliferation. L-selectin is well defined for its adhesion function, it facilitate the initial tethering and rolling of leukocytes, such as neutrophils, across both wall of blood vessels and T lymphocytes across HEVs in the lymph nodes. $CD8^+$ T cells expressing mutant L-selectin showed its impact within *in vivo* environment. A potent immune response depends on cells that can home to lymph nodes and the site of infection. As we have shown during in vivo activation of the $CD8^+$ T cells through a virus infection, Presence of mutant L-selectin is promoted $CD8^+$ T cells homing to sites of infection and, therefore, provided enhanced antiviral immunity.

6.4. Conclusion

In conclusion, L-selectin first identified as a lymph node homing molecule (Gallatin et al. 1983), also, a recent study has further shown that L-selectin is an important molecule for migration of chronic lymphocytic leukaemia cells into the lymph nodes through high endothelial venules (Lafouresse et al. 2015). Beside its characterization as a lymph node homing molecule, L-selectin has been used as a marker of T lymphocyte activation by a wide range of immunologist. L-selectin expression is controlled by two separate mechanisms of ectodomain shedding and gene silencing, it is not a reliable marker to access level of activation very early during $CD8^+$ T cells activation, primary first 7-8 days of primary infection. A recent study has also concluded that percentage L-selectin expression by $CD4^+$ T cells is not a reliable marker to predict the risk of developing progressive multifocal Leukoenchephalopathy since its expression varies on the surface of the $CD4^+$ T cells during the time course of the infection (PML)(Lieberman et al. 2015).

It is time to consider the re-expressed L-selectin on the surface of activated $CD8^+$ T cells as an important molecule for homing into the site of virus infection as well.

6.5. Future studies

In the present study, we have shown that L-selectin is variably expressed on the surface of $CD8^+$ T cells during the course of a primary virus infection, further studies to underpin when the L-selectin ectodomain shedding and gene silencing occur would give interesting results. Then using pharmacologic inhibitors to interfere with the shedding and gene silencing could be a great potential to maintain L-selectin expression on the surface of $CD8^+$ T cells to enhance homing of the virus specific $CD8^+$ T cells to the site of virus infection and, therefore, enhance anti-viral immunity.

It has been shown that viruses such as HIV use mechanisms of L-selectin retaining in the cytoplasm to limit the number of immune cells migrating to the lymph node for priming and activation, using its protein Nef and Vpu (Vassena et al. 2015), it is important to further investigate other viruses as well whether they use different mechanisms to enhance the gene silencing to limit the recruitment of activated $CD8^+$ T cells into sites of infection.

L-selectin is a transmembrane adhesion molecule composed of glycoprotein in nature. L-selectin binds to a wide range of O and N-linked oligosaccharide ligands. L-selectin recognizes its ligands in a glycosylation-dependent manner. The fucose or sialic acid in the oligosaccharide structure has to be sialylated or fucosylated then L-selectin able to bind (Varki 1994). It is clear that the nature of L-selectin binding to its ligand is a complex process and it may happen at different times during the course of a primary virus infection and there may be a peak where their carbohydrates are in the correct form for L-selectin binding, Identifying L-selectin ligands on the inflamed blood vessels supplying blood to the site of infections or within the inflamed virus infected tissues are potential findings

to understand the mechanism of L-selectin dependent homing of virus specific $CD8^+$ T cells to site of infection.

Recombinant soluble L-selectin or antibodies against known ligand may be an ideal approach to study and identify the L-selectin ligands in various virus-infected tissues. This could be done either in vivo, by injecting the soluble L-selectin or antibodies into mice infected with influenza A or vaccinia viruses in order to identify the ligands in the lung and ovary, respectively, or in vitro, by culturing cells from the ovarian tissues and the lung and incubate with soluble L-selectin or antibodies. Bound ligand can be investigated using protein purification techniques to identify what L-selectin binds to in these virus infected tissues.

Further studies to identify sites of infection, other than lung and ovary, that are L-selectin dependent for homing of virus specific $CD8^+$ T cells is important. Re-expressed L-selectin on the surface of virus specific $CD8^+$ T cells may be important for homing to all peripheral tissues. Using other virus infection in mice that target common sites of infection could be another important future studies in order to understand whether the proinflammatory cytokine and chemokine storm play a role in recruiting virus specific $CD8^+$ T cells in L-selectin dependent manner. Also, there is no doubt that response from memory $CD8^+$ T cells is important for vaccination strategies and for recurrent infections with the same virus, whether L-selectin play a pivotal role during virus specific $CD8^+$ T cells recruitment to peripheral tissues is important to investigate.

Bibliography

Ager, A., 2012. ADAMs and Ectododomain Proteolytic Shedding in Leucocyte Migration : Focus on L-Selectin and ADAM17. *Current Immunology Reviews*, 8, pp.1–15.

Ager, A. & May, M.J., 2015. Understanding high endothelial venules: Lessons for cancer immunology. *Oncoimmunology*, 4(6).

Alari-pahissa, E. et al., 2012. CD69 Does Not Affect the Extent of T Cell Priming. *PLOS ONE*, 7(10), pp.1–12.

Alberts, B., Johnson, A. & Lewis, J., 2002. *Molecular Biology of the Cell*,

Allan, W. et al., 1990. Cellular events in the lymph node and lungs in mice with influenza. Consequence of depleting CD4+ T cells. *The Journal of Immunology*, 144, pp.3980–3986.

Allenspach, E., Rawlings, D. & Scharenburg, A., 2003. *Gene Review*,

Alt, F.W. et al., 1992. V D J recombination. *Immunology Today*, 13, p.306.

Althaus, C.L., Ganusov, V. V & De Boer, R.J., 2007. Dynamics of CD8+ T cell responses during acute and chronic lymphocytic choriomeningitis virus infection. *The Journal of Immunology*, 179, pp.2944–2951.

Andrian, U.H. Von & Mackay, C.R., 2000. T-cell function and Migration Two Sides of the Same Coin. *The New England Journal of Medicine*, 343(14), pp.1020–1034.

Aoshi, T. et al., 2011. Innate and adaptive immune responses to viral infection and vaccination. *Current Opinion in Virology*, 1(4), pp.226–232.

Arbornes, M.L. et al., 1994. Lymphocyte Homing and Leukocyte Rolling and Migration Are Impaired in L-Selectin-Deficient Mice. *Immunity*, 1, pp.247–260.

Arstila, T.P. et al., 1999. A direct estimate of the human αβ T cell receptor diversity. *Science*, 286, pp.958–961.

Austrup, F. et al., 1997. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. *Nature*, 385, pp.81–83.

Bachmann, M.F., Ecabert, B. & Kopf, M., 1999. Influenza virus: a novel method to assess viral and neutralizing antibody titers in vitro. *The Journal of Immunology methods*, 225, pp.105–111.

Backer, R.A. et al., 2014. A central role for Notch in effector CD8 + T cell differentiation. *Nature Immunology*, 15(12), pp.1143–1153.

Bartholdy, C., Marker, O. & Thomsen, R., 2000. Migration of activated CD8(+) T lymphocytes to sites of viral infection does not require endothelial selectins. *Blood*, 95(4), pp.1362–1369.

Bender, S.B. et al., 1992. Transgenic mice lacking clas I major histocompatibility complex-restircted T cells have delayed viral clearance and increased mortality after influenza virus challenge. *The Journal of Experimental Medicine*, 175, pp.1143–1145.

Bento, D.C. et al., 2015. High endothelial venules are rare in colorectal cancers but accumulate in extra-tumoral areas with disease progression. *Oncoimmunology*, 4(3), p.e974374.

Berg, E.L. et al., 1993. L-selectin-mediated lymphocyte rolling on MAdCAM-1. *Nature*, 366, pp.695–698.

Bevilacqua, M.P., 1993. Endothelial leukocyte adhesion molecules. *Annual Review Immunology*, 11, pp.767–804.

Blazejewska, P. et al., 2011. Pathogenicity of different PR8 influenza A virus variants in mice is determined by both viral and host factors. *Virology*, 412(1), pp.36–45.

Von Boehmer, H., 1994. von Boehmer. *Cell*, 76, p.219.

Boise, L.H. et al., 1995. CD28 Costimulation Can Promote T Cell Survival by Enhancing the Expression of Bcl-xL. *Immunity*, 3, pp.87–98.

Boulet, S., Daudelin, J.-F. & Labrecque, N., 2014. IL-2 induction of Blimp-1 is a key in vivo signal for CD8+ short-lived effector T cell differentiation. *Journal of immunology*, 193(4), pp.1847–54.

Bowen, B.R., Fennie, C. & Lasky, L.A., 1990. The Mel 14 Antibody Binds to the Lectin Domain of the Murine Peripheral Lymph Node Homing Receptor. *The Journal of Cell Biology*, 110, pp.147–153.

Brandenburg, B. et al., 2013. Mechanisms of Hemagglutinin Targeted Influenza Virus Neutralization. *PLOS ONE*, 8(12), pp.1–14.

Brenner, B. et al., 1996. L-Selectin activates the Ras pathway via the tyrosine kinase p56lck. *PNAS*, 92(26), pp.15376–15381.

Brinkman, C.C., Peske, J.D. & Engelhard, V.H., 2013. Peripheral tissue homing receptor control of naïve , effector , and memory CD8T cell localization in lymphoid and non-lymphoid tissues. *Frontiers in Immunology*, 4, pp.1–8.

Briody, B.A., 1959. Response of Mice to Ectromelia and Vaccinia Viruses. , 23, pp.61–95.

Byrd, D. et al., 2014. Primary Human Macrophages Serve as Vehicles for Vaccinia Virus. *Journal of Virology*, 88(12), pp.6819–6831.

Cáceres, A. et al., 2013. Involvement of the cellular phosphatase DUSP1 in vaccinia virus infection. *PLoS Pathogens*, 9(11), pp.1–21.

Cannon, M.J. et al., 1987. Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells. *Immunology*, 62, pp.133–138.

Caucheteux, S.M., Torabi-parizi, P. & Paul, W.E., 2013. Analysis of naïve lung CD4 T cells provides evidence of functional lung to lymph node migration. *PNAS*, 110(5), pp.1821–1826.

Cerundolo, V. et al., 1997. The proteasome-specific inhibitor lactacystin blocks presentation of cytotoxic T lymphocyte epitopes in human and murine cells. *European Journal of Immunology*, 27, pp.336–341.

Chao, C.-C., Jensen, R. & Dailey, M.O., 1997. Mechanism of L-Selectin Regulation by Activated T cells. *The Journal of Immunology*, 159, pp.1686–1694.

Chen, B.A., Engel, P. & Tedder, T.F., 1995. Structural Requirements Regulate Endoproteolytic Release of the L-Selectin (CD62L) Adhesion Receptor from the Cell Surface of Leukocytes. *The Journal of Experimental Medicine*, 182, pp.519–530.

Cheng, L.E. et al., 2002. Enhanced signaling through the IL-2 receptor in CD8+ T cells regulated by antigen recognition results in preferential proliferation and expansion of responding CD8+ T cells rather than promotion of cell death. *PNAS*, 99(5), pp.3001–3006.

Cornberg, M. et al., 2007. Protection against Vaccinia Virus Challenge by CD8 Memory T Cells Resolved by Molecular Mimicry. *Journal of Virology*, 81(2), pp.934–944.

Cox, M.A., Kahan, S.M. & Zajac, A.J., 2013. Anti-viral CD8 T cells and the cytokines that they love. *Virology*, 435, pp.157–169.

Croft, M. & Dubey, C., 1997. Accessory molecule and costimulation requirements for CD4 T cell response. *Critical Review Immunology*, 17, pp.89–118.

Davies, D.H. et al., 2005. Vaccinia Virus H3L Envelope Protein Is a Major Target of Neutralizing Antibodies in Humans and Elicits Protection against Lethal Challenge in Mice. *Journal of Virology*, 79(18), pp.11724–11733.

Diacovo, T.G. et al., 1996. Platelet-Mediated Lymphocyte Delivery to High Endothelial Venules. *Science*, 273, pp.252–255.

Ding, Z. et al., 2003. L-selectin stimulation enhances functional expression of surface CXCR4 in lymphocytes : implications for cellular activation during adhesion and migration. *Blood*, 101(11), pp.4245–4252.

Dolfi, D. V et al., 2011. Dendritic Cells and CD28 Costimulation Are Required To Sustain Virus-Specific CD8 + T Cell Responses during the Effector Phase In Vivo. *The Journal of Immunology*, 186, pp.4599–4608.

Durrant, D.M., Daniels, B.P. & Klein, R.S., 2014. IL-1R1 signaling regulates CXCL12-mediated T cell localization and fate within the central nervous system during West Nile Virus encephalitis. *The Journal of Immunology*, 193(8), pp.4095–4106.

Dwir, O. et al., 2002. L-selectin Dimerization Enhances Tether Formation to Properly Spaced Ligand. *The Journal of Biological Chemistry*, 277(24), pp.21130–21139.

Edwards, D.R., Handsley, M.M. & Pennington, C.J., 2008. The ADAM Metalloproteinases. *Molecular Aspects Medicine*, 29(5), pp.258–289.

Eichelberger, B.M. et al., 1991. Clearance of Inluenza Virus Respiratory Infection in Mice Lacking Clss I Major Histocompatability Complex-Restricted CD8+ T cells. *The Journal of Experimental Medicine*, 174, pp.875–880.

Epstein, S.L. et al., 1998. Mechanism of Protective Immunity Against Influenza Virus Infection in Mice Without Antibodies. *The Journal of Immunology*, 160, pp.322–327.

Fabre, S. et al., 2008. FOXO1 Regulates L-Selectin and a Network of Human T Cell. *The Journal of Immunology*, 181, pp.2980–2989.

Fisher, D.T. et al., 2011. IL-6 trans-signaling licenses mouse and human tumor microvascular gateways for trafficking of cytotoxic T cells. *The Journal of Clinical Investigation*, 121(10), pp.3846–3859.

Flynn, K.J. et al., 1998. Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. *Immunity*, 8, pp.683–691.

Forsyth, I.A., Hutchings, A. & Butcher, G.W., 2000. A panel of monoclonal antibodies to ovine placental lactogen. *The Journal of Endocrinology*, 165, pp.435–442.

Fruman, D.A. et al., 1999. Impaired B-cell development and proliferation in absence of phosphoinositide 3-kinase p85. *Science*, 283, pp.393–397.

Fu, H. et al., 2014. Self-recognition of the endothelium enables regulatory T-cell trafficking and defines the kinetics of immune regulation. *Nature communications*, 5, p.3436.

Galkina, E. et al., 2003. L-selectin shedding does not regulate constitutive T cell trafficking but controls the migration pathways of antigen-activated T lymphocytes. *The Journal of Experimental Medicine*, 198, pp.1323–1335.

Galkina, E. et al., 2006. Lymphocyte recruitment into the aortic wall before and during development of atherosclerosis is partially L-selectin dependent. *The Journal of Experimental Medicine*, 203, pp.1273–1282.

Galkina, E. et al., 2005. Preferential migration of effector CD8 + T cells into the interstitium of the normal lung. *The Journal of Clinical Investigation*, 115(12), pp.3473–3483.

Galkina, E. et al., 2007. T lymphocyte rolling and recruitment into peripheral lymph nodes is regulated by a saturable density of L-selectin (CD62L). *European Journal of Immunology*, 37, pp.1243–1253.

Gallatin, W.M., Weissman, I.L. & Butcher, E.C., 1983. A cell-surface molecule involved in organ-specific homing of lymphocytes. *Nature*, 304, pp.30–34.

Gallimore, A., Godkin, A. & Ager, A., 2013. High Endothelial Venules, Help or hindrance in the quest for antitumor immunity ? *Oncoimmunology*, 2(5), pp.e24272–1–e24272–3.

Gearing, A.J.H. & Newman, W., 1993. Circulating adhesion molecules in disease. *Immunology Today*, 14, pp.506–512.

Germain, R.N. & Stefanova, I., 1999. The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annual Review Immunology*, 17, pp.467–522.

Ghedin, E. et al., 2005. Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. *Nature*, 437, pp.1162–1166.

Gifford, J.L., Ishida, H. & Vogel, H.J., 2012. Structural Insights into Calmodulin-regulated L-selectin Ectodomain Shedding. *The Journal of Biological Chemistry*, 287(32), pp.26513–26527.

Gilliet, M. et al., 2003. Activation of influenza virus – specific CD4+ and CD8+ T cells : a new role for plasmacytoid dendritic cells in adaptive immunity. *Immunology*, 101(9), pp.3520–3526.

Golden-Mason, L. et al., 2004. Adult human liver contains CD8pos T cells with naive phenotype, but is not a site for conventional alpha beta T cell development. *The Journal of Immunology*, 172, pp.5980–5985.

Goulding, J. et al., 2014. CD8 T Cells Use IFN-γ To Protect against the Lethal Effects of a Respiratory Poxvirus Infection. *The Journal of Immunology*, 192, pp.1–11.

De Graaff, P.M. a et al., 2004. HLA-DP4 presents an immunodominant peptide from the RSV G protein to CD4 T cells. *Virology*, 326(2), pp.220–230.

Graham, M.B. & Braciale, T.J., 1997. Resistance to and Recovery from Lethal Influenza Virus Infection in B Lymphocyte – deficient Mice. *The Journal of Experimental Medicine*, 186(12), pp.2063–2068.

Grailer, J.J., Kodera, M. & Steeber, D.A., 2009. L-selectin : Role in regulating homeostasis and cutaneous inflammation. *Journal of Dermatological Science*, 56, pp.141–147.

Guenin-macé, L. et al., 2011. Mycolactone impairs T cell homing by suppressing microRNA control of L-selectin expression. *PNAS*, 108(31), pp.12833–12838.

Harding, F.A. & Allison, J.P., 1993. CD28-B7 Interactions Allow the Induction of CD8 + Cytotoxic T Lymphocytes in the Absence of Exogenous Help. *The Journal of Experimental Medicine*, 177, pp.1791–1796.

Hay, A.J. et al., 2001. The evolution of human influenza viruses. *The Royal Scociety*, 356, pp.1861–1870.

Hermesh, T. et al., 2011. Anti-viral instruction of bone marrow leukocytes during respiratory viral infections. *Cell Host and Microbe*, 7(5), pp.343–353.

Hickman, H.D. et al., 2015. CXCR3 Chemokine Receptor Enables Local CD8 + T Cell Migration for the Destruction of Virus-Infected Article CXCR3 Chemokine Receptor Enables Local CD8 + T Cell Migration for the Destruction of Virus-Infected Cells. *Immunity*, 42(3), pp.524–537.

Hill, C.A.S., Alexander, S.R. & Walcheck, B., 2003. Indirect capture augments leukocyte accumulation on P-selectin in flowing whole blood. *Journal of Leukocyte Biology*, 73, pp.464–471.

Hirata, T., Furie, B.C. & Furie, B., 2002. P-, E-, and L-selectin mediate migration of activated CD8+ T lymphocytes into inflamed skin. *The Journal of Immunology*, 169, pp.4307–4313.

Hossain, M.J. et al., 2010. Establishment and characterization of a Madin-Darby canine kidney reporter cell line for influenza A virus assays. *Journal of Clinical Microbiology*, 48, pp.2515–2523.

Hynes, R.O., 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*, 69, pp.11–25.

Igarashi, T. et al., 2007. Crystal structures of catrocollastatin/VAP2B reveal a dynamic, modular architecture of ADAM/adamalysin/reprolysin family proteins. *FEBS Lett*, 581, pp.2416–2422.

Ince, W.L. et al., 2013. Reassortment Complements Spontaneous Mutation in Influenza A Virus NP and M1 Genes To Accelerate Adaptation to a New Host. *Journal of Virology*, 87(8), pp.4330–4338.

Ivetic, A., 2013. Signals regulating L-selectin-dependent leucocyte adhesion and transmigration. *International Journal of Biochemistry and Cell Biology*, 45(3), pp.550–555.

Ivetic, A. et al., 2002. The Cytoplasmic Tail of L-selectin Interacts with Members of the Ezrin-Radixin-Moesin (ERM) Family of Proteins. *The Journal of Biological Chemistry*, 277, pp.2321–2329.

Janeway, C.A.J., Travers, P. & Walport, M., 2001. *Immunobiology: The Immune System in Health and Disease*,

Jefferson, T. et al., 2010. Vaccines for preventing influenza in the elderly. The Cochrane Library, 2, pp.1-201.

Jin, C. et al., 2012. Human synovial lubricin expresses sialyl Lewis x determinant and has L-selectin ligand activity. *The Journal of biological chemistry*, 287(43), pp.35922–33.

Johnson, B.J. et al., 2003. Single-cell perforin and granzyme expression reveals the anatomical localization of effector CD8+ T cells in influenza virus-infected mice. *PNAS*, 100(5), pp.2657–2662.

Jones, E. et al., 2003. The influence of macrophage inflammatory protein-1alpha on protective immunity mediated by antiviral cytotoxic T cells. *Immunology*, 109, pp.68–75.

Jung, T.M. & Dailey, M.O., 1990. Rapid modulation of homing receptors (gp90MEL-14) induced by activators of protein kinase C. Receptor shedding due to accelerated proteolytic cleavage at the cell surface. *The Journal of Immunology*, 144, pp.3130–3136.

Justewicz, D.M. & Lecomte, J., 1984. Blocking of Influenza Virus-Induced Cell-Mediated Cytotoxicity by Hemagglutinin-Specific Monoclonal Antibody. *The Journal of Infectious Diseases*, 150(3), pp.348–357.

Jutila, M.A. et al., 1989. Function and regulation of the neutrophil MEL-14 antigen in vivo: comparison with LFA-1 and MAC-1. *The Journal of Immunology*, 143, pp.3318–3324.

Kaech, S.M. et al., 2002. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell*, 111, pp.837–851.

Kaever, T. et al., 2014. Potent Neutralization of Vaccinia Virus by Divergent Murine Antibodies Targeting a Common Site of Vulnerability in L1 Protein. *Journal of Virology*, 88(19), pp.11339–11355.

Kalia, V. et al., 2010. Prolonged Interleukin-2R a Expression on Virus-Specific CD8+ T Cells Favors Terminal-Effector Differentiation In Vivo. *Immunity*, 32(1), pp.91–103.

Kambayashi, T. et al., 2015. Regulation of CD8 + T Cell Proliferation by 2B4/CD48 Interactions. *The Journal of Immunology*, 167, pp.6706–6710.

Kawashima, H. et al., 1999. Identification and characterization of ligands for L-selectin in the kidney . I . Versican , a large chondroitin sulfate proteoglycan , is a ligand for L-selectin. *The Japanese Society for Immunology*, 11(3), pp.393–405.

Kilian, K. et al., 2004. The Interaction of Protein Kinase C Isozymes and with the Cytoplasmic Domain of L-selectin Is Modulated by Phosphorylation of the Receptor. *The Journal of Biological Chemistry*, 279(33), pp.34472–34480.

Kishimoto, T.K. et al., 1989. Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. *Science*, 245, pp.1238–1241.

Klinger, A. et al., 2009. Cyclical expression of L-selectin (CD62L) by recirculating T cells. *International Immunology*, 21(4), pp.443–455.

Kotturi, M.F. et al., 2007. The CD8+ T-cell response to lymphocytic choriomeningitis virus involves the L antigen: uncovering new tricks for an old virus. *Journal of virology*, 81(10), pp.4928–4940.

Krieg, C. et al., 2010. Improved IL-2 immunotherapy by selective stimulation of IL-2 receptors on lymphocytes and endothelial cells. *PNAS*, 107(26), pp.11906–11911.

Kristensin, N. et al., 2004. Cytokine production by virus-specific CD8+ T cells varies with activation state and localization, but not with TCR avidity. *Journal of general virology*, 85, pp.1703–1712.

Ku, C.C. et al., 2000. Control of homeostasis of CD8 memory T cells by opposing cytokines. *Science*, 288, pp.675–678.

Lafouresse, F. et al., 2015. L-selectin controls trafficking of chronic lymphocytic leukemia cells in lymph node high endothelial venules in vivo. *Blood*, 126(11), pp.1336–1345.

Laouar, A. et al., 2008. Concurrent generation of effector and central memory CD8 T cells during vaccinia virus infection. *PLoS ONE*, 3(12).

Lefrancois, L., 2006. Development, trafficking and function of memory T-cell subsets. *Immunological Reviews*, 211, pp.93–103.

Lenardo, M.J., 1991. Interleukin-2 programs mouse T lymphocytes for apoptosis. *Nature*, 353, pp.858–861.

Ley, K. et al., 2007. Getting to the site of inflammation : the leukocyte adhesion cascade updated. *Nature Reviews*, 7, pp.678–689.

Li, O. et al., 2006. CD62L is required for the priming of encephalitogenic T cells but does not play a major role in the effector phase of experimental autoimmune encephalomyelitis. *Scandinavian journal of immunology*, 64(2), pp.117–24.

Lieberman, L. et al., 2015. CD62L (L-selectin) is Not a Reliable Biomarker for Predicting Risk of Progressive Multifocal Leukoencephalopathy in Natalizumab-Treated Multiple Sclerosis Patients. *Neurology*, 84. no. 14 supplement P4.039.

Lim, K. et al., 2015. Neutrophil trails guide influenza- specific CD8 + T cells in the airways. *Science*, 349(6252), pp.1071–1082.

Lin, L.C.W., Flesch, I.E.A. & Tscharke, D.C., 2013. Immunodomination during Peripheral Vaccinia Virus Infection. *PLoS Pathogens*, 9(4), pp.1–12.

Lukacher, A.E., Braciale, V.L. & Braciale, T.J., 1984. In Vivo Effector Function of Influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *The Journal of Experimental Medicine*, 160, pp.814–826.

Mace, T.A. et al., 2011. Differentiation of CD8 T cells into effector cells is enhanced by physiological range hyperthermia. *Journal of Leukocyte Biology*, 90, pp.951–962.

Mackenzie, C.D., Taylor, P.M. & Aksonas, B.A., 1989. Rapid recovery of lung histology correlates with clearance of influenza virus by specific CD8 + cytotoxic T cells. *Immunology*, 67, pp.375–381.

Masopust, D. & Schenkel, J.M., 2013. The integration of T cell migration, differentiation and function. *Nature reviews. Immunology*, 13(5), pp.309–20.

Mathew, A. et al., 2008. Robust intrapulmonary CD8 T cell responses and protection with an attenuated N1L deleted vaccinia virus. *PLoS ONE*, 3(10), pp.1–9.

McAdam, A.J., Schweitzer, A.N. & Sharpe, A.H., 1998. The role of B7 co-stimulation in activation and differentiation of CD4+ and CD8+ T cells. *Immunological Reviews*, 165, pp.231–247.

McGill, J., Van Rooijen, N. & Legge, K.L., 2008. Protective influenza-specific CD8 T cell responses require interactions with dendritic cells in the lungs. *The Journal of experimental medicine*, 205(7), pp.1635–1646.

McKenzie, I.F.C., 1978. Transfer of Specific Cutotoxic T Lymphocytes Protects Mice Inoculated With Influenza Virus. *Nature*, 273, pp.238–239.

Mckimm-Breschkin, J.L., 2012. Influenza neuraminidase inhibitors : antiviral action and mechanisms of resistance. *Respiratory Viruses*, 7, pp.25–36.

McNicholl, I.R. & McNicholl, J.J., 2001. Neuraminidase Inhibitors: Zanamivir and Oseltamivir. *The Annals of Pharmacotherapy*, 35, pp.57–70.

Medzhitov, R. & Janeway, C.A.J., 2000. Innate immunity. *The New England Journal of Medicine*, 343(5), pp.338–344.

Michiels, B. et al., 2011. A systematic review of the evidence on the effectiveness and risks of inactivated influenza vaccines in different target groups. *Vaccine*, 29(49), pp.9159–9170.

Michiels, B. et al., 2013. The Value of Neuraminidase Inhibitors for the Prevention and Treatment of Seasonal Influenza : A Systematic Review of Systematic Reviews. *PLOS ONE*, 8(4), pp.1–17.

Mobley, J.L. & Dailey, M.O., 1992. Regulation of adhesion molecule expression by CD8 T cells in vivo. I. Differential regulation of gp90MEL-14 (LECAM-1), Pgp-1, LFA-1, and VLA-4 alpha during the differentiation of cytotoxic T lymphocytes induced by allografts. *The Journal of Immunology*, 148, pp.2348–2356.

Mora, J.R. & Andrian, U.H. Von, 2006. T-cell homing specificity and plasticity : new concepts and future challenges. *Trends in Immunology*, 27(5), pp.235–243.

Moscona, A., 2005. Neuraminidase Inhibitors for Influenza. *The New England Journal of Medicine*, 353, pp.1363–1373.

Moutaftsi, M. et al., 2006. A consensus epitope prediction of murine T CD8+ -cell responses to vaccinia virus. *Nature Biotechnology*, 24(7), pp.817–819.

Mozdzanowska, K. et al., 1997. $CD4^+$ T Cells Are Ineffective in Clearing a Pulmonary Infection with Influenza Type A Virus in the Absence of B Cells. *Virology*, 239, pp.217–225.

Mozdzanowska, K., Maiese, K. & Gerhard, W., 2000. Th cell deficient mice control influenza virus infection more effectively than Th-and B cell-deficient mice: Evidence for a Th-independent contribution by B cells to virus clearance. *The Journal of Immunology*, 164, pp.2635–2643.

Murakawa, Y. et al., 1992. Association of Human Lymph Node Homing Receptor (Leu 8) With The TCR/CD3 Cpmlex. *The Journal of Immunology*, 148(6), pp.1771–1776.

Nakanishi, Y. et al., 2010. CTL mobilization to virus-infected tissue requires CD4+ T cell help. *Nature*, 462, pp.510–513.

Needham, L.K. & Schnaar, R.L., 1993. The HNK-1 reactive sulfoglucuronyl glycolipids are ligands for L-selectin and P-selectin but not E-selectin. *Biochemistry*, 90, pp.1359–1363.

Nelson, B.H. & Willerford, D.M., 1998. Biology of interleukin-2 receptor. *Advanced Immunology*, 70, pp.1–81.

Nishijima, K. et al., 2005. Costimulation of T-cell proliferation by anti- L -selectin antibody is associated with the reduction of a cdk inhibitor p27. *Immunology*, 116, pp.347–353.

Okkenhaug, K. & Vanhaesebroeck, B., 2003. PI3K IN LYMPHOCYTE DEVELOPMENT , DIFFERENTIATION AND ACTIVATION. *Nature*, 3, pp.317–330.

Olson, M.R., Mcdermott, D.S. & Varga, S.M., 2012. The Initial Draining Lymph Node Primes the Bulk of the CD8 T Cell Response and Influences Memory T Cell Trafficking after a Systemic Viral Infection. *PLoS Pathogens*, 8(12).

Onrust, S. V et al., 1996. Modulation of L-Selectin Ligand Expression during an Immune Response Accompanying Tumorigenesis in Transgenic Mice. *The Journal of Clinical Investigation*, 97, pp.54–64.

Palframan, R.T. et al., 2001. Inflammatory Chemokine Transport and Presentation in HEV : A Remote Control Mechanism for Monocyte Recruitment to Lymph Nodes in Inflamed Tissues. *The Journal of Experimental Medicine*, 194(9), pp.1361–1373.

Pardigon, N. et al., 1998. Role of co-stimulation in CD8+ T cell activation. *International Immunology*, 10(5), pp.619–630.

Pavalko, F.M. et al., 1995. The Cytoplasmic Domain of L-Selectin Interacts with Cytoskeletal Proteins Via α-Actinin : Receptor Positioning in Microvilli Does Not Require Interaction with alpha-Actinin. *The Journal of Cell Biology*, 129(4), pp.1155–1164.

Pepinsky, B. et al., 1992. Structure/function studies in vascular cell adhesion molecule-1. *The Journal of Biological Chemistry*, 276, pp.17820–17826.

Precopio, M.L. et al., 2007. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *Journal of Experimental Medicine*, 204(6), pp.1405–1416.

Preece, G., Murphy, G. & Ager, A., 1996. Metalloproteinase-mediated regulation of L-selectin levels on leucocytes. *The Journal of Biological Chemistry*, 271, pp.11634–11640.

Price, A.A. et al., 1997. Alpha 6 integrins are required for Langerhans cell migration from the epidermis. *The Journal of Experimental Medicine*, 186, pp.1725–1735.

Richards, H. et al., 2008. CD62L (L-selectin) Down-Regulation Does Not Affect Memory T Cell Distribution but Failure to Shed Compromises Anti-Viral Immunity. *The Journal of Immunology*, 180, pp.198–206.

Rodriguez, M. et al., 2003. Gamma Interferon Is Critical for Neuronal Viral Clearance and Protection in a Susceptible Mouse Strain following Early Intracranial Theiler's Murine Encephalomyelitis Virus Infection. *Journal of Virology*, 77(22), pp.12252–12265.

Roe, K., Orillo, B. & Verma, S., 2014. West Nile Virus-Induced Cell Adhesion Molecules on Human Brain Microvascular Endothelial Cells Regulate Leukocyte Adhesion and Modulate Permeability of the In Vitro Blood-Brain Barrier Model. *PLOS ONE*, 9(7), pp.1–12.

Rosen, S.D., 2004. Ligands for L-selectin: Homing, Inflammation, and Beyond. *Annual Review Immunology*, 22, pp.129–156.

Salek-Ardakani, S. et al., 2008. OX40 Drives Protective Vaccinia Virus-Specific CD8 T Cells. *The Journal of Immunology*, 181(11), pp.7969–7976.

Sallusto, F. et al., 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*, 401, pp.708–712.

Sánchez-puig, J.M. et al., 2004. Susceptability of different leukocyte cell types to vaccinia virus infection. *Virology Journal*, 1(10), pp.1–7.

Schatz, . G, Oettinger, M.A. & Schlissel, M.S., 1992. V(D)J recombination: molecular biology and regulation. *Annual Review Immunology*, 10, p.359.

Schenkel, J.M., 2014. Resident memory CD* T cells trigger protective innate and adaptive immune response. *Science*, 98, pp.98-101.

Schluns, K.S. et al., 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nature Immunology*, 1, p.426.

Schuster, K. et al., 2009. Homeostatic proliferation of naive CD8 1 T cells depends on CD62L / L-selectin-mediated homing to peripheral LN. *European Journal of Immunology*, 39, pp.2981–2990.

Schwartz, R.H., 1990. Culture Model for Lymphocyte Clonal Anergy. *Science*, 148, pp.1349–1356.

Shahinian, A. et al., 1993. Differential T Cell Costimulatory Requirements in CD28-Deficient Mice. *Science*, 261, pp.609–612.

Sher, A. & Coffman, R.L., 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Annual Review Immunology*, 10, p.385.

Shuford, B.W.W. et al., 1997. 4-1BB Costimulatory Signals Preferentially Induce CD8 T Cell Proliferation and Lead to the Amplification In Vivo of Cytotoxic T Cell Responses. *The Journal of Experimental Medicine*, 186(1), pp.47-55.

Shyjan, A.M. et al., 1996. Human mucosal addressin cell adhesion molecules-1 (MAdCAM-1) demonstrates structural and functional similarities to the alpha 4 beta 7-integrin binding domains of murine MAdCAM-1, but extreme divergence of mucin-like sequences. *The Journal of Immunology*, 156, pp.2851–2857.

Simon, J. & Werner, G.T., 1979. Vaccinia Virus Infection of the Central Nervous System in X-Irradiated Mice. *Infection and Immunity*, 25(3), pp.1035–1042.

Sinclair, L. V et al., 2008. Phosphatidylinositol-3-OH and nutrient-sensing mTOR pathways control T lymphocyte trafficking. *Nature Immunology*, 9(5), pp.513–521.

Smalley, D.M. & Ley, K., 2005. L-selectin: mechanisms and physiological significance of ectodomain cleavage. *Journal of Cellular and Molecular Medicine*, 9(2), pp.255–266.

Smith, G.L., Vanderplasschen, A. & Law, M., 2002. The formation and function of extracellular enveloped vaccinia virus. *Journal of General Virology*, 83, pp.2915–2931.

Smolen, J.E. et al., 2000. L-Selectin Signaling of Neutrophil Adhesion and Degranulation Involves p38 Mitogen-activated Protein Kinase. *The Journal of Biological Chemistry*, 275(21), pp.15876–15884.

Stadtmann, A. et al., 2013. The PSGL-1 – L-selectin signaling complex regulates neutrophil adhesion under flow. *The Journal of Experimental Medicine*, 210(11), pp.2171–2180.

Stamper, H.B. & Woodruff, J.J., 1976. Lymphocyte Homing into Lymph Nodes: In Vitro Demonstration of The Selective Affinity of Recirculating Lymphocytes for High Endothelial Venules. *The Journal of Experimental Medicine*, 144, pp.828–833.

Steeber, D.A. et al., 1997. Ligation of L-selectin Through Conserved Regions Within the Lectin Domain Activated Signal Transduction Pathways and Integrin Function in Human, Mouse, and Rat Leukocytes. *The Journal of Immunology*, 159, pp.952–963.

Streeter, P.R., Rouse, B.T.N. & Alto, P., 1988. Immunohistologic and Functional Characterization of a Vascular Addressin Involved in Lymphocyte Homing into Peripheral Lymph Nodes. *The Journal of Cell Biology*, 107, pp.1853–1862.

Subramanian, H. et al., 2012. Signaling through L-Selectin Mediates Enhanced Chemotaxis of Lymphocyte Subsets to Secondary Lymphoid Tissue Chemokine. *The Journal of Immunology*, 188, pp.3223–3236.

Sullivan, J.A. et al., 2012. FOXO3 Regulates CD8 T Cell Memory by T Cell-Intrinsic Mechanisms. *PLoS Pathogens*, 8(2), pp.32–43.

Taylor, A. et al., 2006. Mechanisms of immune suppression by interleukin- 10 and transforming growth factor-β : the role of T regulatory cells. *Immunology*, 117, pp.433–442.

Taylor, A.R., 1944. Chemical Analysis of The Influenza Virus A (PR8 Strain) and B (Lee Strain) and The Swine Inluenza Virus. *The Journal of Biological Chemistry*, 153, pp.675–686.

Taylor, P.M. & Askonas, B.A., 1986. Influenza nucleoprotein-specific cytotoxic T-cell clones are protective in vivo. *Immunology*, 58, pp.417–420.

Thimme, R. et al., 2003. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *Journal of virology*, 77(1), pp.68–76.

Tolonen, N. et al., 2001. Vaccinia Virus DNA Replication Occurs in Endoplasmic Reticulum-enclosed Cytoplasmic Mini-Nuclei. *Molecular Biology of the Cell*, 12, pp.2031–2046.

Tomala, J. et al., 2009. In Vivo Expansion of Activated Naive CD8+ T cells amd NK Cells Driven By Complexes of IL-2 and Anti-IL-2 Monoclonal Antibody As Novel Approach of Cancer Immunotherapy. *The Journal of Immunology*, 183, pp.4904–4912.

Topham, D.J. & Doherty, P.C., 1998. Clearance of an Influenza A Virus by CD4 T Cells Is Inefficient in the Absence of B Cells. *Journal of Virology*, 72(1), pp.882–885.

Townsend, A.R. et al., 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell*, 44, pp.959–968.

Tscharke, D.C. et al., 2005. Identification of poxvirus CD8 T cell determinants to enable rational design and characterization of smallpox vaccines. *The Journal of Experimental Medicine*, 201(1), pp.95–104.

Turner, G.S., 1967. Respiratory Infection of Mice with Vaccinia Virus. *Journal of General Virology*, 1, pp.399–402.

Unsinger, J. et al., 2009. Differential lymphopenia-induced homeostatic proliferation for CD4+ and CD8+ T cells following septic injury. *Journal of Leukocyte Biology*, 85(3), pp.382–390.

Varki, A., 1994. Selectin ligands. Proc. Natl. Acad. Sci., 91, pp.7390–7397.

Vassena, L. et al., 2015. HIV-1 NEF and VPU interfere with L-selectin (CD62L) cell surface expression to inhibit adhesion and signalling in infected CD4+ T lymphocytes. *Journal of Virology*, 89, pp.5687-5700.

Vazquez, M.I., Catalan-Dibene, J. & Zlotnik, A., 2015. B cells responses and cytokine production are regulated by their immune microenvironment. *Cytokine*, 74(2), pp.318–326.

Vestweber, D., 2007. Adhesion and signaling molecules controlling the transmigration of leukocytes through endothelium. *Immunological Reviews*, 218, pp.178–196.

Villarroel, V.A. et al., 2014. CXCR3-Mediated Skin Homing of Autoreactive CD8 T Cells Is a Key Determinant in Murine Graft-Versus-Host Disease. *Journal of Investigative Dermatology*, 134(6), pp.1552–1560. Available at: http://dx.doi.org/10.1038/jid.2014.2.

Volz, A. et al., 2014. Rapid Expansion of CD8+ T Cells in Wild-Type and Type I Interferon Receptor-Deficient Mice Correlates with Protection after Low-Dose Emergency Immunization with Modified Vaccinia Virus. *Journal of Virology*, 88(18), pp.10946–10957.

Vorobyeva, A.G. et al., 2014. Cyclopamine modulates gamma-secretase mediated cleveage of amyloid precursor protein by altering its subcellular trafficking and lysosomal degradation. *The Journal of Biological Chemistry*, pp.1–31.

Walcheck, B. et al., 2003. ADAM17-independent shedding of L-selectin. *Journal of Leukocyte Biology*, 74, pp.389–394.

Wang, Y. et al., 2009. Regulation of Mature ADAM17 by Redox Agents for L-Selectin Shedding. *The Journal of Immunology*, 182, pp.2449–2457.

Watson, M.L. et al., 1990. Genomic organization of the selectin family of leukocyte adhesion molecules on human and mouse chromosome 1. *The Journal of Experimental Medicine*, 172, pp.263–272.

Webster, R.G. et al., 1992. Evolution and Ecology of Influenza A Viruses. *Microbiological Reviews*, 56(1), pp.152–179.

Wedepohl, S. et al., 2012. L-Selectin – A dynamic regulator of leukocyte migration. *European Journal of Cell Biology*, 91(4), pp.257–264.

Wherry, E.J. et al., 2007. Correction Molecular Signature of $CD8^+$ T Cell Exhaustion during Chronic Viral Infection. *Immunity*, 27, pp.670–684.

Wherry, E.J. & Ahmed, R., 2004. Memory CD8 T-Cell Differentiation during Viral Infection. *Journal of Virology*, 78(11), pp.5535–5545.

Whittle, J.R.R. et al., 2011. Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. *PNAS*, 108(34), pp.1–6.

Williamson, J.D. et al., 1990. Biological characterization of recombinant vaccinia viruses in mice infected by the respiratory route. *Journal of General Virology*, 71, pp.2761–2767.

Wolint, P. et al., 2004. Immediate cytotoxicity but not degranulation distinguished effector and memory subsets of CD8+ T cells. *The Journal of Experimental Medicine*, 199(7), pp.925–936.

Wong, P. & Pamer, E.G., 2014. Disparate In Vitro and In Vivo Requirements for IL-2 During Antigen-Independent CD8 T Cell Expansion. *The Journal of Immunology*, 172, pp.2171–2176.

Wu, H. et al., 2010. Sustained viral load and late death in Rag2- / - mice after influenza A virus infection. *Virology Journal*, 7(172), pp.1–6.

Xiao, Z. et al., 2007. The CD8 T cell response to vaccinia virus exhibits site-dependent heterogeneity of functional responses. *International Immunology*, 19(6), pp.733–743.

Xu, J. et al., 1996. Impaired primary T cell responses in L-selectin-deficient mice. *The Journal of experimental medicine*, 183(2), pp.589–98.

Xu, R. et al., 2004. Cellular and Humoral Immunity against Vaccinia Virus Infection of Mice. *The Journal of Immunology*, 172, pp.6265–6271.

Yoon, H. et al., 2007. Sequential Activation of CD8 + T Cells in the Draining Lymph Nodes in Response to Pulmonary Virus Infection. *The Journal of Immunology*, 179, pp.391–399.

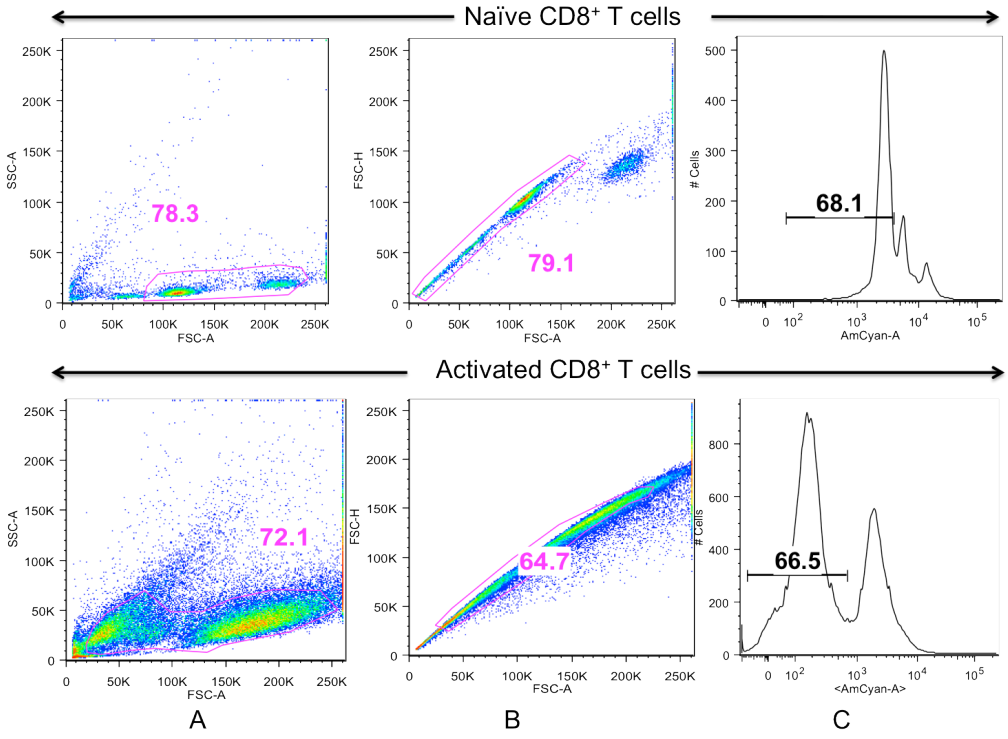
Yuen, T.J. et al., 2010. Analysis of A47 , an Immunoprevalent Protein of Vaccinia Virus , Leads to a Reevaluation of the Total Antiviral CD8 T Cell Response. *Journal of Virology*, 84(19), pp.10220–10229.

Zajac, A.J., Dye, J.M. & Quinn, D.G., 2003. Control of lymphocytic choriomeningitis virus infection in granzyme B deficient mice. *Virology*, 305, pp.1–9.

Zhao, Y., Adams, Y.F. & Croft, M., 2011. Preferential Replication of Vaccinia Virus in the Ovaries is Independent of Immune Regulation Through IL-10 and TGF-β. *Adaptive Immunity*, 24(5), pp.387–396.

Appendix A

Gating strategy for flow cytometry analysis



For flow cytometry analysis, population of interest was analyzed by creating a Boolean gate which was made of cell, based on the forward scatter (FSC-A) versus side scatter (SSC-A), singlet, based on the foreword scatter height (FSC-H) versus (SSC-A) and live based on the cells staining with aqua live dye (amcyan).

Appendix B