2-7-2013

Mechanisms of Lung Surveillance by Pathogen-Specific Cytotoxic T Lymphocytes

Young-Tae Lee
leechard75@gmail.com

Follow this and additional works at: http://digitalcommons.uconn.edu/dissertations

Recommended Citation
http://digitalcommons.uconn.edu/dissertations/4
MECHANISMS OF LUNG SURVEILLANCE
BY PATHOGEN-SPECIFIC CYTOTOXIC T LYMPHOCYTES

Young-Tae Lee, Ph.D.
University of Connecticut, 2013

Cytotoxic T lymphocyte (CTL)-mediated immunity plays a critical role in clearance of influenza virus-infected cells through contact-dependent interactions. Recent studies have described stationary tissue-resident memory (Trm) T cells which maintain CD69 and CD103 expression in peripheral tissues after local infections. These Trm cells are believed to contribute to protection after local reinfection, because they stay near the site of inoculation. Parabiosis studies previously showed that some non-migratory virus-specific CD8 T cells expressed both CD69 and CD103 in the airways after influenza virus infection. Since the TGFβ and TcR signaling pathways induce CD103 and CD69 expression on CD8 T cells in other models, I hypothesized that environmental factors and residual viral products support sustained surveillance of the lungs by pathogen-specific CTLs. In chapter 3, I used mice that express a dominant negative form of the TGFβRII (dnTGFβRII) to investigate the role of TGFβ signals in regulation of virus-specific CTL responses in the lungs after influenza virus infection. My data showed that TGFβ signals were necessary to reduce numbers of KLRG1+ virus-specific Teff cells which were capable of making effector cytokines during early infection. In addition, TGFβ signals were required for CD103 expression on virus-specific CTLs that were retained in the lungs during the
recovery stage of the infection. In chapter 4, I examined how antigen-persistence changes the virus-specific CTL response in the lungs using recombinant influenza viruses that differ in their ability to support prolonged antigen presentation. Comparisons of the CTL that responded to these viruses showed that prolonged antigen presentation reinforced expression of CD69 on virus-specific CTLs in the lung tissues, but not in the airways. I also used transfer studies to determine how CD69 influenced CD8 T cell migration in the lungs. Confocal microscopy showed that some CD69KO CTLs were collected around the blood vessels during the late stage of the infection. My data provide new insights into the generation and maintenance of virus-specific Trm cells in the lungs. CD69 and CD103 are both required for maintenance of long-lived Trm cells in the local tissues where they can provide immediate protective immunity against serologically distinct strains of influenza viruses.

Young-Tae Lee – University of Connecticut 2013
MECHANISMS OF LUNG SURVEILLANCE
BY PATHOGEN-SPECIFIC CYTOTOXIC T LYMPHOCYTES

YOUNG-TAE LEE

B.A., Dankook University, Korea, 2001

M.S. Dankook University, Korea, 2003

A Dissertation
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy
At the
University of Connecticut
2013
APPROVAL PAGE

Doctor of Philosophy Dissertation

MECHANISMS OF LUNG SURVEILLANCE
BY PATHOGEN-SPECIFIC CYTOTOXIC T LYMPHOCYTES

Presented by

Young-Tae Lee, B.A., M.S.

Major Advisor
Linda S. Cauley, Ph.D.

Associate Advisor
Robert Clark, M.D.

Associated Advisor
Robert Cone, Ph.D.

Associated Advisor
Stefan Brocke, M.D., Ph.D.

University of Connecticut
2013
ACKNOWLEDGEMENTS

This thesis would not have been possible without the help, support and patience of my adviser, Dr. Linda Cauley. I would like to thank for her for effort that she put into my education and training. I have learned a lot of molecular and cellular immunology and developed some scientific expertise in analyzing data. It was great pleasure working with her.

I also thank to my committee members, Dr. Robert Clark, Dr. Robert Cone and Dr. Stefan Brocke, who always encouraged me and also provided guidance and support for this project. I was really happy to discuss my data with them. I thank Dr. Mary Jo Turk who was my external reviewer for her positive feedback on my work as well as some important revisions.

I would like to thank our former and current lab members, Keith Bouchard, Tao Wu, Jenny Suarez, Yinghong Hu, Jason Redman and Carolina Aguila for their kindness, friendship and help. I thank Dr. Leo’s and Dr. Khanna’s labs for helpful discussion and advice. I also thank to my other colleagues and friends in the department.

The CCAM, FACS and histology facilities of the University of Connecticut Health Center have been indispensable. They generously helped me when I had problems. I also thank the administrators in the department of immunology for their support and assistance. The OVA MHCI tetramers that were used in my project were made at the University of Connecticut. All other tetramers were supplied by the NIH Tetramer Facility (Emory University Vaccine Center at Yerkes, Atlanta GA). I thank Dr. Clark and Dr. Hadley for providing the dnTGFβRII and CD103KO mice, respectively. Without these reagents this work would not have been possible.

I would like to thank my wife, Hee Seung Lee, for her personal support and great patience at all times. I also thanks to my son, Jihyuk Lee, who has brought us enormous happiness. I would like to acknowledge my other family members in Korea who have given me their unequivocal support.
TABLE OF CONTENTS

ABBREVIATIONS ........................................................................................................... vi
LIST OF FIGURES and TABLES ................................................................................. vii
CHAPTER I. INTRODUCTION ......................................................................................... 1
  1. CHARACTERISTICS OF INFLUENZA VIRUS ....................................................... 2
     A. INFLUENZA VIRUS AND HUMAN HEALTH .................................................... 2
     B. INFLUENZA VIRUS ENTRY AND REPLICATION IN THE LUNGS .......... 4
  2. IMMUNUE RESPONSES AGAINST INFLUENZA INFECTION ......................... 5
     A. INNATE IMMUNITY ........................................................................................... 5
        i. Sensing of influenza virus by innate recognition receptors .......... 5
        ii. Influenza viruses interfere with the innate immune system .... 6
        iii. The role of innate immune cells in response to influenza virus
             infection ........................................................................................................... 7
     B. CYTOTOXIC T LYMPHOCYTE IMMUNITY ......................................................... 9
        i. Signals that lead to naïve CD8 T cell activation ................. 9
        ii. Molecules that control CTL migration into the lungs ........... 9
        iii. CTL effector function .......................................................... 11
        iv. Heterosubtypic immunity .................................................... 15
        v. Heterogeneity of memory CD8 T cells ............................ 15
     C. CD4 T CELL RESPONSES .................................................................................. 16

CHAPTER II. ANIMALS, MATERIALS AND METHODS ............................................ 18

CHAPTER III. THE INFLUENCE OF TGFβ ON CTL RESPONSES IN THE LUNGS .................................................................................................................................. 23
  ABSTRACT ................................................................................................................. 24
  INTRODUCTION ......................................................................................................... 26
  RESULTS .................................................................................................................... 29
  DISCUSSION ............................................................................................................. 37
  FIGURES ................................................................................................................... 42
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>dnTGFβRII</td>
<td>Dominant-negative form of TGF beta receptor II</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HEV</td>
<td>High endothelial venule</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intercellular adhesion molecules 1</td>
</tr>
<tr>
<td>IEL</td>
<td>Intraepithelial lymphocyte</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL-7Rα</td>
<td>Interleukin-7 receptor alpha</td>
</tr>
<tr>
<td>KLRG1</td>
<td>Killer cell lectin-like receptor subfamily G member 1</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen1</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLN</td>
<td>Mediastinal lymph node</td>
</tr>
<tr>
<td>MPECs</td>
<td>Memory precursor effector cells</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>PA</td>
<td>Polymerase acidic protein</td>
</tr>
<tr>
<td>S1PR1</td>
<td>Sphingosine 1-phosphate receptor-1</td>
</tr>
<tr>
<td>SLECs</td>
<td>Short-lived effector cells</td>
</tr>
<tr>
<td>Tcm</td>
<td>Central memory cells</td>
</tr>
<tr>
<td>TcR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Teff</td>
<td>Effector T cells</td>
</tr>
<tr>
<td>Tem</td>
<td>Effector memory T cell</td>
</tr>
<tr>
<td>Trm</td>
<td>Tissue resident memory T cell</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
</tbody>
</table>
LIST OF FIGURES AND TABLES

Figure 3-1. The numbers of KLRG1+ virus-specific effector CTLs were increased in the absence of TGFβ signals. .................................42

Figure 3-2. CD103 is selectively expressed on KLRG1- virus-specific CTLs. .................................................................45

Figure 3-3. The numbers of cytokine-producing CTLs in the lungs were increased when TGFβ signaling was blocked. ..................47

Figure 3-4. Increased numbers of infiltrating leukocytes in the absence of TGFβ regulation in the lungs. .................................50

Figure 3-5. TGFβ signaling is required for CD103 expression. ..........52

Figure 3-6. Large numbers of KLRG1+ virus-specific CTL were distributed around the alveoli during the peak of the CTL response. ......54

Figure 3-7. CD103 helps virus-specific CD8+ T cells stay in the lungs. .....56

Figure 3-8. CD103 is required for retention of virus-specific CD8 T cells in the lungs. .................................................................58

Figure 3-9. Model to show that TGFβ signaling induces different responses from KLRG1- and KLRG1+ virus-specific CTLs. ..........61

Figure 4-1. Different influenza virus strains vary in their ability to support antigen persistence in the mediastinal lymph node (MLN). .....86

Figure 4-2. The presence of antigen changes the phenotypes of virus-specific CTL in the lung. .................................................................88

Figure 4-3. The antigen persistence increases the numbers of activated virus-specific CTL in the lung. .................................92

Figure 4-4. Interactions with MHCI molecules keep OVA-specific CTL activated during the late stage of the infection. ..................94

Figure 4-5. There is mild inflammation in the lungs during late antigen presentation. .................................................................96

Figure 4-6. CD69-deficiency does not have a major impact on CTL activation or IFNγ production. ..................................................98

Figure 4-7. CD69 expression is required for efficient CTL migration to the lungs. .................................................................101
Figure 4-8. Dual CD69/CD103KO induces inefficient migration of virus-specific CD8 T cells into the lungs. .....................................................104

Figure 4-9. The imaris software is used to count the transferred CD8 T cells. ...........................................................106

Figure 4-10. Dual transfers were used to show that CD69 deficiency reduces the numbers of virus-specific CD8 T cells in the lungs. .................................................................108

Figure 4-11. Confocal microscopy was used to analyze the distribution of dual CD69/CD103KO cell in the lungs. ......................110

Figure 4-12. Confocal microscopy shows that many CD69KO cells are close to the blood vessels during late antigen presentation. ..............................................................................112

Table 4-1. The numbers of virus-specific effector CTLs colocalized with the blood vessels. .....................................................116

Figure 4-13. The route of infection changes the distribution of virus-specific CD8 T cells in mucosal tissues. ..............................118

Figure 4-14. Bystander virus-specific memory CTLs are recruited in the small intestine during local infection with LM-OVA. ..........120

Figure 5-1. Parabiosis has been used to follow CD8 T cell migration in the blood and the spleen. .............................................130

Figure 5-2. CD69 may regulate virus-specific CTL migration to the peripheral tissues. .............................................................132
CHAPTER I:

INTRODUCTION
1. CHARACTERISTICS OF INFLUENZA VIRUSES

A. Influenza virus and human health

Influenza viruses are highly contagious pathogens which cause severe respiratory disease with symptoms such as fever, nausea, headache and pulmonary congestion. Seasonal variants cause high levels of morbidity and mortality annually, which mostly include elderly and sick people. These viruses cause 36,000 deaths and more than 200,000 hospitalizations in the United States each year \cite{1,2} with around 300,000 - 500,000 deaths and 3 - 5 million hospitalizations around the world \cite{3}. In addition to these annual epidemics, new strains of influenza virus can cross between species and cause occasional pandemics which can spread through human populations around the world. The viruses continually change due to high mutation rates and a segmented viral genome which can reassort to produce new strains \cite{4}.

Influenza viruses are from the A genus of the Orthomyxoviridae family which are common pathogens of the respiratory track \cite{4,5}. Based on antibody reactivity with their surface glycoproteins, which are hemagglutinin (HA) and neuraminidase (NA), influenza A viruses can be divided into several serotypes. Many serotypes have been identified in birds (H1-H16 and N1-N9), but only a few subtypes replicate efficiently in humans \cite{6}. Currently, most seasonal infections are caused by influenza A H1N1 and H3N2 subtypes and influenza B viruses. Evolutionary pressure causes constant antigenic drift leading to escape mutations which avoid the host’s previous antibody response \cite{7}. In addition when two different viruses infect the same cell the segmental genome can create a third reassortant strain. This process can be helped by an intermediate host such as the pig \cite{8}. To protect humans from influenza virus infections antibody based vaccines must be designed for different subtypes.
Major influenza pandemics have occurred three times in the twentieth century and once in the twenty first century. Between 1918-1919 the first documented H1N1 influenza virus caused an outbreak known as the "Spanish influenza pandemic", which resulted in more than 40 million deaths around the world and was the most devastating outbreak of influenza so far [9]. The HA gene in the 1918 virus did not contain some highly virulent sequence motifs which have been identified in the H5N1 avian influenza virus that is currently prevalent in birds [10,11]. It has been demonstrated that the variant of HA that was encoded in the 1918 virus played a pivotal role in the pathogenicity of the infection, while the NA protein did not play a major role [12]. Variants of the H1N1 strain still circulate in human populations, but in 1957 a second pandemic called the Asian influenza H2N2 strain developed, when the HA (H2), N2 and PB1 genes were replaced with genes from a new avian strain [13, 14]. Another pandemic developed in Hong Kong in 1968, which was caused by a reassortant A/H3N2 subtype. This virus contained new avian HA (H3) and PB1 genes in the background of the human 1957 H2N2 virus [8]. The most recent pandemic developed in 2009 and was caused by a new A/H1N1 variant which originated from swine leading to the first pandemic in the 21st century [4]. Other avian influenza viruses can also infect humans but do not spread efficiently between people including H7N7, H7N3, H9N2 and H10N7 [8, 15], as well as the new H5N1 strain that began to infect some humans in 1997. This is a highly pathogenic avian influenza virus (HPAI) can be transmitted to humans from domestic poultry [16, 17].

Some influenza virus infections are complicated by secondary bacterial infections which can lead to lethal pneumonia [8]. Many people who died during the 1918 pandemic had bacterial infections in the lungs. In addition, more than two-third of the deaths during the 1957 pandemic were associated with bacterial pneumonia [19]. Current data suggest that influenza viruses cause immune suppression, which leads to
secondary infections with common gram-negative bacteria including *Staphylococcus aureus, Haemophilus influenza* and *Streptococcus pneumonia* [18-20].

**B. Influenza virus entry and replication in the lungs**

Influenza A viruses contain eight negative-stranded RNA segments which encode 11 different proteins, which are hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), polymerase acidic protein (PA), matrix protein 1 (M1), matrix protein 2 (M2), non-structural protein 1 (NS1), nuclear export protein (NEP; NS2), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and PB1-F2 which is encoded by a shifted reading frame in the PB1 gene. The virus envelope contains two major glycoproteins which are HA and NA [8], which are the major targets of the humoral immune response [7]. Some conserved internal proteins such as the polymerase acidic protein (PA), polymerase basic protein 2 (PB2) and nucleoprotein (NP) contain epitopes that are targeted by the cellular immune response [8].

The respiratory track has a very large surface area which provides an entry point for a wide range of pathogens to enter the body [21]. The lungs are protected by many mechanisms including ciliated epithelial cells which help expel inhaled antigens via the mucociliary escalator [22]. When the virus enters the host cells, HA binds to sialic acid residues with terminal oligosaccharides which are primarily found on the lung epithelial cells. Avian influenza viruses preferentially interact with sialic-acids with galactose with $\alpha$-2,3 linkages, while human influenza viruses preferentially bind to sialic acid residues with $\alpha$-2,6 linkages [23]. The lung epithelial cells are an important target for influenza virus infection because they express a trypsin-like protease which is required for cleavage of hemagglutinin (HA0) into HA1 and HA2. This cleavage releases the carboxyl terminus of HA1 and produces the amino terminus HA2 protein which is essential for
membrane fusion and to release the viral genome into the cytoplasm of the host cells during the production of infectious influenza virus \(^{24-26}\). The three external proteins that are located in the lipid bilayer of the viral envelope are HA, NA and M2 which play a pivotal role in the life cycle of influenza A virus. The M2 protein forms a channel which induces endosome acidification, leading to uncoating of the virion \(^{8,27}\). In addition, the acidic pH in the endosome induces a conformational change of HA, to allow the exposure of internal amino acid groups, which is required for the insertion of fusion peptides into the host membrane. Eventually, the membrane breaks and a fusion pore allows individual viral RNPs (vRNAs) to be released into the host cytoplasm. Viral genomes are transported into the nucleus via nuclear localization signals (NLS) which interact with the NP protein \(^{7}\). When the NP, NS1 and NS2 proteins are newly synthesized they are transported into the nucleus and viral replication begins from a template RNA that is used to make full-length complementary RNA (cRNA) via a primer-independent mechanism. The M1, NS2 and NP proteins help transport the vRNPs into the cytoplasm. Once inside the host cells these proteins move to the plasma membrane where the HA and NA proteins accumulate and form a budding site. Finally, the NA glycoprotein helps release the progeny viruses from the infected host cells via cleavage of sialic acids which are bound to new HA proteins \(^{7}\).

2. IMMUNE RESPONSE AGAINST INFLUENZA INFECTION

A. Innate immunity

i. Sensing of influenza virus by innate recognition receptors

Influenza virus infection elicits host immune responses which are crucial to stop viral replication. The innate immune system provides the first line of defense against influenza virus infection. When influenza viruses enter the host cells, they are
recognized by pattern recognition receptors (PRRs) such as toll-like receptor 7 (TLR7), retinoic acid inducible gene I (RIG-I) and the NOD-like receptor family pyrin domain containing 3 (NLRP3) protein, which can recognize pathogen-associated molecular patterns (PAMPs) of influenza virus \[28\]. Endosomal TLR7 can detect single-stranded RNA and cytoplasmic RIG-I recognizes 5’-triphosphate viral RNA\[29\]. Furthermore, activated RIG-I binds to the mitochondrial antiviral signaling protein (MAVS) which activates NF-kB to induce proinflammatory cytokines \[29\]. Signals from these receptors result in the production of proinflammatory and antiviral cytokines such as IL-6, IL-12, and type I interferon α/β \[30-32\]. Proinflammatory IL-6 and IL-12 play a role in the cytotoxic response by enhancing virus-specific CTL activity to produce interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) \[33\]. In addition, recent studies have demonstrated that NLRP3 inflammasome activation plays a crucial role in the detection of influenza viruses when caspase-1 activates the immature forms of pro-IL-1β and pro-IL-18 \[34,35\]. Production of IL-1β and IL-18 is important to recruit monocytes and neutrophils into the lung through a process that requires two signals such as TLR (signal 1) and various stress signals including potassium efflux and cell membrane perturbation (signal 2) \[29\]. A recent study has shown that the M2 ion channel plays an important role in inflammasome activation by the induction of ion imbalance in cytoplasm (signal 2) \[36\]. These data show that influenza virus can provide both signal 1 (TLR7) and signal 2 which result in inflammasome activation \[29\].

**ii. Influenza viruses interfere with the innate immune system**

Some influenza virus encoded proteins can interfere with the host immune response. The non-structural protein (NS1) disturbs the nuclear export of mRNA by forming a complex with the nuclear-export machinery \[37\]. In addition, NS1 targets the ubiquitin ligase TRIM25 which is essential for RIG-I mediated antiviral activity, resulting in evasion
from recognition of retinoic acid-inducible gene I (RIG-I) [38] and reduces production of the interferon α/β and proinflammatory cytokines [39,40]. Recent studies have demonstrated that NS1 is capable of preventing infected DC from maturating, suggesting that its activity influences both innate and adaptive immune responses [41,42]. The influenza virus PB1-F2 is an 87-amino acid protein which is encoded by an alternate reading frame of the PB1 gene. It plays a role in triggering apoptosis of infected cells via mitochondrial permeabilization, resulting in the release of cytochrome C that interacts with caspase-9 [43]. In addition, PB1-F2 inhibits production of type I interferon by binding the MAVS adaptor protein [44].

iii. The role of innate immune cells in response to influenza virus infection

During influenza virus infection many innate immune cells are involved in the clearance of the virus or virus infected cells. Natural killer (NK) cells contribute to the elimination of some infected cells and produce large amounts of proinflammatory cytokines such as IFNγ and TNFα. In fact, Fc receptor-bearing NK cells can recognize antibody-bound infected cells and lyse them by releasing perforins and granzymes [45]. In addition, NKp44 and NKp46 which are cytotoxicity receptors can bind to hemagglutinins and lyse influenza-infected cells by enhancing granzyme B and perforin secretion [46-48].

There are two major types of phagocyte cells in the lungs, which are alveolar macrophage and dendritic cells (DCs). Resting alveolar macrophages produce low levels of inflammatory cytokines. Upon influenza virus infection, the numbers of alveolar macrophages increase and they phagocytose influenza virus-infected cells. Activated alveolar macrophages also produce TNFα and nitric oxide synthase 2 (NOS2) which is plays a role in inflammation. In the latter case macrophages can contribute to pathology.
in the lungs \[^{49,50}\]. Alveolar macrophages are thought to have regulatory function by production of IL-10 and transforming growth factor-β (TGFβ) which are anti-inflammatory cytokines \[^{51}\].

DCs are professional antigen presenting cells (APCs) which play a pivotal role in connecting the innate and adaptive immune responses. DCs are innate immune cells that have the capacity to recognize PAMPs using PRRs including TLR and NOD-like receptors (RLRs) \[^{52}\]. In the murine model there is evidence that CD11b+ DCs mainly express TLR2 and TLR7, while CD103+ DC mainly express TLR3 \[^{53}\]. However, both CD11b+ and CD103+ DCs express low levels of TLR4. Upon influenza virus infection, the conventional DCs are thought to utilize RIG-I to recognize influenza virus, but plasmacytoid DCs (pDCs) which make type I IFN α/β are thought to use TLR 3, 7 and 9 \[^{54}\]. Interferons are key molecules that induce DC maturation. The conventional DCs produce a lot of proinflammatory cytokines such as IL-6, IL-12, TNFα, IL-8 \[^{55}\]. A recent study showed that TNFα/inducible nitric oxide synthase (iNOS) producing DCs (tipDCs) can be recruited into the lungs using CCR2 during early influenza virus infection and present antigens to virus-specific CTLs \[^{56}\].

Different subsets of DCs have been identified in the lungs using characteristics such as anatomical distribution, surface markers and migratory patterns \[^{57,58}\]. The respiratory DCs include two migratory subsets which are CD11b+ and CD103+ DCs play a pivotal role in the initiation of adaptive immune responses. Both DCs reside underneath the airway epithelium and continuously sample antigens from the airway lumen by extending their dendrites \[^{69,60}\]. Once the DCs uptake antigens, they migrate into the mediastinal lymph node (MLN) through the afferent lymphatic vessels using CCR7, which is lymph node homing receptor. During migration the DCs become
activated and upregulate costimulatory molecules such as CD80 and CD86 on their surface [61]. The mature DCs present antigens as small peptides in the context of major histocompatibility complex (MHC) and elicit robust adaptive immune responses in the MLN and spleen [62,63].

B. Cytotoxic T lymphocyte immunity

i. Signals that lead to naïve CD8 T cell activation

CD8 T cells are important cells for immune surveillance of the body against a variety of infections, because they can migrate into many different tissues where virus infections begin. Naïve CD8 T cells express L-selectin (CD62L), chemokine receptor 7 (CCR7) and leukocyte function antigen-1 (CD11a) which play different roles in the rolling, adhesion, and T cell extrasvasation into the peripheral lymph nodes via HEV [64]. Naïve CD8 T cells circulate through secondary lymphoid organs until they meet APCs with their cognate antigen. Cognate antigen recognition and costimulatory signals are required for naïve to become effector CD8 T (Teff) cells. At this time, T cell receptor (TcR ; signal 1) on naive CD8 T cells interacts with processed peptides in the context of MHC class I molecules and CD28 (signal 2) on CD8 T cells also interact with the costimulatory molecules including CD80 and CD86 on DCs [65]. In addition, a third signal such as IL-12 is required for full differentiation and effector functions [66]. After antigen encounter, the naive CD8 T cells undergo the phenotypic changes to become Teff cells which migrate to the lungs during influenza virus infection [67].

ii. Molecules that control CTL migration into the lungs

Antigen stimulation plays a central role in the generation of Teff and memory T cells which play a role in protective immunity. Once T cells become activated, they migrate into infected site, using many different integrins and chemokine receptors, and ultimately
kill infected cells by contact-dependent interactions \cite{21}. Integrins are cell surface receptors that form adhesions between migrating cells and the extracellular matrix. The family includes 18 $\alpha$ subunits and 8 $\beta$ subunits which pair $\alpha\beta$ subunits \cite{68}. Integrins play crucial roles in leukocyte activation, extravasation and cytotoxic killing activity. Activated CD8 T cells express high levels of $\beta_2$-integrin lymphocyte function-associated antigen-1 (LFA-1; $\alpha$L$\beta_2$ integrin) and $\alpha_4$ integrin very late antigen-4 (VLA-4; $\alpha_4\beta_1$) \cite{69}. LFA-1 binds to intercellular adhesion molecules 1 and 2 (ICAM1 and ICAM2) which are constitutively expressed on the vascular endothelium and in the interstitium \cite{70}. The intracellular signals by CCL19 and CCL21 increase the integrin affinity by inducing its conformational change \cite{71}. VLA-4 interacts with vascular cell-adhesion molecule 1 (VCAM1) and mucosal vascular addressin cell-adhesion molecule 1 (MADCAM-1). A previous study demonstrated that LFA-1 plays a role in retention of activated CD8 T cells in the lungs, rather than delayed the endothelial transmigration, but there is no effect on cell adhesion after blockade of VLA-4 \cite{69}. When influenza specific CTLs arrive at the airways, CD11a is down-regulated within 40 hours, it can be re-expressed when the cells are transferred into other mice \cite{72;73}. A recent study reported a mechanism by which nearly the entire extracellular domain of CD11a is cleaved by metalloproteinase-9 (MMP-9) from macrophages \cite{74}. Since influenza virus-specific CTLs lose the ability to recirculate after they reach the airways \cite{75}, it is likely that they are removed by mucociliary escalator. The CD11a$^{low}$ cells in the airways are functional in proliferation, but they are impaired in cytolytic activity during respiratory virus infection \cite{76;77}. However, these cells may contribute to protective immunity by stabilizing the interactions between CTL and the target cells during cytolysis using interactions between CD103 and E-cadherin which play a role in cytolytic function and by secretion of proinflammatory cytokines \cite{78;79}. 
Another study has shown that α1 integrin VLA-1 (very late antigen-1; α1β1), which binds to type IV collagen, is highly expressed on the influenza virus-specific CTLs that can be collected from the airways by bronchoalveolar lavage (BAL). This study demonstrated that VLA-1 expressing CD8 T cells have higher ability to secrete IFNγ, than influenza virus-specific CD8 T cells which lack VLA-1 expression, and are retained in the lungs where they provide protective immunity against secondary challenge \([80,81]\). The VLA-1+ CD8+ T cells in the lungs expressed CD69+, and CD44^{high} but lacked CD62L, suggesting that they were Teff cells.

Chemokine receptors are G-protein-coupled receptors which have a 7 transmembrane structure. There are 19 distinct chemokine receptors in mammals which can be divided into 4 different subfamilies based on the number and disposition of cystein residues (CC, CXC, CX3C and XC) in near their amino terminus. Chemokine receptors play a central role in chemotaxis and recruit T cells to inflamed sites in response to chemokine ligands \([82]\). A recent study demonstrated that CXCR3 (the receptor for CXCL10) plays a role in virus-specific CTL migration to the lungs during acute respiratory infection. However, CCR5 expression, but not CXCR3, plays a role in recruitment of memory CTL into the airways after secondary infection with another strain \([83]\). Furthermore, CXCR3 and CCR5 deficiency caused increase a numbers of memory precursor cells in the lungs and spleen, which may be due to limited access to antigen in the lungs which can cause activation-induced cell death \([84]\).

### iii. CTL effector function

Cytotoxic T lymphocytes (CTLs) play a key role in eliminating influenza virus infected cells by inducing apoptosis through contact-dependent interactions. Therefore, influenza virus-specific CTLs must be recruited to the lung epithelial cells that are
releasing the new infectious virus. After engagement of the T cell receptors with cognate antigen in the context of MHC I, the CTLs secrete cytolytic granules into the immunological synapse. CTL can destroy more than one infected cell during sequential interactions with multiple cells. This killing activity does not require costimulatory signals.

Infections with lymphocytic choriomeningitis virus (LCMV) and Listeria monocytogenes (LM) induce heterogenous populations of Teff cells which can be identified using different surface markers such as IL-7Rα (CD127) and killer lectin-like receptor G1 (KLRG1). CD127+KLRG1- antigen specific CD8 T cells is memory precursor effector cells (MPECs) which are characterized by high levels of CD127, CD44, CD62L, CD27, CD122 and Bcl-2 expression. The MPECs produce larger amounts of IL-2, IFNγ and TNFα, than CD127- KLRG1+ CD8 T cells, after exposure to antigen \(^{[85]}\). MPECs are capable of self-renew and to proliferate in response to IL-7 and IL-15 \(^{[86]}\). In contrast KLRG1 is a marker for short-lived effector cells (SLECs) which are able to proliferate in response to antigen and can make cytokines such as IL-2 and IL-15, but they do not develop into long-lived memory CD8 T cells \(^{[87]}\). In addition, during acute infection with LCMV MPEC and SLECs have similar effector functions, including cytolytic activity and IFNγ production \(^{[87,88]}\). In addition, CD127+KLRG1+ CD8 T cells which are double-positive effector cells (DEPCs) revealed intermediate life span and proliferation compared to MPECs and SLECs \(^{[87]}\).

Others have suggested that the generation and survival of SLECs and MPECs is regulated by many different factors such as transcription factors, cytokines, and co-stimulatory molecules. The transcription factors T-bet and Blimp-1 play a role in the development of SLECs. High levels of T-bet expression decrease IL-7Rα, but low level is
sufficient for IL-2/IL-15Rβ (CD122) expression [87,89,90]. In addition, IL-12 is able to increase T-bet expression during pathogen specific CTL differentiation [91].

Two major mechanisms of CTL-mediated killing have been indentified, which are Ca^{2+} -dependent perforin/granzyme mediated apoptosis and Ca^{2+} -independent Fas/Fas ligand-mediated apoptosis [92]. A recent study demonstrated that perforin induces pores, which increase Ca^{2+} influx into the target cells. In turn granzymes undergo endocytosis and are released into the cytosol of the target cells [93]. There are 10 different granzymes in mice including granzyme A and B which are the most abundant molecules [94]. It has been shown that granzyme A and B play different role in CTL function. Granzyme B induces an apoptotic pathway by cleaving capspases-3 and -9, followed by DNA fragmentation [95], while granzyme A is part of a caspase independent pathway which induces oligosomal DNA degradation [96]. Following influenza virus infection, two different subsets of virus-specific CTLs can be identified based on granzyme A and B expression. During early infection (6dpi), there were more virus-specific CTLs that were producing granzyme A together with granzyme B in the lungs, than virus-specific CTLs which were producing granzyme B alone. However, during peak of responses (10dpi), virus-specific CTLs which were making granzyme B alone were outnumbered granzyme A/B-producing virus-specific CTLs in the lungs [97].

During antigen stimulation Teff cells upregulate Fas ligand (CD95L) which interacts with Fas (CD95) on the target cells. The Fas molecule is a member of the TNF receptor superfamily of molecules that contain an intracellular death domain to initiate caspase-dependent apoptosis. CD95/CD95L engagement activates caspase-8 directly, which can cleave Bid into its active form tBid which induces permeabilization of the mitochondria membrane to release cytochrome c, which in turn activates caspase 9 [98].
Virus-specific Teff functions, including cell-mediated cytotoxicity and proinflammatory cytokines, can damage the host during acute infection. To inhibit excessive immune responses several different mechanisms have been shown to shutdown the Teff responses by inducing apoptosis. Apoptosis is regulated by intrinsic factors that are the B cell lymphoma-2 (Bcl-2) protein family such as prosurvival (Bcl-2, Bcl-XL, Mcl-1, and Bcl-w) and proapoptotic molecules (Bax, Bak, Bad, Bim, Bid, and Puma). The prosurvival factors Bcl-2 and Bcl-XL inhibit Bim and Puma which release of cytochrome c from the mitochondrial membrane to activate caspase-9. The balance of both prosurvival and proapoptotic molecules maintains the integrity of the mitochondrial membrane and control apoptosis [99;100]. After viral clearance, the majority of effector CTLs undergoes massive programmed death, which is called the contraction phase and small numbers of memory CTLs survive and confer long-term protection against reinfection. During the contraction phase the proapoptotic molecules Bim and Puma play a role in induction of apoptosis [101;102]. In addition reduced amount of cytokines such as IL-2, IL-4, IL-7, and IL-15 promote the Teff cell death [103], since the signals from these cytokine are able to increase Bcl-2 and Bcl-XL levels.

When activated CD8 T cells are repeatedly exposure to antigens, apoptosis is induced by the Fas pathway resulting in activation-induced cell death (AICD) [104]. However, others suggested that CD4 and CD8 T cells use different death pathways [105]. This study suggested that the Fas signal-induced AICD may limit in CD4 T cells, rather than CD8 T cells which die by other mechanisms included TGFβ. TGFβ is a pleiotrophic cytokine which its signal controls a variety of cellular processes, such as cell fate decisions, proliferation and survivals and also acts as both proinflammatory and immunosuppressive cytokines [106]. A recent study demonstrated that TGFβ signaling
plays a role in selective induction of SLEC apoptosis during early clonal expansion by reducing Bcl-2 \[^{107}\].

iv. Heterosubtypic immunity

Influenza viruses undergo continuous antigenic mutation which allows variants to escape from the antibody response. Since CTLs can recognize conserved internal proteins, they can provide short-term heterosubtypic immunity between different subtypes \[^{108,109}\]. Memory CTLs kill target cells faster than naïve CD8 T cells and thus promote faster viral clearance than primary infection. Antigen-specific CD8 T cell numbers are greatly expanded after secondary challenge in the upper respiratory tract \[^{110}\]. Adoptive transfer of NP-specific CD8 T cells into the trachea can protect mice from challenge of a lethal influenza virus infection \[^{111}\]. After primary influenza infection, some Tem cells remain in the peripheral tissues and can mediate an accelerated response to reinfection \[^{112,113}\].

v. Heterogeneity of memory CD8 T cells

After viral clearance, a large numbers of Teff cells undergo contraction phase, however small numbers of CD8 T cells survive and become memory T cells which express CD127. These memory cells can be subdivided into different subsets such as central memory cells (Tcm : CCR7\(^{hi}\)CD62L\(^{hi}\)) and effector memory cells (Tem : CCR7\(^{lo}\)CD62L\(^{lo}\)) based on the expression of the lymphoid homing receptors, CCR7 and CD62L \[^{112}\]. Some Tem cells are preferentially found in the peripheral tissues, but Tcm cells are mainly located in the secondary lymphoid organs. Compare to Tcm cells, Tem cells mediate rapid cytotoxic activity after encounter antigen stimulation \[^{112,113}\].

Recent studies have demonstrated the third subsets of memory T cells which are
tissue-resident memory (Trm) CD8 T cells in peripheral tissues such as the skin, gut, salivary gland, lung and brain in different infectious models \cite{80,114-118}. Most Trm cells maintain an activated phenotype similar to Tem cells. In addition Trm cells express CD69 and CD103. These Trm cells are less proliferative, than Tcm cells, and poorly replenished from the circulation. In addition, there is evidence that some Trm are able to making cytokines after \textit{in vitro} stimulation. For example, Trm cells in the lungs also have the ability to produce cytokines including TNF\(\alpha\), IFN\(\gamma\), IL-4, IL-13, and IL-17 in the presence of heat killed influenza virus pulsed APCs \cite{115}. In addition, Trm cells from the brain or the salivary glands are able to produce granzyme B and IFN\(\gamma\), respectively \cite{116,118}. These data suggest that Trm cells exist as long-lived memory pools and mediate protective immune responses during local infections \cite{116,119,120}.

**C. CD4 T cell responses**

It has been shown that activation of CD4 T cells require cognate antigen stimulation by interacting peptides in the context of MHCII molecules presented by antigen presenting cells (APCs) in the draining lymph nodes (DLNs) \cite{121}. The activated CD4 T cells undergo clonal expansion and differentiate into distinct subsets of Teff cells in the presence of different cytokines. When naïve CD4 T cells differentiate in the presence of IL-4, these cells become T helper 2 (Th2) cells which produce IL-4, IL-5, and IL-13. These Th2 cytokines stimulate B cells to produce IgG1 and IgE antibodies \cite{122}. However, in the presence of IL-12 and IFN\(\gamma\) naïve CD4 T cells differentiate into Th1 cells which can mediate cellular immunity \cite{123}.

Previously, others demonstrated that Th1 cells were more effective than Th2 cells in protection against a lethal dose of influenza virus infection, as demonstrated by adoptive transfer of either Th1 or Th2 cells isolated from influenza virus-infected mice \cite{124,125}.
Some HA-specific CD4 T cells that are recovered from the BAL and lungs are capable of making high levels of Th1 cytokines IFNγ and TNFα ex vivo, but Th2 cytokines such as IL-4 are undetectable [126]. In addition, previous study demonstrated that CD4 T cells acquired perforin-mediated cytotoxicity and increased B cell responses which produce neutralizing antibodies during influenza virus infection [127].

However, influenza virus-specific CD4 T cells undergo more rapid contraction phase in the lung parenchyma and the airways than influenza virus-specific CD8 T cells [128]. Therefore, smaller numbers of virus-specific CD4 T cells persist in the lungs, compared to virus-specific CD8 T cells, but there was no difference in spleen and lymph nodes. After secondary challenge virus-specific memory CD4 T cells are greatly expanded in the upper respiratory tract [110]. These memory CD4 T cells have a role in innate immunity because they are able to produce large amounts of cytokines including IFNγ, IL-12 and IL-6 within 48 hours after influenza virus infection [129]. In addition, memory CD4 cells also promote the maturation of antigen presenting cells by production of proinflammatory cytokines such as IL-2 [130], which further enhances virus-specific CD8 T cell responses such as CTL activation and trafficking. Therefore, CD4 helper cells play an important role in regulating heterosubtypic immunity.
CHAPTER II:

ANIMALS, MATERIALS AND METHODS
Mice

C57BL/6 and congenic CD45.1+ mice were purchased from Charles River through the NCI animal program. The CD69KO [131], CD103KO [132], dnTGFβRII [133], GFP+2.3 [134] and Ubiquitin-GFP+ mice [135] were generously provided by other investigators and cross-bred with F5 and OTI TcR transgenic mice lines at the University of Connecticut Health Center. All experiments were performed in accordance with regulations by the American Association for Accreditation of Laboratory Animal Care, as well as federal and state agencies.

Bone marrow chimeras

Between 4 - 6 weeks of age female mice were lethally irradiated (1000 rads) and one day later treated with 200g anti-CD8 antibodies (Clone 2.43) to deplete remaining host-derived CD8 T cells. Bone marrow cells from dnTGFβRII+ and wild type mice were mixed at different ratios (total 10^7) before transfer to lethally irradiated C57BL/6 recipients. Congenic CD45.1 and CD45.2 markers were used distinguish the mutant and wild CD8 T cells. Recipient mice were maintained for 6 - 8 weeks before viral infection.

Viruses and infections

Mice were anesthetized by i.p. injection with 250 – 300 µl (1.6 g/ml avertin) (2,2,2-tribromoethanol) and received either 600 50% egg infectious doses (EID₅₀) E61-13-H17 influenza virus, or 10^6 EID₅₀ X31-OVA [136], or 600 EID₅₀ HKx31, or 10³ pfu WSN-OVAI [67] intranasally (i.n.). The fertilized chicken eggs were purchased from the Charles River Laboratory and virus stocks were grown, titered and stored as described previously [137].
Transfer experiments

Spleens and peripheral lymph nodes were dissociated to form single cell suspensions and depleted of red blood cells. For in vivo competition assays recipient animals received a total of $10^6$ mixed lymphocytes by tail vein injection. Alternatively, lymphocytes were labeled with 1μM carboxy fluorescein diacetate succinimidyl ester (CFSE)-dye at 37°C for 10mins and 2x10^5 cells were transferred to the recipients [138].

Sample preparation for tetramer analysis

Non-adherent cells were collected from the lungs by lavage 5 times in Hank's balanced saline solution (HBSS). Mice were exsanguinated. Ground glass microscope slides were used to make cell suspensions from spleens and peripheral lymph nodes. Red blood cells were lysed with ammonium chloride and lymphocytes were filtered through cell strainers. Lymphocytes were released from chopped lung tissue by digestion with 150 U/ml collagenase (Life Technologies, Rockville, MD) in RPMI, 1 mM MgCl₂, 1 mM CaCl₂ and 5% FCS, at 37°C for 90 mins. Cells were pushed through strainers and spun on 44/67% Percoll gradients at 400g for 20 mins. Lymphocytes were washed with PBS. Washed lymphocytes were stained with tetramers that are specific for the influenza virus NP₃₆₆₋₃₇₄/Dᵇ and PA₃₂₄₋₃₃₃/Dᵇ epitopes as described previously [139;140]. Lymphocytes were stained with APC-conjugated tetramers and anti-CD8 (clone 53.6.72) for 1 h. at room temperature. For flow cytometer analysis all other markers were stained at 4°C using mAb specific for CD45.1, CD45.2, CD44, CD69, CD103, CD127, PD-1, KLRG1 and CD62L (eBioscience or BD Pharmingen, San Diego CA). The samples were analyzed on a Becton-Dickinson FACSCalibur or LSR-II flow cytometer and analyzed using Flowjo software (Tree Star Inc.). Dying cells were identified using a fluorescent dye that interacts with free amines (Invitrogen). To block antigen presentation, mice were treated twice with 25D1.16 or control antibodies, 20dpi (100ug i.n. plus 400ug i.v.)
Confocal microscopy

Visualize transferred OTI cells *in situ* fragments of lung tissues were fixed in 2% (1% used for GFP+ cells) paraformaldehyde (PFA) for 20 mins at 4°C. Lung tissue was stained for 6hr at 4°C with the antibodies, including Ep-CAM, CD31, CD45.1 and anti-GFP antibodies (eBioscience or BD Pharmingen, San Diego CA). The washed tissues were mounted on slides using Shandon Immu-Mount or 50% glycerol in 2x PBS 5mM MgCl₂ for GFP+ cells. Images were collected using a Zeiss LSM-780-Meta confocal microscope with an automated Axiovert 100M platform with xyz control. Image data was analyzed using Metamorph® software (Universal Imaging Corp, Downingtown, PA) and Imaris® Suite (Bitplane Inc). Colocalization and isosurfacing were applied to multiple images using Imaris imaging software.

Intracellular cytokine staining analysis

Lymphocytes were enriched from the Spleen and lungs. For *in vitro* peptide stimulation, lymphocytes (total 5x10⁶ cells/well) were cultured in the presence of NP or PA peptide (1μg/ml) with Brefeldin A (BFA) (20μg/ml) for 5 hrs at 37°C incubator. Stimulated lymphocytes were stained with antibodies, including CD45.1, CD45.2 and CD8 and fixed with 2% PFA on ice. Lymphocytes were permeabilized with 0.1% saponin and stained for cytokines, including IFNγ and TNFα

Statistics

Unless otherwise stated, all experiments were repeated at least twice using a minimum of 3 animals per group. Statistical significance was determined using an unpaired two-tailed Student T test. The comparisons that were used to generate P
values are indicated by horizontal lines (* P <0.05, ** P <0.01, *** P <0.001).
CHAPTER III.

THE INFLUENCE OF TGFβ ON CTL RESPONSES IN THE LUNGS
ABSTRACT

Large percentages of the virus-specific CD8 T cells that express CD103 stay in the lungs during the recovery from influenza virus infection. Since TGF\(\beta\) is known to induce CD103 expression on CD8 T cells in other models, we crossed F5 TcR transgenic mice with dnTGF\(\beta\)RII mice that express a dominant negative form of the TGF\(\beta\)RII (dnTGF\(\beta\)RII) to investigate whether TGF\(\beta\) induced CD103 expression in the lungs. When I transferred the F5-WT and F5-dnTGF\(\beta\)RII+ cells into the same recipient mice, I found that very few F5-dnTGF\(\beta\)RII+ cells expressed CD103 when the TGF\(\beta\) signaling pathways were blocked. These data showed that TGF\(\beta\) signals are required for CD8 T cells to express CD103 in the lungs.

To determine how TGF\(\beta\) influences the other virus-specific CTL responses in the lungs, we made chimeric mice with mixed bone marrow cells from the dnTGF\(\beta\)RII and wild type animals. When the TGF\(\beta\) signals were blocked, there were dramatically increased numbers of KLRG1+ virus-specific Teff cells in all tissues 10dpi, including the lungs. Since virus-specific CTLs can damage the lungs, I tested the ability of the CD8 T cells from wild type and dnTGF\(\beta\)RII+ chimeric mice to make proinflammatory cytokines. After \textit{in vitro} stimulation larger numbers of dnTGF\(\beta\)RII+ CD8 T cells produced IFN\(\gamma\) alone, or IFN\(\gamma\) together with TNF\(\alpha\), as compared to the wild type CD8 T cells. Histological analysis also showed that there were more extensive areas of inflammation in the dnTGF\(\beta\)RII chimeric mice, than in the WT B6 chimeric mice. When I used confocal microscopy to compare the distribution of KLRG1+ and KLRG1- virus-specific CD8 T cells in the lungs, the majority of OTI-dnTGF\(\beta\)RII+ cells which expressed KLRG1 were located in the tissues around the alveoli 10dpi, but there were very few mutant cells in the lumen of the airways. At later time points some OTI-WT cells which expressed
CD103 were located in the airways. These studies showed that TGFβ signaling selectively reduced the numbers of KLRG1+ virus-specific Teff cells in the lungs, while upregulated CD103 expression on many surviving virus-specific CTLs in the airways.

Since CD103 is an adhesion molecule which can promote interactions with epithelial cells that express E-cadherin, I used transfer experiments to directly investigate whether CD103 expression helps retain virus-specific CD8 T cells in the lungs. After OTI TcR transgenic mice were crossed with CD103KO mice, I transferred equal numbers of OTI-WT together with OTI-CD103KO cells to the recipient mice 2 days before infection with WSN-OVAi virus. During the peak of the CTL response I found approximately equal ratios of CD103KO and wild type CD8 T cells in all tissues. As the infection progressed the ratios in the lungs changed and there were less OTI-CD103KO than OTI-WT cells. In addition, confocal microscopy showed that both WT and CD103KO cells were distributed throughout the lungs during the peak of CTL response. Later in the infection the percentages changed and there were less of CD103KO, than wild type, cells in the airways.
INTRODUCTION

Naïve CD8 T cells expand and differentiate into Teff cells after they encounter their cognate antigen on APCs in the draining lymph node. These antigen-activated CTLs must then migrate to the sites of viral replication to promote viral clearance. The homing receptors that are required for efficient CTL migration to the infected tissues include integrins which promote adhesive interactions between T cells and other cell types.

Integrins are a family of transmembrane proteins, which play a role in adhesive interactions that are important for T cell recirculation, migration and recognition of foreign antigens [68]. Several studies have demonstrated that specific integrins are involved in CTL migration to the mucosal tissues. For example, α4β7 integrin interacts with mucosal addressin cell adhesion molecule-1 (MadCAM-1) and plays a role in mediating the entry of T cells into the intestine [141,142]. The expression of α4β7 is down regulated and αEβ7 (CD103) is upregulated when pathogen-specific CD8 T cells arrive in the intestinal mucosa where TGFβ is made [143]. Another integrin known as the lymphocyte function-associated antigen 1 (LFA-1 or αLβ2; CD11a/CD18) also plays a role in CTL migration to peripheral tissues including the lungs, by promoting interactions with intracellular adhesion molecule-1 (ICAM-1) on endothelial cells. These interactions are regulated by chemokines CCL12 and CXCL21 which induce a conformation change in the integrin which increases its affinity for ICAM-1ligand [144]. In addition, the very late antigen-1 (VLA-1; α1β1) is a collagen receptor which promotes retention of the antigen-specific CTL in the lungs and plays a role in protective immunity against secondary infection [21]. In this study I will focus on CD103, since many virus-specific CD8 T cells express CD103 in the airways after influenza virus infection [145,146]. In addition, since TGFβ signaling is required for CD103 expression on CD8 T cells in other tissues including the
kidney \(^{[147]}\), I also investigated the role of TGF\(\beta\) in regulating the T cell response. Since we believe that virus-specific memory CD8 T cells in the lungs play a key role in heterosubtypic immunity, it is important to understand the role of TGF\(\beta\) signaling in the generation of antigen-specific tissue resident memory (Trm) CD8 T cells \(^{[148]}\).

During CD8 T cell differentiation, antigen-specific effector CD8 T cells (Teff) can be subdivided into memory precursor and short-lived Teff cells based on CD127 (IL-7R\(\alpha\)) and KLRG1 expression. The memory precursor Teff cells (MPECs) are CD127+KLRG1-CD8 T cells which have the capacity for robust proliferation and long-term survival in the absence of antigen \(^{[85]}\). In contrast, CD127- KLRG1+ CD8 T cells are short-lived Teff cells (SLEC) that are impaired in proliferation, but are capable of immediate effector functions \(^{[149]}\). A third subset of Trm cells has recently been identified in peripheral tissues, including the skin, brain, gut and lungs \(^{[114-116;119]}\). These Trm cells maintain an activated phenotype by expressing molecules such as CD69 as well as CD103 which is regulated by TGF\(\beta\). A recent study demonstrated that TGF\(\beta\) signaling also reduces the survival of KLRG1+ virus-specific CTLs after LCMV infection \(^{[107]}\), it is largely unknown how TGF\(\beta\) signaling impacts virus-specific CTL responses in the lungs during influenza virus infection.

In this chapter, I have used mice that express a dominant-negative form of the human transforming growth factor beta receptor II (dnTGF\(\beta\)RII) under control of the CD4 promoter without the CD8 silencer \(^{[150]}\), to understand how TGF\(\beta\) signaling changes the responses of influenza virus-specific CD8 T cells. I also used CD103-deficient mice \(^{[132]}\) to directly determine how CD103 expression on antigen-specific CTLs impacts their migration and/or retention in the lungs. My data demonstrate that large numbers of KLRG1+ virus-specific CTL accumulate in the lungs in the absence of TGF\(\beta\) signaling.
Furthermore, TGFβ is required for CD103 expression on KLRG1- virus-specific CTLs in the airways during late stage of the infection.

The data that are presented in this chapter are divided between three specific aims. Each aim addresses a specific question about the way that environmental factors (TGFβ) influence the numbers and phenotype of virus-specific CD8 T cells in the lungs. The questions are outlined below.

Aim 1.1: Does TGFβ signaling influence the numbers of virus-specific CTL in the lungs?

Aim 1.2: Is TGFβ signaling required for CD103 expression on virus-specific CTL in the lungs?

Aim 1.3: How does CD103 change the distribution of virus-specific CTLs inside the lungs?
RESULTS

Aim 1.1: Does TGFβ signaling influence the numbers of virus-specific CTL in the lungs?

Blockade of TGFβ signaling increases the numbers of KLRG1+ virus-specific CTLs in the lungs.

TGFβ is a pleiotropic cytokine which can influence cell fate decisions, proliferation and survival \cite{106}. A recent study demonstrated that TGFβ signaling reduced the survival of KLRG1+ virus-specific CTLs during early clonal expansion \cite{107}, however, it is largely unknown how TGF β signaling influences the CTL response in the lungs during influenza virus infection. To determine whether TGFβ signaling influenced the numbers of virus-specific CTL in the lungs, I performed collaborative experiments with other members of our laboratory. C57BL6 mice were irradiated and bone marrow cells (5x10^6) from B6 and dnTGFβRII mice (mixed at 1:1) were transferred into irradiated B6 mice. After 8 weeks the reconstituted mice were infected with the HKx31 virus and CD45.1/CD45.2 congenic markers were used to compare the numbers of NP- and PA-specific WT and dnTGFβRII+ CD8 T cells by MHCI tetramer analysis. I found larger numbers of NP- and PA-specific CD8 T cells which expressed KLRG1 in the BAL, lungs and spleen 10dpi when the dnTGFβRII+ was present (Figure 3-1B and 3-1C). High numbers of tetramer-specific dnTGFβRII+ CD8 T cells which expressed KLRG1 were also found in the lungs and spleen 20dpi, but there were very few CD8 T cells in the BAL (Figure 3-1B and 3-1C). These data suggest that TGFβ plays a role in reducing the numbers of KLRG1+ virus-specific Teff cells in the lungs during acute viral infection, and further suggest that CTLs which lack CD103 are not efficiently retained in the airways.
**TGFβ induces CD103 expression on KLRG1- virus-specific CTLs in the lungs.**

The data suggest that TGFβ signaling induces different responses from KLRG1- and KLRG1+ virus-specific CTL (Figure 3-1). To investigate how TGFβ signals impact CD103 expression on the KLRG1- and KLRG1+ subsets of virus-specific CTLs in the lungs, we subdivided the virus-specific CTL into two subsets using KLRG1 antibodies shown in Figure 3-1. Each subset of KLRG1+ and KLRG1- of virus-specific CD8 T cells was then further analyzed for CD69 and CD103 expression (Figure 3-2). We found that CD103 was only expressed on KLRG1- NP-specific WT CD8 T cells in the lungs 10dpi, but was not present on KLRG1+ cells. Small numbers of KLRG1- CD8 T cells also expressed CD103 in the lungs which suggested that dnTGFβRII did not outcompete the endogenous TGFβRII on some CD8 T cells. In addition, there were some KLRG1- NP-specific WT CD8 T cells expressed CD69 alone or CD103 together with CD69 in the lungs, but small numbers of KLRG1+ cells that expressed CD69 alone. However, there were similar percentages of NP-specific dnTGFβRII+ CD8 T cells which expressed CD69 alone between KLRG1+ and KLRG1- subsets in the lungs. By 20dpi we found some KLRG1- NP-specific WT CD8 T cells which expressed CD69 alone or CD103 together with CD69 in the lungs, but small numbers of the KLRG1+ cells expressed only CD69. In contrast, small numbers of KLRG1+ and KLRG1- subsets expressed CD69 when TGFβ was blocked. This suggests that TGFβ selectively induces CD103 expression on KLRG1- CTL in the wild type mice during influenza virus infection.

**Virus-specific CTLs produce cytokines in the absence of TGFβ signaling.**

Tumor models show that TGFβ can be secreted from tumor cells and prevents CTL from making cytokines without causing cell death [151]. To investigate whether TGFβ signaling influenced the effector function of virus-specific CTLs during influenza virus infection, I performed intracellular cytokine staining after cell culture in the presence of...
synthetic NP31 and PA peptides. Bone marrow cells from either B6 WT or dnTGFβRII mice were transferred into the separate groups of irradiated B6 mice. CD8 depletion antibodies were administrated to the recipient mice one day after the bone marrow transfers to deplete residual host CD8 T cells. The recipient animals were infected with HKx31 virus ~8 weeks after reconstitution. Lymphocytes were recovered from lungs and spleens and the cells were cultured (5x10^6 /well) in the presence of 1μg NP or PA peptides. The cells were fixed and analyzed for IFNγ and TNFα production 10 and 21dpi (Figure 3-3). These data showed that larger numbers of dnTGFβRII+ CD8 T cells made IFNγ alone, or IFNγ together with TNFα, in response to peptide stimulation 10dpi, as compared to the WT CD8 T cells (Figure 3-3B). I also found larger numbers of dnTGFβRII+ than WT CD8 T cells that expressed IFNγ alone, or IFNγ together with TNFα, in the lungs and spleen after stimulation with PA peptides 20dpi, than the WT CD8 T cells. However there was no significantly different between the two groups when NP peptides were used for stimulation (Figure 3-3C). These data indicate that TGFβ signaling reduces the numbers of IFNγ-producing CTLs in the lungs after influenza virus infection.

Lack of TGFβ signaling results in increased numbers of infiltrating leukocytes in the lungs.

Since IFNγ and TNFα can cause severe immunopathology [152,153] and I found a lot of cells that were capable of making these proinflammatory cytokines in the lungs when TGFβ signals were blocked, I used histological analysis to determine how TGFβ influenced the local inflammatory response in the lungs. Bone marrow cells from B6 WT or dnTGFβRII mice were transferred to irradiated B6 mice, which were infected with HKx31 virus ~8 weeks later. The lungs were harvested from groups of 3 mice 10dpi and infused with paraformaldehyde (PFA) and cut into sections for staining with hematoxylin
and eosin (H&E). These studies showed large numbers of infiltrating lymphocytes in the lungs of both groups of animals 10dpi, however there were more extensive areas of inflamed lung when the mice were made with bone marrow cells from dnTGFβRII+ mice (Figure 3-4). These data indicate that TGFβ signaling plays a role in reducing inflammatory immune responses in the lungs after influenza virus infection.

**Aim 1.2. Is TGFβ signaling required for CD103 expression on virus-specific CTL in the lungs?**

*TGFβ signaling induces CD103 expression on virus-specific CD8 T cell in the lungs.*

Since TGFβ is required for CD103 expression on CD8 T cells in other tissues \[^{147}\], I investigated whether TGFβ signaling was required for CD103 expression on influenza virus-specific Teff cells in the lungs. We crossed F5 TcR transgenic mice with dnTGFβRII mice \[^{150}\], and equal numbers of F5-WT and F5-dnTGFβRII+ cells (total $10^6$) were transferred to the recipient mice one day before E61-13-H17 infection (Figure 3-5). The CD45.1/CD45.2 congenic markers were used to compare the ratios of transferred cells in different tissues. Since human DNA was used to make the dnTGFβRII construct, the transferred dnTGFβRII+ cells are rejected from the host between 10-20dpi \[^{154}\], so I was only able to track the transferred F5-dnTGFβRII+ cells until 9 dpi.

In this experiment I detected larger percentages of F5-dnTGFβRII+ cells in the MLN and spleen, compared to F5-WT cells (Figure 3-5B). In addition, there were higher ratios of F5 WT than F5-dnTGFβRII+ cells in the airways and lungs. I found dramatically reduced CD103 expression on F5-dnTGFβRII+ cells in the lungs 9dpi, compared to the F5-WT cells as shown by overlaid histograms. Since the mutant dnTGFβRII must compete with the endogenous TGFβRII on CD8 T cells, small percentages of F5-
dnTGFβRII+ cells expressed CD103 in the lungs 9dpi, which suggested that the mutation may be slightly leaky. These data indicate that TGFβ is required for CD103 expression on virus-specific CTLs in the lungs after influenza virus infection.

**Aim 1.3: How does TGFβ signaling change the distribution of virus-specific CTLs inside the lungs?**

_KLRG1+ cells exhibits different location in the lungs during the recovery phase._

The flow cytometry data showed large numbers of KLRG1+ cells in the lungs when the dnTGFβRII was expressed 10dpi (Figure 3-1B), but there were very few KLRG1+ cells in the BAL 20dpi (Figure 3-1C). I compared the distribution of KLRG1- and KLRG1+ virus-specific CD8 T cells inside the lungs. To prevent the dnTGFβRII + cells from being rejected, I made chimeric recipients using CD45.2+ bone marrow cells from WT B6 and dnTGFβRII mice (mixed 10:1 ratio). After ~8 weeks reconstitution I transferred either CD45.1 OTI-WT (10³) or CD45.1 OTI-dnTGFβRII+ (10³) cells to separate groups of chimeric mice and two days later infected them with the WSN-OVAi virus (Figure 3-6A). The CD45.1 congenic marker was used to tract the transferred OTI cells during imaging. To stain the endothelial cells in the blood vessels, I gave CD31 antibody by intravenous injection 15mins before the lungs were harvested. Fragments of lung tissue were fixed with PFA and further stained with CD45.1, KLRG1 and Ep-CAM antibodies. The outer boundary of airway wall was identified with the Ep-CAM antibodies (red), as indicated by the white dashed lines. The lung tissues were analyzed by confocal microscopy (Figure 3-6B).

The results showed that large numbers of KLRG1+ OTI-dnTGFβRII+ cells were located around the alveoli 10dpi, (Figure 3-6B and 3-6C). In contrast, many KLRG1-
OTI-WT cells were located in the walls of the airways (Figure 3-6D and 3-6E). The numbers of KLRG1+OTI-WT cells declined in the lungs 20dpi, but substantial numbers of KLRG1+OTI-dnTGFβRII+cells were still found around the alveoli 20dpi. Interestingly, most KLRG1- OTI-WT cells which expressed CD103 were embedded in the airways at the late time points (Figure 3-6F). These data suggest that TGFβ induced expression of CD103 on virus-specific CD8 T cells and thus helps remain the cells in the lungs during influenza virus infection.

*CD103 expression plays role in retention of CD8 T cells in the lungs.*

Previous studies have implicated CD103 for a role in CTL migration or retention in mucosal tissues [132,147]. To directly determine whether CD103 plays a similar role in the lungs, we generated OTI-CD103KO mice by crossing OTI mice with CD103KO mice [132]. I adoptively transferred equal numbers of OTI-WT and OTI-CD103KO (total10⁵) in the same recipients two days before WSN-OVAi infection. I used the CD45.1/CD45.2 congenic markers to compare the ratios of OTI-WT and OTI-CD103KO cells in different tissues at 10, 20 and 35dpi (Figure 3-7A).

This experiment showed almost equal ratios of OTI-WT and OTI-CD103KO cells in all tissues including the lungs, spleen and MLN 10dpi, which indicates that CD103 did not have a major impact on virus-specific CTL migration to the lungs during the peak of the CTL response (Figure 3-7B). At later time points (20-35dpi) there were higher ratios of OTI-WT cells (~60%), as compared to OTI-CD103KO cells (~40%) in the BAL and lung parenchyma. In this experiment I found that some CD103KO virus-specific CD8 T cells accumulated in the spleen during the late stage of infection, however, other experiments did not show the same pattern. This suggests that CD103 deficiency does not influence migration to the lungs during the Teff response, but leads to impaired
retention of virus-specific Teff cells in the lungs during the late stage of the infection. By 10dpi the transferred cells expressed similar levels of CD69, but there was a selective loss of CD69+ CTL at later time points when CD103 was not expressed. These data indicate that CD103 helps retain virus-specific CTLs in the lungs during late antigen presentation.

We also generated F5-CD103KO mice by crossing F5 TcR transgenic mice with CD103KO mice and I transferred equal numbers of F5-WT and F5-CD103KO cells (total 10^6) to the recipient mice two days before infection with E61-13-H17. The CD45.1/CD45.2 congenic markers were used to compare the ratios of mutant and WT cells in different tissues. I found equal ratios of F5-WT and F5-CD103KO cells in the lungs 10dpi, but the F5-WT cells (~ 70%) outnumbered the F5-CD103KO cells (~ 30%) 20 and 30dpi (Figure 3-7C). These data also support the idea that CD103 is required for prolonged retention of F5-WT cells in the lungs.

**CD103 expression changes the distribution of virus-specific CTL inside the lungs.**

Since my data show that CD103 expression increases the numbers of virus-specific CTL in the airways during the late stage of the infection, I used scanning confocal microscopy to investigate whether CD103-deficiency changed the distribution of virus-specific CTLs in different anatomical compartments within the lungs. To produce OTI cells that could be visualized after transfer, we crossed OTI-WT and OTI-CD103KO mice with Ubquitin-GFP mice which express green fluorescent protein (GFP) in all cell types [135]. I used GFP+ 2.3 mice as recipients since they express GFP in the bone marrow and will tolerate the GFP+ T cells [134]. I transferred equal numbers of GFP+ OTI-CD103KO (10^3) and CD45.1+ OTI-WT (10^3) cells into GFP+ 2.3 mice two days before infection with WSN-OVAi virus. Confocal microscopy was used to compare the
distribution of CD45.1+ and GFP+ cells in the lungs at different time points (Figure 3-8A). By 10dpi I detected no difference in the location or ratios of OTI-WT and OTI-CD103KO cells in the lungs by microscopy and flow cytometry analyses (Figure 3-8B). By 19 and 30dpi I compared the ratios by counting cell numbers at the airways and the lung parenchyma from several images. There were less OTI-CD103KO cells than OTI-WT cells in the airways and lung parenchyma 19dpi (Figure 3-8C). By 30dpi I obtained significantly reduced numbers of OTI-CD103KO cells, as compared to OTI-WT cells in the airways, but there was no difference in the lung parenchyma (Figure 3-8D). These data indicate that CD103 helps retain virus-specific CTL in the lung airways where E-cadherin is highly expressed during the recovery phase of the infection.
DISCUSSION

In this chapter I have shown that some CD103+ virus-specific CD8 T cells are located in the airways during late antigen presentation and that TGFβ induces CD103 expression on these virus-specific CTL. TGFβ is a pleiotrophic cytokine which regulates many cellular processes including T cell proliferation and survival [106]. My data show that the numbers of KLRG1+ virus-specific Teff cells were greatly increased when TGFβ signaling was blocked (Figure 3-1). This is consistent with a previous study which showed that TGFβ signaling over-rides IL-15 induced expression of the prosurvival molecule Bcl-2 (B-cell lymphoma 2) and selectively induces apoptosis of short-lived Teff cells during clonal expansion in LCMV infected mice [107].

In tumor models TGFβ is secreted from the tumor cells and directly acts on CTLs to inhibit production of cytokines and other effector molecules such as IFNγ, granzyme A and B [151], suggesting that TGFβ signals can inactivate some CTL without killing them. My data also showed that a blockade in the TGFβ signaling pathway increased the numbers of virus-specific Teff cells that were capable of making IFNγ alone, or IFNγ together with TNFα (Figure 3-3). In this model, my data indicate that the decrease in cytokine-producing CTL was due to apoptosis of the virus-specific Teff cells, as measured using a fluorescent dye that crosses the membranes of dead cells and binds to free amines in the cytoplasm. In addition, my data show that the peak of the CTL response (10dpi) corresponds with large numbers of infiltrating leukocytes in the lungs in the absence of TGFβ signaling (Figure 3-4). Although this implies that large numbers of cytokine-producing Teff cells have the potential to cause severe lung damage, we did not see any dead animals after infection with the HKx31 virus, which is a mildly pathogenic strain. Other studies have shown that large quantities of proinflammatory
cytokines can cause high mortality rates in humans during infections with more pathogenic strains, including H5N1 viruses, as well as the H1N1 strain that was responsible for the 1918 pandemic \cite{152,155-157}. During these infections a cytokine storm contributed to several disease symptoms including fever, viral pneumonia, respiratory failure and lymphopenia \cite{158,159}. My data suggest that TGFβ signaling down regulates the inflammatory response by reducing the numbers of virus-specific Teff cells in the lungs during infection with a less pathogenic strain.

Large amounts of TGFβ are produced in the lungs after infection with some influenza and other respiratory viruses \cite{160}. TGFβ can be expressed by a variety of cells including fibroblasts and stromal cells, macrophages, T cells, dendritic cells and mouse tracheal epithelial cells \cite{106,161}. The TGFβ is secreted as an inactivated homodimeric proprotein known as the latency-associated polypeptide (LAP) which must be activated to release a short peptide that is responsible for receptor binding \cite{162}. The cleavage reaction can be induced by a variety of molecules with protease activity, including thrombospondin-1 (TSP-1) which binds to N-terminal region of LAP and induces a conformational change which makes active TGFβ1 accessible its receptor. The integrin αvβ6, which is expressed on lung epithelial cells, can also bind to a specific arginine-glycine-aspartic acid (RGD) sequence in the LAP and activate TGFβ1 \cite{163}. Alternatively the influenza virus neuraminidase protein (NA) has enzymatic activity and can cleave LAT-TGFβ into its active form, although the efficiency of the cleavage reaction varies between different strains \cite{164}. Since the active peptide contains a hydrophobic domain, it has a very short half-life in vivo and must be quantified using immunoblot analysis \cite{165}.

KLRG1 is a cell surface molecule that can inhibit virus-specific Teff function. This molecule belongs to the C-type lectin-like superfamily and contains an immunoreceptor
tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain. It has been shown that E-cadherin is a ligand for KLRG1 and their interaction recruits SH2 (Src homology 2)-containing inositol phosphatase-1 (SHIP-1) that acts as a negative regulator for T cell proliferation \(^{[166;167]}\). My data show that large numbers of KLRG1+ virus-specific Teff cells accumulate in the lungs 10dpi when TGFβ signaling is blocked (Figure 3-6). Since any surviving KLRG1+ virus-specific Teff cells do not express CD103, they may interact with lung epithelial cells which express E-cadherin.

Interestingly, the engagement of KLRG1 with E-cadherin, together with the interaction between CD3/TCR:MHCI complex, inhibits the nuclear factor of activated T-cells (NFAT) pathway that plays a role in expression of cytokines and cell surface molecules including IL-2, TNFα and Fas ligand (CD94L) \(^{[168]}\). In addition, cross-linking of KLRG1 with monoclonal antibodies decreases IFNγ production and cytolytic function in NK cells \(^{[169]}\). Therefore, these data suggest that the interaction between E-cadherin and KLRG1 may inhibit excessive virus-specific Teff function when TGFβ is not present in the lungs and thus provide an alternative pathway to decrease Teff cell-mediated inflammation 10dpi.

A recent study revealed different distributions of KLRG1- and KLRG1+ subsets of pathogen-specific Teff cells in the secondary lymphoid organs during acute LCMV infection \(^{[170]}\). The KLRG1+ Teff cells preferentially accumulated in the red pulp of spleen, while the KLRG1- Teff cells, which expressed the CCR7 homing receptor, were found in the B cell zone and white pulp. However, the distribution of these two subsets of virus-specific Teff cells in the peripheral tissues is not known. My confocal microscopy analysis showed that many KLRG1+ virus-specific Teff cells were located around the alveoli 10dpi with WSN-OVA\(_i\) virus (Figure 3-6B and 3-6C). Late after infection some
KLRG1- virus-specific Teff cells which expressed CD103 were located in the airways (Figure 3-6F). These data indicate that CD103 expression changes the distribution of the KLRG1- subset of virus-specific Teff cells in the lungs.

My data supported the idea that TGFβ-induced expression of CD103 is required for retention of newly activated virus-specific CTL in the lungs during the recovery phase of the infection (Figure 3-7B). Thus, virus-specific Teff cells which lack CD103 expression are able to migrate into the lungs during acute infection, but they are inefficiently retained in the airways during the recovery phase. These data are consistent with previous finding that CD103 deficiency induced less accumulation host-specific Teff cells in the intestinal epithelium during the graft-versus-disease (GVHD) which is also a mucosal site [147].

Several studies have shown that Teff-like Trm cells persist in the peripheral tissues such as skin, salivary gland, lung and small intestine after local infections, where they express CD103 [80,114-118]. Some of these studies suggested that local antigen stimulation was required for induction and maintenance of CD103 on Trm cells in the brain and lungs [115,116,171]. These Trm cells require CD103 expression to retain in the brain and the lungs [116,171]. Previously, members of our laboratory performed parabiosis experiments to follow CTL migration in the blood stream. This study showed that some virus-specific CTLs which expressed CD69 together with CD103 stayed in the lungs during the late stage of the infection [145]. This supports the idea that CD103 on virus-specific CTLs is maintained by local antigen stimulation in the lungs. In contrast, others demonstrated that local antigen stimulation was not required for CD103 expression on Trm cells in the skin and salivary gland [118,119]. Interestingly, E-cadherin is contributed to retention of pathogen-specific Trm cells in the salivary glands, which E-cadherin may induce
homotypic adhesive interaction between Trm cells and epithelial cells \cite{118}. These data suggest that maintenance of CD103 on Trm cells is dependent on local antigen or tissue microenvironment.

In this chapter, my data showed that environmental-derived signals (TGFβ) tightly regulate the responses of virus-specific Teff and Tem cells in the lung. The TGFβ induces different responses from KLRG1- and KLRG1+ subsets of virus-specific Teff cells by selectively inducing apoptosis of short-lived Teff cells, but it induces survival of long-lived Teff cells via CD103 adhesion molecule in the lungs where are protected by the mucociliary escalator (Figure 3-9). Therefore these data provide a better understanding how TGFβ signaling pathway maintains virus-specific Teff and Tem cells in the lungs during influenza virus infection. In next chapter, I will discuss how TcR receptor-derived signals influence the phenotypes and numbers of activated virus-specific Teff cells using two different strains such as persisting WSN-OVA\textsubscript{I} and non-persisting X31-OVA viruses.
Figure 3-1A:

- CD45.1 C57BL/6
- CD45.1/2 dnTGFβRII
- Irradiated C57BL/6
- HKx31
- Time points: 0, +10d, +20d
- ~8 weeks
Figure 3-1:

B

NP

C

PA

KLRG1+

KLRG1−
Figure 3-1. The numbers of KLRG1+ virus-specific effector CTLs were increased in the absence of TGFβ signals.

(A) Experimental protocol and timeline. Mixed bone marrow cells (mixed 1:1 ratio) from B6 (WT) and dnTGFβRII (Mut) mice were transferred into B6 mice that were lethally irradiated. The recipient mice were infected with HKx31 virus ~8 weeks after reconstitution. MHCI tetramers were used to analyze influenza virus-specific CD8 T cells in different tissues 10 and 20dpi. The CD45.1/CD45.2 congenic markers were used to distinguish between host, WT and mutant cells. The tetramer positive cells in the lungs and spleen were divided into KLRG1+ (gray) and KLRG1- (white) subsets.

(B) The numbers of NP-specific CTL and (C) the numbers of PA-specific CTL. Data from a pool of 3 animals is shown. Duplicate experiments gave similar results.
Figure 3-2:

WT Mut

KLRG1+ KLRG1- KLRG1+ KLRG1-

10dpi

20dpi

CD69

CD103
Figure 3-2. CD103 is selectively expressed on KLRG1- virus-specific CTLs.

Mixed bone marrow cells (mixed 1:1 ratio) from B6 (WT) and dnTGFβRII (Mut) mice were transferred into B6 mice that were lethally irradiated. The recipient mice were infected with HKx31 virus ~8 weeks after reconstitution. MHCI tetramers were used to analyze influenza virus-specific CD8 T cells in different tissues 10 and 20dpi. The CD45.1/CD45.2 congenic markers were used to distinguish between host, WT and mutant cells. Separate subsets of KLRG1+ and KLRG1- NP-specific CD8 T cells were analyzed for CD69 and CD103 expression. Data from 3 animals per group are shown. Duplicate experiments gave similar results.
Figure 3-3A:

CD45.2 C57BL/6

CD45.2 dnTGFβRII

Irradiated C57BL/6

~8 weeks

HKx31

0 +10d +21d
Figure 3-3:

B 10dpi

NP31 pep

PA pep

WT Mutant

# cells (x 10^5)

Lung

Spleen

C 21dpi

NP31 pep

PA pep

WT Mutant

# cells (x 10^4)

Lung

Spleen
Figure 3-3. The numbers of cytokine-producing CTLs in the lungs were increased when TGFβ signaling was blocked.

(A) Experimental protocol and timeline. Bone marrow cells from B6 or dnTGFβRII mice were transferred into separate groups of B6 mice that were lethally irradiated. Bone marrow chimeric mice were infected with HKx31 virus ~8 weeks after reconstitution.

(B) Lymphocytes were enriched 10dpi (3 mice per group) for peptide stimulation in the presence of brefeldin A.

(C) Lymphocytes were enriched 21dpi (3 mice per group) for peptide stimulation in the presence of brefeldin A. The numbers of CD8 T cells expressing IFNγ alone (white), TNFα alone (black), or both cytokines (hatched) are shown. Statistical significance was determined using an unpaired students T test. Mean±SD from 3 mice per group are shown. *P<0.05, **P<0.01.
Figure 3-4:
Figure 3-4. Increased numbers of infiltrating leukocytes in the absence of TGFβ regulation in the lungs.

Irradiated C57BL/6 mice were reconstituted with bone marrow cells from wild type or dnTGFβRII+ donor mice ~8 weeks before HKx31 infection. Inflated lungs were embedded in paraffin 10dpi and 4 micron sections were stained with hematoxylin and eosin. (A) Mixed donor cells no infection. (B & D) Wild type donor cells. (C & E) dnTGFβRII+ donor cells 10dpi. Photographs were taken at original magnification 10x and 60x. Duplicate experiments gave similar results.
Figure 3-5:

A

CD45.1
F5-WT

CD45.1/2
F5-dnTGFRβII+

-2d
0
+9d

C57BL/6

E61-13-H17

B

9dpi

BAL

Lung

MLN

Spleen

% Cells

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100

0
25
50
75
100
Figure 3-5. TGFβ signaling is required for CD103 expression.

(A) Experimental protocol and timeline. Equal numbers (10⁶) of F5-WT and F5-dnTGFβRII cells were transferred to B6 mice two days before E61-13-H17 infection.

(B) The transferred F5 cells were analyzed using CD45.1 and CD45.2 congenic markers 9dpi. The bar graphs indicate the ratios of F5-WT (gray bar) and F5-dnTGFβRII cells (open bar) in different tissues from 3 mice. Overlaid histograms show gated populations of transferred F5-dnTGFβRII (solid line) or F5-WT (gray shading) using representative animals from groups of 3. The percentages of cells that are positive for each marker are displayed for F5-WT (above the line) and F5-dnTGFβRII (below the line) cells. Statistical significance was determined using an unpaired students T test. Mean±SD from 3 mice per group are shown (*P<0.05 **P<0.01 and ***P<0.001). Duplicate experiments gave similar results.
Figure 3-6:

A. Diagram illustrating the experimental setup for the study. CD45.2 C57BL/6 mice are used as recipients for CD45.2 dnTGFβRII mice. The recipients are infected with influenza virus at 0, +10d, and +20d time points. CD45.1+ OTI-WT mice are used for comparison.

B-F. Images showing the results of the experiment. Each panel (B to F) represents a different time point or condition.
Figure 3-6. Large numbers of KLRG1+ virus-specific CTL were
distributed around the alveoli during the peak of the CTL response.

(A) Experimental protocol and timeline. Mixed bone marrow cells (10:1 ratio) from B6
or dnTGFβRII mice were transferred to irradiated B6 mice. Chimeric mice were
received 10^3 naïve CD45.1+ (B & D) OTI-WT cells or (C & E) OTI-dnTGFβRII+ cells
48 hours before infection with WSN-OVAi virus. The CD31 antibody was injected to
stain endothelial cells 15mins before the lungs were harvested. Fragments of fixed
lung tissue were stained with antibodies to Ep-CAM (red), CD31 (yellow) CD45.1
(blue) and KLRG1 (green) 10 dpi. (b-e) Enlargements of the boxed regions. The
Imaris imaging software was used to identify areas of overlapping blue and green
staining (white regions) in the boxed regions.

(F) Naïve CD45.1+ wild type OTI cells (blue) were analyzed for CD103 expression
20dpi (green) with (f) overlapping blue and green staining on cells in the boxed
region (white). Z-stack images were taken at original magnification 20x. Duplicate
experiments gave similar results.
Figure 3-7:

A

CD45.1
OTI-WT

CD45.1/2
OTI-CD103KO

C57BL/6

WSN-OVA

-2d 0 +10d +20d +35d

B

10dpi

KO WT

20dpi

KO WT

35dpi

KO WT

CD69

Percentage of transferred cells (%)

BAL

Lung

MLN

Spleen

F5-CD103KO

F5-WT

CD69

Percentage of transferred cells (%)

C

10dpi

KO WT

20dpi

KO WT

30dpi

KO WT

CD69

Ratios of the cells (%)

BAL

Lung
Figure 3-7. CD103 helps virus-specific CD8+ T cells stay in the lungs.

(A) Experimental protocol and timeline. (B) Equal numbers (total $10^6$) of OT-WT and OTI-CD103KO cells were transferred to B6 mice two days before WSN-OVA<sub>i</sub> infection. The transferred OTI cells were analyzed using CD45.1 and CD45.2 congenic markers. The bar graphs indicate the frequencies of OTI-WT (black) and OTI-CD103KO cells (white) within the transferred cell population. Histograms from representative animals show gated populations of transferred OTI-CD103KO (white) or OTI-WT (black).

(C) Equal numbers (total $10^6$) of F5-WT and F5-CD103KO cells were transferred to B6 mice two days before E61-13-H17 infection. The mutant and wild type F5 cells were identified using CD45.1/CD45.2 congenic markers. The bar graphs indicate the ratios of F5-WT (black) and F5-CD103KO cells (white) in different tissues of 3 mice. Overlaid histograms from representative animals of each group show F5-CD103KO (white) or F5-WT (black). Statistical significance was determined using an unpaired students T test. Mean±SD from 3 mice per group are shown (*P<0.05 **P<0.01 and ***P<0.001). Duplicate experiments gave similar results.
Figure 3-8:

A

CD45.1
OTI-WT

GFP+
OTI-CD103KO

GFP+2.3

WSN-OVA

B

10dpi

WT CD103KO Ep-CAM

10dpi

20dpi

30dpi
Figure 3-8:

C

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>CD103KO</th>
<th>WT</th>
<th>CD103KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>20dpi</td>
<td>96</td>
<td>31</td>
<td>112</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>21</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>182</td>
<td>53</td>
<td>167</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>63</td>
<td>27</td>
<td>65</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>204</td>
<td>72</td>
<td>353</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>46</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>20</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>79</td>
<td>59</td>
<td>52</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>40</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>14</td>
<td>45</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>78</td>
<td>49</td>
<td>131</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>16</td>
<td>59</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>28</td>
<td>240</td>
<td>131</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>CD103KO</th>
<th>WT</th>
<th>CD103KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>30dpi</td>
<td>6</td>
<td>3</td>
<td>37</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>22</td>
<td>68</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>19</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>194</td>
<td>55</td>
<td>28</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>88</td>
<td>25</td>
<td>33</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>86</td>
<td>30</td>
<td>23</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>137</td>
<td>29</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>15</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>22</td>
<td>46</td>
<td>22</td>
</tr>
</tbody>
</table>

D

20dpi

30dpi

% of transferred cells

AW | P

**** | ****

**** | n.s.
Figure 3-8. CD103 is required for retention of virus-specific CD8+ T cells in the lungs.

(A) Experimental protocol and timeline. Equal numbers of CD45.1 OT-WT (10^3) and GFP+OTI-CD103KO (10^3) cells were transferred to GFP+2.3 mice two days before WSN-OVA_i infection. The transferred OTI cells were analyzed using confocal microscopy using CD45.1 and GFP 10, 20 and 30dpi. Each color indicates OTI-WT (Blue), OTI-CD103KO (Green) and Ep-CAM (Red). The Ep-CAM antibody was used to mark the outer boundary of the airway.

(B) Images show representative areas from each group 10, 20 and 30dpi.

(C) The numbers of transferred cells were counted inside and outside the white line.

(D) Colored dots (black, blue and red) indicate data from different animals. Statistical significance was determined using an unpaired students T test. Mean±SD from multiple images of 4 mice per group are shown (*P<0.05 **P<0.01 and ***P<0.001). Duplicate experiments gave similar results.
Figure 3-9:

Influenza

KLRG1+

CD8

CD8

KLRG1-

CD8

CD103

CD103

TGFβ

Apoptosis

(1)

(2)
Figure 3-9. Model to show that TGFβ signaling induces different responses from KLRG1- and KLRG1+ virus-specific CTLs.

(1) TGFβ plays a role in reducing the numbers of virus-specific CTLs which express KLRG1 in the lungs during acute viral infection

(2) TGFβ-induced expression of CD103 is required for retention of newly activated virus-specific CTLs which do not express KLRG1 in the lungs during the recovery phase of the infection
CHAPTER IV:

THE ROLE OF ANTIGEN IN CD8 T CELL MIGRATION TO THE LUNGS
ABSTRACT

WSN-OVA\textsubscript{i} and X31-OVA are recombinant influenza viruses which express the SIINFEKL epitope. Both viruses induce strong immune responses in the lungs during the peak of the infection. When naïve CD8 T cells from OTI TcR transgenic mice were used to determine how long the SIINFEKL peptides were present \textit{in vivo} they proliferated in the MLN more than 30dpi with WSN-OVA\textsubscript{i} virus, while no SIINFEKL peptides detected in the X31-OVA infected mice. Histological analysis revealed mild inflammatory responses in the lungs and MLN of both groups of animals 30dpi. We used MHCI tetramer analysis to compare the phenotypes of the endogenous virus-specific CD8 T cells in these animals. Larger percentages of OVA-specific CTL expressed CD69 and CD103 in the lungs when the SIINFEKL peptides were present. In addition, when the WSN-OVA\textsubscript{i} infected mice were treated with antibodies that recognize H-2K\textsuperscript{b}/peptide complexes, the percentages of OVA-specific CTL that expressed CD69 and CD103 decreased in the lungs.

As CD69 is a marker of early antigen stimulation, I investigated whether CD69 expression helps activated CD8 T cells reach the lungs during influenza virus infection. First we crossed F5 and OTI TcR transgenic mice with CD69KO mice to produce cells for transfer studies. When I transferred naïve CD8 T cells from the progeny animals to B6 mice before influenza virus infection, there were similar numbers of CD69KO and wild type CD8 T cells in most tissues 10dpi. In contrast, there were much larger numbers of wild type, than CD69KO, cells in the lungs. These data indicate that CD69 is required for efficient migration to the lungs. To visualize the transferred cells \textit{in situ} we crossed the F5-WT and F5-CD69KO mice with other mice that express GFP in all cell types 10dpi. When I used confocal microscopy to compare the distribution of wild type and
CD69KO cells in the lungs at 10 and 25dpi, there were no differences between F5-WT and F5-CD69KO. I also found that large numbers of CD69KO cells were in close association with the blood vessels in the lungs during the recovery phase of the infection. These data suggest that CD69 expression influences CTL migration in peripheral tissues such as the lungs.
INTRODUCTION

Epithelial cells in the airways play an important role in viral replication. Cytotoxic T lymphocytes (CTLs) promote viral clearance from the lungs by inducing apoptosis through contact-dependent interactions [172]. Since direct interactions are required to induce apoptosis, the influenza virus-specific CTLs must migrate to the site of viral replication to kill the infected epithelial cells before they release new infectious virus particles.

CD8 T cells undergo multiple steps of maturation after they respond to antigen stimulation [173]. During differentiation the activated CTLs change several surface markers which are required for activated T cells to move between different tissues in the body. Naïve and Tcm CD8 T cells respond to antigen stimulation in the lymphoid tissues and must therefore express CCR7 and CD62L to enter into the lymph nodes via the high endothelial venules (HEV) [64]. Both subsets of cells also express CD127 (i.e. Interleukin-7 receptor alpha or IL-7Rα) which is important for survival and homeostatic proliferation [85]. The naïve CD8 T cells undergo multiple rounds of cell division after antigenic stimulation and differentiate into effector CD8 T cells (Teff) which have cytotoxic activity and produce cytokines [174]. During T cell activation CCR7, CD62L and CD127 are down regulated, while CD11a and CD44 are upregulated and promote extravasation to peripheral tissues, through interactions with intercellular adhesion molecule-1 (ICAM-1) and hyaluronic acid, respectively [175;176]. Most of the activated CTLs become short lived Teff cells, which die during the contraction phase of the response. The mechanisms that control the contraction are not completely defined, but data from our laboratory indicate that TGFβ-induced apoptosis plays an important role during influenza virus infection (Submitted for publication).
Members of our laboratory previously used parabiosis experiments to follow CTL migration in the blood stream and found that some non-migrating CD8 T cells stay in the lungs and MLN long after viral clearance, where they maintain stable CD69 and CD103 expression\(^{[145]}\). Some of these virus-specific CD8 T cells also express PD-1 at low levels, which is a marker that is transiently expressed on all T cells soon after TcR signaling, but can also be a marker of functional exhaustion in situations of chronic inflammation\(^{[177;178]}\).

Several studies have shown that some virus-specific CTLs maintain stable CD69 expression in the lungs after respiratory infections\(^{[73;145;179]}\), and as well as selection of other peripheral tissues including the skin, kidney, brain, salivary glands and guts\(^{[114;118;119]}\). This marker, which is also known as the early leukocyte activation antigen, is a type II membrane glycoprotein from the C-type lectin family\(^{[180]}\). The cytoplasmic tail of CD69 contains a calcium binding domain and cross linking leads to activation of ERK1/2 in CD4+ T cells\(^{[181]}\). The ligand for CD69 has not been clearly defined, but some studies suggest that CD69 interacts with complex oligosaccharides\(^{[182]}\). CD69 expression can be transiently induced on CD8 T cells by activation of the TcR signaling pathway\(^{[183]}\) or by exposure to type I interferon\(^{[184;185]}\). In an asthma model blockade of CD69 reduced the numbers of CD4 T cells in the lungs, which corresponded with reduced inflammation and suggests that CD69 is involved in CD4 T cell migration\(^{[186]}\). Some studies suggested that CD69 slows the rate of T cell exit from the peripheral lymph node by down regulation shpingosine-1-phosphate receptor 1 (S1PR1) following T cell activation\(^{[187;188]}\). The S1PR1 binds to CD69 and is required for T cells to enter the blood vessels in response to high concentrations shpingosine-1-phosphate (S1P)\(^{[189]}\). However, the role of CD69 in CTL migration into the peripheral tissues is largely
unknown. Other studies have shown that chemokine receptor CCR7 is also required for T cells leave peripheral tissues and enter the peripheral lymph nodes (pLNs) \[^{190}\].

In this chapter, I will discuss how antigen persistence impacts the phenotypes of CD8 T cell in the lungs at different times during the response to influenza virus infection. Furthermore, I will discuss how antigen-induced CD69 expression regulates virus-specific CTL migration and distribution in the lungs during the recovery phase of the infection. Members of our laboratory previously demonstrated that processed viral antigens persist in the respiratory tract (MLNs) for at least two months after WSN-OVA\_i infection, as demonstrated by weak proliferative responses by carboxyfluorescein succinimidyl ester (CFSE)-labeled naïve OTI cells in transfer studies \[^{145}\]. Some endogenous virus-specific Teff cells also showed signs of a response to chronic antigen stimulation in the infected mice, including sustained PD-1 expression \[^{191}\]. Other groups have also reported evidence of prolonged antigen presentation after infection with other strains of influenza virus \[^{192,193}\]. In contrast, when naïve OTI cells were used to map antigen presentation after infection with the X31-OVA they could not detect SIINFEKL peptides beyond 15dpi \[^{136}\]. I have used the persisting WSN-OVA\_i and non-persisting X31-OVA strains of influenza virus to determine how sustained presentation of pathogen-derived peptide antigens impacts the phenotype and migration patterns of influenza-specific Teff cells in the lungs and other tissues during memory development.

The data that are presented in this chapter are divided between three specific aims. Each aim addressed a specific question about the way that antigen stimulation influences the numbers and phenotypes of virus-specific CD8 T cells in the lungs.
Aim 2.1. How does antigen persistence influence the resident T cell responses in the lungs?

Aim 2.2. How does CD69 deficiency impact virus-specific CD8 T cell activation and distribution?

Aim 2.3: How does the route of infection influence the local CTL response?
RESULTS

Aim 2.1. How does antigen persistence influence the resident T cell responses in the lungs?

Antigen persistence in the lungs after two distinct influenza virus infections.

Previously members of our laboratory demonstrated that WSN-OVA<sub>1</sub> infection induced prolonged antigen presentation until 60dpi<sup>[145]</sup>, but others showed that the X31-OVA does not induced antigen persistence more than 15dpi<sup>[136]</sup>. I infected separate groups of B6 mice which were infected with either WSN-OVA<sub>1</sub> or X31-OVA virus to determine how long viral peptides persist in vivo. The mice were maintained for 20 and 30 days before I transferred 2x10<sup>5</sup> naïve CD45.1+ OTI cells to each mouse by i.v. injection. One week later I recovered lymphocytes from the MLN and analyzed the transferred cells for CFSE dilution, using the CD45.1 marker to gate on the proliferating OTI cells. Although very small numbers of transferred OTI cells divided in the MLN of the X31-OVA infected mice, there were many dividing OTI cells in the WSN-OVA<sub>1</sub> infected mice 26 - 36dpi (Figure 4-1). These data show that SIINFEKL peptides persist after WSN-OVA<sub>1</sub> infection, but not after infection with X31-OVA virus.

MHCI tetramer analysis was used to phenotype endogenous virus-specific CD8 T cells in the lungs.

Several groups have shown that large numbers of virus-specific CD8 T cells maintain stable CD69 expression in the lungs after infection with different strains of influenza virus including our studies with WSN-OVA<sub>1</sub> virus<sup>[146;179]</sup>. Since the experiments with transferred OTI cells showed that the WSN-OVA<sub>1</sub> and X31-OVA viruses differ in their ability to support prolonged antigen presentation<sup>[136;145]</sup>, I used MHCI tetramer
I infected separate groups of mice with either WSN-OVA_i or X31-OVA and used tetramers that are specific for the NP and PA epitopes \(^{140}\) to identify endogenous virus-specific T eff cells in different tissues 10, 20 and 30dpi. Other antibodies were used to measure CD44, CD62, CD69, CD103, CD127 and PD-1 (Figure 4-2). The overlaid histograms show gated populations of virus-specific CD8 T cells from WSN-OVA_i (dashed line) or X31-OVA (gray shading) infected mice. The percentages of tetramer-specific CD8 T cells in the marked regions are displayed above the line for WSN-OVA_i and below the line for X31-OVA virus. The tetramer-negative CD8 T cells were used to set the gates for phenotypic analysis. I used an unpaired student T-test to determine statistical significance among groups of 5 animals.

This experiment showed that both viruses induced large numbers of OVA-, NP- and PA-specific CD8 T cells in the tissues by 10dpi, which shared very similar phenotypes (Figure 4-2A). By 20dpi some differences in the phenotypes of the cells from the two groups of animals became apparent. Specially, smaller numbers of tetramer specific CD8 T cells expressed CD69, CD103 and PD-1 in the lungs of the X31-OVA infected mice, than in the WSN-OVA_i infected mice (Figure 4-2B). These differences were more significant by 30dpi (Figure 4-2C and 4-3A). These data suggest that prolonged antigen presentation induces more activated phenotype for virus-specific T eff cells in the lungs during the recovery phase of influenza virus infection.
4-3B). When I compared the ratios of cells in the lungs and lymphoid tissues from each animal 10dpi, I found that more than 70% of the total OVA-specific CTL population was located in the lungs of both groups of animals (Figure 4-3C). The percentages of OVA-specific CTLs in the lungs of the WSN-OVAi infected mice remained the same until 30dpi. In contrast the percentage of the OVA-specific CTLs in the lungs of the X31-OVA infected animals declined to 35% by 30dpi (Figure 4-3C). These data show that a larger portion of the total OVA-specific CTL population stayed in the lungs when the SIINFEKL peptides were present.

What is the evidence that antigen plays a role in prolonged T cell activation?

To further confirm whether chronic antigen stimulation contributed to the activated phenotype of the pathogen-specific Teff cells in the lungs, I used a blocking antibody (25D1.16) which can bind to the peptide SIINFEKL (OVA peptide 257-264) when it is bound to a H-2Kb MHCI molecule. This antibody prevents interactions between the MHCI complex and the TcR \(^{[194]}\). The mice were infected with WSN-OVAi virus and treated twice with the 25D1.16 blocking antibodies (or an isotype control) at 20dpi (100μg i.n. plus 400μg i.v.) and 23dpi (250μg i.v.). MHCI tetramers and other antibodies were used to phenotype the virus-specific Teff cells in the lungs 29dpi. Overlaid histograms show gated populations of OVA-specific CD8 T cells in the lungs from isotype (dashed line) or 25D1.16 (gray shading) antibody treated mice (Figure 4-4A). The percentages of OVA-specific CD8 T cells that were positive for CD69 and CD103 are displayed for isotype (above the line) and 25D1.16 (below the line) treated mice. This experiment showed that the total numbers of OVA-specific CD8 T cells in the lungs of mice that were treated with the blocking antibody were reduced, as compared to that in the lungs of isotype antibody-treated mice (Figure 4-4B). The blocking antibodies also decreased the percentages of CD69 and CD103+ OVA-specific CD8 T cells in the lungs.
Although CD103 was down-regulated on OTI cells in the BAL, CD69 expression was unchanged. In contrast, the phenotype and numbers of NP-specific CD8 T cells were similar in the 25D1.16 and isotype-treated groups. These data indicate that recent antigen stimulation reinforces the activated phenotypes of some virus-specific Teff cells in the lungs.

Histology was used to examine the inflammatory response in the lungs.

Since viral peptides were detected in the MLN long after infection with WSN-OVA<sub>i</sub> virus, I used histological analysis to examine the inflammatory responses in the lungs during the recovery phase of infection. Again two groups of mice were infected either WSN-OVA<sub>i</sub> or X31-OVA virus and the lungs were harvested from groups of 3 mice 10 and 30dpi. The inflated lungs were cut into sections and stained with H&E. These studies showed large numbers of leukocytes in the lungs 10dpi with both viruses. Many leukocytes were clustered in and around the airways and in the alveolar spaces (Figure 4-5A and 4-5B). By 30dpi the leukocyte infiltration declined, but some mild inflammation was still detected in the lungs (Figure 4-5C and 4-5D). These data show that antigen persistence does not cause robust inflammatory responses in the lungs.

Aim 2.2. How does CD69 deficiency impact virus-specific CD8 T cell activation and distribution?

Does CD69 deficiency impact the kinetics of virus-specific CD8 T cell activation?

A recent study demonstrated that CD69 can interact with sphingosine-1-phosphate receptor 1 (S1PR1) which is required for lymphocyte egression from secondary lymphoid tissues and may thus influence the kinetics of the CTL response [187,188]. To determine whether CD69 expression influenced virus-specific CD8 Teff cell
activation, I used F5 mice that were crossed with CD69KO mice to generate F5-CD69KO mice. To compare the rate of cell division \textit{in vivo}, I labeled a mixed population of naïve CD8 T cells from F5-WT and F5-CD69KO cells with CFSE and transferred a total 10^6 F5 cells to each recipient, two days before infection with E61-13-H17 virus. The F5-WT and F5-CD69KO cells in the BAL, Lung, MLN and spleen were analyzed for CFSE-dilution on days 3, 5, 7 and 9 after infection, using the CD45.1/CD45.2 congenic markers. This experiment did not reveal any differences in the rate of cell proliferation by the two transferred populations at any time point analyzed (Figure 4-6A).

Furthermore, I tested whether CD69-deficiency influenced virus-specific Teff function. A mixed population of F5-WT and F5-CD69KO cells (10^6) was transferred to the recipient mice two days before E61-13-H17 infection as before. Enriched CD8 T cells were recovered from the lungs and spleen 3 and 4dpi and stimulated for 5hr with synthetic F5 peptide (1μg/ml) and brefeldin A (BFA). Intracellular cytokine staining showed that CD69-deficient CTL can make IFNγ after \textit{in vitro} stimulation (Figure 4-6B).

\textit{Transfer studies were used to determine whether CD69 expression changes the CTL response in the lungs.}

Several studies have shown that virus-specific CTLs which maintain stable CD69 expression become greatly enriched in the lung airways after viral clearance [145,146]. To determine whether CD69 plays a role in CD8 T cell migration, I transferred a mixed population of F5-WT and F5-CD69KO cells (10^6) into the same recipients two days before E61-13-H17 infection. It was necessary to transfer both populations to the same recipients to ensure that they were exposed to the same inflammatory conditions and concentrations of antigen. At different times after infection (10, 20 and 30dpi) I used
the CD45.1/CD45.2 congenic markers to compare the ratios of F5-WT and F5-CD69KO cells in different tissues (Figure 4-7A).

Although I found almost equal ratios of F5-WT and F5-CD69KO cells in the spleen and MLN, there were consistently higher numbers of F5-WT cells in the lungs at all time points analyzed (Figure 4-7B). I also found that some F5-CD69KO cells accumulated in the spleen 20 and 30dpi. Other experiments did not reveal an accumulation of F5-CD69KO in the spleen. When I analyzed gated populations of F5 cells for CD103 expression, I found that the absence of CD69 expression resulted in smaller percentages of CD103+ CD8 T cells than wild type cells in the lungs (Figure 4-7B). These data indicate that the absence of CD69 causes inefficient CD8 T cell migration into the lungs.

In addition, I investigated whether the absence of CD69 expression influenced the response of OVA-specific CTL in the lungs. Equal numbers of OTI-WT and OTI-CD69KO cells (10^6 total) were transferred to recipient mice two days before WSN-OVA_i infection and the CD45.1/CD45.2 congenic markers were used to track their response on different days after infection (i.e. 10, 20 and 30dpi). I found lower frequencies of OTI-CD69KO (~ 35%) in the lungs, as compared to the OTI-WT cells (~ 65%), at all time points analyzed (Figure 4-7C). Together, these data indicate that CD69 deficiency induce inefficient migration of virus-specific CD8 T cells to the lungs and consequently there is a selective reduction in the numbers of CD103+ cells in the lungs.

Since CD69 and CD103 are expressed by the same cells, we generated CD69/CD103 doubleKO (DKO) cells by crossing F5-CD69KO with CD103KO mice. I transferred equal numbers (total 10^6) of F5-WT and F5-DKO cells into the same recipient
mice and analyzed the ratios of WT and mutant cells using the CD45.1 and CD45.2 markers. I observed larger percentage of F5 WT cells compared to F5-DKO cells in the lungs 10dpi, which was similar to the CD69KO mice (Figure 4-7B). By 20dpi more F5 WT cells were observed than F5-DKO cells in the BAL, but both cell ratios were similar in other tissues. There were equal ratios of transferred cells in the lungs and MLN 30dpi, but not in the spleen (Figure 4-8). My data showed that CD69 helps virus-specific CTL migration to the lungs during influenza virus infection. However, it is unknown why CD69 deficiency did not influence virus-specific CTL migration in the absence of CD103.

*Does CD69 expression influence influenza virus-specific CTL location in the lungs?*

My data indicate that antigen-induced CD69 expression promotes efficient virus-specific CTL migration into the lungs, as demonstrated by the *in vivo* competition studies. Next I also used scanning confocal microscopy to investigate whether CD69-deficiency changed the distribution of virus-specific CTLs in different anatomical compartments inside the lungs. To generate F5 cells that could be visualized after transfer, we crossed F5-CD69KO cells with Ubquitin-GFP mice which express GFP in all cell types [135]. Because GFP is recognized as a foreign antigen in B6 mice, I used GFP+ 2.3 mice as recipients, since they express GFP in the bone marrow and will tolerate the transferred GFP+ T cells [134]. Equal numbers of CD45.1+ F5-WT and GFP+ F5-CD69KO (2x10⁴) cells were transferred into GFP+ 2.3 mice two days before E61-13-H17 infection. I used the CD45.1 marker and GFP to compare the distributions of transferred cells at different days after infection. To visualize the endothelial cells in the blood vessels, I gave CD31 antibody via intravenous injection 15mins before lung harvest. Fragmented lung tissues were further stained with Ep-CAM antibody and the lung tissues were analyzed by confocal microscopy (Figure 4-10).
To compare the frequencies of transferred F5-WT and F5-CD69KO cells in the lung, I used the Imaris imaging software to make each blue and green cell in multiple images as shown in Figure 4-9. The light green beads mark GFP+ cells, while light blue beads mark the CD45.1 cells. The results of this study were consistent with the flow data and CD69-deficiency resulted in reduced numbers of F5 cells in the lungs at all time points analyzed (10 and 25dpi) and supported the idea that CD69 promotes influenza virus-specific CD8 T cells migrate into the lungs (Figure 4-10B and 4-10C). During the peak of CD8 T cell response larger numbers of F5-WT cells were embedded in the airways, as compared to the F5-CD69KO cells, but there was no difference in the numbers of F5-WT and F5-CD69KO cells in the lungs parenchyma (Figure 4-10B). By 25 dpi larger numbers of F5-WT cells were detected in the airways and parenchyma compared to the F5-CD69KO cells, but the distribution between two cell types was not significantly different (Figure 4-10C). Furthermore, when equal numbers of CD45.1+ F5-WT and GFP+ F5-DKO (2x10⁴) cells were transferred into GFP+ 2.3 mice two days before E61-13-H17, infection more F5-WT cells were found in the lungs 10dpi and 25dpi, as compared to the F5-DKO cells (Figure 4-11A and 4-11B). Interestingly, by 25dpi many F5-DKO cells were positioned close to the blood vessels, as compared to the F5-WT cells (Figure 4-11B).

To analyze CD69 deficiency influenced their location between WT and CD69KO cells in the airways during the recovery phase of infection, the 10⁴ CD45.1 F5-WT, or GFP+ F5-CD69KO, or GFP+ F5-DKO cells were transferred into each recipient two days before E61-13-H17 infection. CD45.1 and GFP were used to track distribution of transferred F5 cells. I injected CD31 antibody by i.v. 15 mins before lungs were harvested to stain endothelial cells. The lung tissues were further stained with Ep-CAM to stain lung epithelial cells 10 and 25dpi. The lung tissues were analyzed by confocal
microscopy (Figure 4-12). The imaging software was used to measure distances between the transferred cells and blood vessels. Colocalization was also used to detect cells that are touching or localized inside the blood vessels. To understand 3-dimentional space, isosurfacing was applied to give a constant volume of the blood vessels on the images, which provides more visualization of the small blood vessels. The frequencies of colocalized cells in the airways were calculated from multiple images of F5-WT, F5-CD69KO and F5-DKO transferred groups by inserting beads as before. There was no different distribution between F5-WT, F5-CD69KO and F5-DKO cells in the airways10dpi (Figure 4-12A). My data showed that higher percentages of F5-CD69KO and F5-DKO cells were closely colocalized with the blood vessels 25dpi, compared to the F5-WT cells in the airways (Figure 4-12B and 4-12C). Table 1 indicates the cell numbers that are touching blood vessels in the airways using multiple images 25dpi. These suggest that CD69 helps influenza specific CTL migration efficiently and may play a role in egression/retention of virus-specific CTLs in the lungs during late stage of infection.

**Aim 2.3: How does the route of infection influence the local CTL response?**

*CTL migration was analyzed after the different route of infection.*

A recent study suggested that the mucosal route of infection increased CD8 T cell migration into the lung airways after Sendai virus infection [195]. To further determine how the route of infection influenced the size and phenotype of the pathogen-specific CTL populations in the lungs, I infected B6 with recombinant Lister monocytogenes (LM-OVA) by oral infection. For comparison another group of mice was infected intranasally with WSN-OVA virus. At different times after infection I used MHCI tetramer analysis to compare the numbers of OVA-specific CD8 T cells in the tissues.
I found large numbers of OVA-specific CD8 T cells in the tissues from both groups of mice 10 and 20dpi, but smaller percentages of the OVA-specific CD8 T cells in the LM-OVA infected mice were found in the lungs, as compared to the WSN-OVA infected mice (Figure 4-13A). Furthermore, the respiratory infection induced higher frequencies of OVA-specific CD8 T cells that expressed CD69 alone, CD103 alone and CD69 together with CD103 in the lungs, than the oral infection (Figure 4-13B). These data support the idea that a local route of infection caused larger percentages of the total pathogen-specific CD8 T cells to enter the lungs.

I next used serial infections to investigate whether some circulating virus-specific memory CD8 T cells were recruited into the gastrointestinal tract during an oral LM-OVA infection and whether they became CD69 and/or CD103 in the absence of local antigen stimulation. The mice were first infected with HKx31 (i.n.) to generate virus-specific memory CD8 T cells. Six months later same mice were infected with LM-OVA by oral feeding. MHCI tetramer analysis showed that there were very few of NP- and PA-specific CD8 T cells in the epithelium or lamina propria of the small intestine before oral infection, but some NP- and PA-specific CD8 T cells were located in the Peyer’s patches (Figure 4-14).

After LM-OVA infection the numbers of NP-specific CD8 T cells in the epithelium of the small bowel increased, but the numbers of PA-specific CD8 T cells were unchanged. The numbers for both populations of influenza virus-specific memory CD8 T cells were increased in the lamina propria by 9dpi, while most virus-specific CD8 T cells had disappeared from the Peyer’s patches. As inflammation decreased the numbers of NP- and PA-specific CD8 T cells in the epithelium and lamina propria decreased and some virus-specific CD8 T cells reappeared in the Peyer’s patches by 15dpi (Figure 4-14B).
These data showed that some NP-specific CD8 T cells were recruited into the IEL and LP compartment during peak of inflammation. Most of the NP-specific CD8 T cells in the IEL compartments expressed both CD69 and CD103 at all three time points. In contrast the NP-specific CD8 T cells in the LP included approximately equal numbers of CD69-single positive and CD69/CD103 double positive CTL, while the Peyer’s patches contained heterogeneous populations of NP and PA-specific CTL (Figure 4-14C). These data indicate that some NP-specific CD8 T cells can be recruited into the IEL and LP compartments by non-specific inflammation, where they express CD69 and CD103 without local antigen stimulation, but the numbers quickly decrease as inflammation declines.
DISCUSSION

Previously, members of our laboratory have shown that processed viral antigens persist in the MLN more than one month after infection with WSN-OVA virus [145]. In addition, this paper showed that CD11b+DCs present peptides to naive virus-specific CD8 T cells in the MLN during the late stage of the response [191]. These data are consistent with a recent finding that viral RNA is present in the lungs during the time that the viral peptides were present, as shown using nested PCR analysis [193]. Together these data suggest that new peptides may be produced from the persisting viral genome in the lungs. In contrast, others found that viral peptides do not persist more than 15 days after infection with the X31-OVA virus [136]. I used these viruses to study how antigen persistence influences the phenotypes and numbers of virus-specific CTL in the lungs. My data showed that large percentages of endogenous influenza virus-specific CTL maintained an activated phenotype during late antigen presentation as shown by stable CD69 and CD103 expression in the lungs (Figure 4-2). CD69 as a marker for activation is transiently expressed by TcR stimulation [183]. Thus, these data suggest that persisting antigen presentation maintains activated Tem cells in the lungs, which have an important role in controlling secondary influenza virus infection.

When I used 25D1.16 antibodies to block SIINFEKL with MHCI molecules, the blocking antigen presentation did not change the percentage of CD69+ OVA-specific CTLs in the airways, so it is possible that CD69 expression can be maintained by an antigen-independent mechanism at that site [72,196]. Although type I IFN can induce transient CD69 expression on CD8 T cells [184,185], another member of our laboratory did not find IFN mRNA by polymerase chain reaction (PCR) during the late stage of the
response (Tao Wu Unpublished data). These data support the idea that local antigen stimulation increases the numbers of CD69+ antigen-specific CTL in the lungs.

Other studies demonstrated that CD69+ virus-specific Tem cells are greatly enriched in the airways and the lungs during recovery from respiratory infections including Sendai and influenza viruses \[^{145;146}\]. The CD69 is a type II C-type lectin which is involved in lymphocyte migration \[^{180}\]. However, it has not been investigated whether CD69 plays a role in influenza virus-specific CD8 T cell migration into the lungs or other peripheral tissues. A study has suggested that CD69 interacts with complex oligosaccharides which are expressed by epithelial cells \[^{182}\]. In addition, there is evidence that CD69 has a role in CD4 T cell migration to the lungs \[^{186}\], suggesting that CD69 expression may play a role in virus-specific CD8 T cell migration. My data are consistent with this idea, since I found that CD69 induces efficient virus-specific Teff cell migration into the lungs after influenza virus infection.

My phenotypic data show that CD69 was expressed on tetramer-specific CTLs in the airways during the recovery phase from influenza virus infection as shown in Figure 4-2. Recent studies have demonstrated that CD69 directly interacts with S1PR1, which delay T cells egress from the secondary lymphoid organs \[^{187}\]. This suggests that CD69 deficiency may influence the kinetics of virus-specific Teff cell activation in the draining lymph node \[^{187;188}\]. However, others also showed that the CD69-deficient CD8 T cells are able to proliferate at similar rate to WT CD8 T cells as demonstrated by BrdU (5-bromo-2-deoxyuridine) incorporation \[^{197}\]. In addition, my data showed that there was no significantly different between CD69KO and WT virus-specific Teff cells in the MLN. In addition, CD69KO virus-specific Teff cells are able to produce IFN\(\gamma\) after in vitro stimulation (Figure 4-6). Thus, CD69 deficiency does not influence influenza virus-
specific CTL activation during early infection. It also has been implicated that S1PR1 plays a role in T cell egress from the lymphoid organs and the skin tissue [187,198]. The finding that influenza virus-specific CD8 T cells maintained high levels of CD69 expression in the lungs during late stage of infection suggests that the expression of S1PR1 on the cell surface is down regulated by direct CD69 interaction. My data demonstrated that more CD69KO virus-specific Teff cells are colocalized with the blood vessels in the airways, compared to the wild type virus-specific Teff cells. This suggests that more CD69KO virus-specific Teff cells are attracted toward S1P ligand gradient in the blood vessels (Figure 4-12). However, it is unknown how virus-specific Teff cells which express high levels of S1PR1 can reach the S1P ligand from the blood vessels. Since high concentration of S1P is maintained in the blood and lymphatic vessels [199], it is possible that influenza virus infection induces lung injury and S1P ligand leaks from the blood vessels. Since a S1P ligand plays a role in enhancing endothelial barrieral function in the lungs [200], high levels of S1P ligand may be produced by some endothelial cells when the endothelial cells are injured. Thus these data suggest that CD69 expression promotes Tem cells retain in the peripheral tissues.

Recent work has shown that tissue-specific resident memory T cells (Trm) which are characterized by CD69 expression can persist in several different tissues such as the skin, gut, salivary gland, lung and brain in mouse models and human [80,114-118]. For example, a recent study showed that resident memory CTLs which express CD69 and VLA-1 are detected in the epithelial layer of the skin after herpes simplex virus (HSV) clearance [119]. Previously, members of our laboratory showed that CD69+ virus-specific Tem cells are maintained in the lungs several months after influenza virus infection [145]. These suggest that the activated virus-specific Teff cells are mostly located in the tissues that have recovered from a recent viral infection. However, others showed that
local antigen stimulation is not required for CD69 expression on Trm cells. A recent study has shown the naïve CD8 T cells can enter IEL compartment of the GI tract during homeostatic proliferation and become CD69+ CD8 T cells which does not express PD-1 which is a marker for recent antigenic stimulation \[^{114}\]. These data suggest that the generation and maintenance of Trm cells vary in different tissues.

In contrast to Trm, virus-specific Tem cells can be recruited from the circulation into the lungs following heterologous virus infection \[^{73}\]. My data showed that NP-specific memory CD8 T cells are recruited into both the IEL and LP 9dpi with LM-OVA infection, but PA-specific CTLs increase in numbers only in the LP (Figure 4-14). These recruited virus-specific Tem cells in the GI tract express high percentages of CD69 and CD103 without antigen stimulation. It is consistent with data that CD8+ intraepithelial lymphocytes express CD69 in the absence of cognate antigen stimulation in the gut \[^{201}\], implying that microenvironment provides specific signal to induce CD69 expression \[^{114}\]. When inflammation declined the numbers of NP- and PA-specific Tem cells are decreased in the small intestines, suggesting that local antigen presentation enhances virus-specific Tem retention in the peripheral tissues.

In this chapter, my data showed that antigen persistence in the lungs increases the numbers of antigen-specific Tem cells which express CD69 and CD103. In vivo competition studies demonstrated that CD69 deficiency induces inefficient influenza specific-CTL migration to the lungs. Confocal microscopy analysis demonstrated that CD69 deficiency induces different distribution of virus-specific Tem cells which are closely located or colocalized in the blood vessels. Thus, the role of CD69 expression promotes efficient migration of antigen-specific Teff cells into the lungs and may enhance virus-specific Tem cells stay in the airways long after infection by down-
regulating S1P1 receptors. Interestingly, bystander influenza virus-specific Tem cells which express CD69 without cognate antigen stimulation can be recruited into the GI tracks during small intestine inflammation.
Figure 4-1:

[Histograms showing cell numbers for 20dpi and 30dpi for WSN-OVAi, X31-OVA, and Uninfected groups.]

- WSN-OVAi: 74% (±0.2) at 20dpi, 47% (±2) at 30dpi
- X31-OVA: 22% (±2) at 20dpi, 7% (±0.8) at 30dpi
- Uninfected: 0% at 20dpi, 13% at 30dpi
Figure 4-1. Different influenza virus strains vary in their ability to support antigen persistence in the mediastinal lymph node (MLN).

CFSE-labeled naïve OTI-WT cells were transferred to each group of mice 20 and 30 days after infection with either WSN-OVA<sub>i</sub> or X31-OVA. The OTI cells were analyzed using CD45.1 congenic. The MLNs of 3 mice per group were analyzed for CFSE dilution 7 days after transfer. Histograms from representative animals show gated populations of transferred OTI cells. Duplicate experiments gave similar results.
Figure 4-2:

A

<table>
<thead>
<tr>
<th>OVA-tet (10dpi)</th>
<th>CD62L</th>
<th>CD69</th>
<th>CD103</th>
<th>CD127</th>
<th>PD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL</td>
<td><img src="image1" alt="Histograms" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td><img src="image2" alt="Histograms" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLN</td>
<td><img src="image3" alt="Histograms" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NP31-tet (10dpi)</th>
<th>CD62L</th>
<th>CD69</th>
<th>CD103</th>
<th>CD127</th>
<th>PD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL</td>
<td><img src="image4" alt="Histograms" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td><img src="image5" alt="Histograms" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLN</td>
<td><img src="image6" alt="Histograms" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PA-tet (10dpi)</th>
<th>CD62L</th>
<th>CD69</th>
<th>CD103</th>
<th>CD127</th>
<th>PD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL</td>
<td><img src="image7" alt="Histograms" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td><img src="image8" alt="Histograms" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLN</td>
<td><img src="image9" alt="Histograms" /></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4-2:

B

<table>
<thead>
<tr>
<th></th>
<th>CD62L</th>
<th>CD69</th>
<th>CD103</th>
<th>CD127</th>
<th>PD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OVA-tet (20dpi)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL</td>
<td>8</td>
<td>67</td>
<td>34</td>
<td>43</td>
<td>58</td>
</tr>
<tr>
<td>Lung</td>
<td>10</td>
<td>62</td>
<td>138</td>
<td>51</td>
<td>75</td>
</tr>
<tr>
<td>MLN</td>
<td>54</td>
<td>22</td>
<td>45</td>
<td>72</td>
<td>33</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>CD62L</th>
<th>CD69</th>
<th>CD103</th>
<th>CD127</th>
<th>PD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NP31-tet (20dpi)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL</td>
<td>1</td>
<td>74</td>
<td>24</td>
<td>22</td>
<td>82</td>
</tr>
<tr>
<td>Lung</td>
<td>5</td>
<td>68</td>
<td>33</td>
<td>29</td>
<td>38</td>
</tr>
<tr>
<td>MLN</td>
<td>22</td>
<td>51</td>
<td>27</td>
<td>41</td>
<td>71</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>CD62L</th>
<th>CD69</th>
<th>CD103</th>
<th>CD127</th>
<th>PD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PA-tet (20dpi)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL</td>
<td>6</td>
<td>76</td>
<td>68</td>
<td>49</td>
<td>50</td>
</tr>
<tr>
<td>Lung</td>
<td>6</td>
<td>77</td>
<td>64</td>
<td>56</td>
<td>57</td>
</tr>
<tr>
<td>MLN</td>
<td>160</td>
<td>28</td>
<td>45</td>
<td>78</td>
<td>23</td>
</tr>
</tbody>
</table>
Figure 4-2:
Figure 4-2. The presence of antigen changes the phenotypes of virus-specific CTL in the lung.

(A-C) C57BL/6 mice were infected with either WSN-OVA<sub>i</sub> or X31-OVA virus. MHCI tetramers were used to analyze influenza virus-specific CD8<sup>+</sup> T cells in different tissues 10, 20 and 30dpi. Histograms indicate representative animals from each group of 5 mice. Overlaid Histograms show gated populations of tetramer-specific CD8<sup>+</sup> T cells from WSN-OVA<sub>i</sub> (dashed line) or X31-OVA infected mice (gray shading). The percentages of cells that are positive for each marker are displayed for WSN-OVA<sub>i</sub> (above the line) or X31-OVA infected mice (below the line). Statistical significance was determined using an unpaired students T test. Mean±SD from 3-5 mice per group are shown. (*P<0.05, **P<0.01 and ***P<0.001). Duplicate experiments gave similar results.
Figure 4-3:

A

<table>
<thead>
<tr>
<th>OVA+/tet</th>
<th>CD103</th>
<th>CD69</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSN-OVA</td>
<td>2 (±0.7)</td>
<td>32 (±3.3)</td>
</tr>
<tr>
<td>I</td>
<td>30 (±11.8)</td>
<td>32 (±3.3)</td>
</tr>
<tr>
<td>X31-OVA</td>
<td>4 (±1.7)</td>
<td>15 (±1.3)</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>OVA+/tet</th>
<th># of OVA+ cells (x 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td></td>
</tr>
<tr>
<td>MLN</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>WSN-OVA</td>
<td>28 (±2.1)</td>
</tr>
<tr>
<td>X31-OVA</td>
<td>32 (±3.3)</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>OVA+/tet</th>
<th>% of OVA+ cells in the lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSN-OVA</td>
<td></td>
</tr>
<tr>
<td>X31-OVA</td>
<td></td>
</tr>
</tbody>
</table>

30dpi

* 0.5
** 1.0
*** 1.5
** 2.0
30dpi

** 120
80
** 80
40
20
Figure 4-3. The antigen persistence increases the numbers of activated virus-specific CTL in the lung.

(A) The OVA-specific CD8 T cells from the WSN-OVA₁ or X31-OVA infected mice were analyzed for CD69 and CD103 expression. MHC-I tetramers were used to analyze influenza virus-specific CD8 T cells in the lungs 30dpi.

(B) The numbers of OVA-specific CD8 T cells were counted in different tissues 10 and 30 dpi with WSN-OVA₁ (white) or X31-OVA (black) virus. Bar graphs indicate the number of OVA-specific CD8 T cells in different tissues.

(C) Comparison of OVA-specific CD8 T cells in the lungs and secondary lymphoid organs. The percentages were calculated from the total OVA-specific CD8 T cells in the tissues after infection with WSN-OVA₁ (white bar) or X31-OVA (black bar) infected mice. Statistical significance was determined using an unpaired students T test. Mean±SD from 3-5 mice per group are shown. (*P<0.05, **P<0.01 and ***P<0.001). Duplicate experiments gave similar results.
Figure 4-4:
**Figure 4-4. Interactions with MHCI molecules keep OVA-specific CTL activated during the late stage of the infection.**

**(A)** The WSN-OVA<sub>i</sub>-infected mice were injected with blocking 25D1.16 or isotype antibodies 20 and 23 dpi. The numbers and phenotypes of virus-specific CD8 T cells were analyzed 6 days later in the lungs. Histograms indicate representative animals from each group of 3 mice. Overlaid Histograms show gated populations of tetramer-specific CD8 T cells after treatment with isotype (dashed line) or 25D1.16 antibodies (gray shading). The percentages of cells in the marked region are displayed for isotype (above the line) and 25D1.16 (below the line).

**(B)** Bar graphs indicate the numbers of virus-specific CD8 T cells in the lungs from isotype (white bar) or 25D1.16 (gray bar) antibody treated mice. Statistical significance was determined using an unpaired students T test. Mean±SD from 3-5 mice per group are shown. (*P<0.05, **P<0.01 and ***P<0.001). Duplicate experiments gave similar results.
Figure 4-5:

WSN-OVAi  X31-OVA

10dpi

A  aw

B  aw

30dpi

C  aw

D  aw

E  aw

Uninfected
Figure 4-5. There is mild inflammation in the lungs during late antigen presentation.

Mice were infected with (A & C) WSN-OVA, or (B & D) X31-OVA virus. Inflated lungs were embedded in paraffin 10dpi and 4 micron sections were stained with H&E (A & B) 10dpi and (C & D) 30dpi.

(E) The lung tissues from uninfected B6 mice were used as a control group. Photographs were taken at 10x magnification (aw = airway). Similar results were obtained from two independent experiments. Duplicate experiments gave similar results.
Figure 4-6:
Figure 4-6:

B

<table>
<thead>
<tr>
<th></th>
<th>F5 WT</th>
<th>F5 CD69KO</th>
<th></th>
<th>F5 WT</th>
<th>F5 CD69KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>3dpi</td>
<td></td>
<td></td>
<td>4 dpi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFSE</td>
<td></td>
<td></td>
<td>CFSE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td></td>
<td></td>
<td>IFNγ</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lung

MLN

Spleen

Control (no peptide)
Figure 4-6. CD69-deficiency does not have a major impact on CTL activation or IFNγ production.

(A) Equal numbers of CFSE-labeled naïve F5-WT and F5-CD69KO cells (total 10^6) were transferred to the same recipient mice two days before infection with E61-13-H17. The dividing F5 cells were analyzed using CD45.1 and CD45.2 markers in different tissues 3, 5, 7 and 9dpi. The dot plots show a representative animal from 3 mice.

(B) Equal numbers of CFSE-labeled F5-WT and F5-CD69KO cells (1x10^6) were adoptively transferred to the same recipient mice two days before E61-31-H17 infection. The dividing F5 cells were analyzed using CD45.1 and CD45.2 markers in the different tissues 3 and 4dpi. Lymphocytes were enriched in the lungs, MLN and spleen after peptide stimulation in the presence of brefeldin A. The percentages of IFNγ producing CD8 T cells are shown. Duplicate experiments gave similar results.
Figure 4-7:

A

CD45.1
F5-WT

CD45.1/2
F5-CD69KO

-2d 0 +10d +20d +30d

C57BL/6

E61-13-H17

B

Percentages of transferred cells

KO WT

CD103

BAL

Lung

MLN

Spleen

KO WT

CD103

F5-CD69KO

F5-WT

10dpi

20dpi

30dpi
Figure 4-7:

C

![Graph showing percentages of transferred cells for OTI-CDE8KO and OTI-WT at 10dpi, 20dpi, and 30dpi across BAL, Lung, MLN, and Spleen compartments.](image-url)
Figure 4-7. CD69 expression is required for efficient CTL migration to the lungs. 

(A) Experimental protocol and timeline.

(B) Equal numbers of F5-WT and F5-CD69KO cells (total $10^6$) were transferred to B6 mice two days before E61-13-H17 infection. The transferred F5 cells were analyzed using CD45.1 and CD45.2 congenic markers in different tissues 10, 20 and 30dpi. The bar graphs indicate the frequencies of F5-WT (black) and F5-CD69KO (white) cells within the transferred cell population. Histograms from representative animals show gated populations of transferred F5-CD69KO (white) or F5-WT (black). Statistical significance was determined using an unpaired students T test. Mean±SD from 3 mice per group are shown (*P<0.05 **P<0.01 and ***P<0.001). Similar results were obtained from three independent experiments.

(C) Equal numbers (total $10^6$) of OT-WT and OTI-CD69KO cells were transferred to B6 mice two days before WSN-OVA i infection. The mutant and wild type OTI cells were identified using CD45.1/CD45.2 congenic markers in different tissues 10, 20 and 30dpi. The bar graphs indicate the ratios of OTI-WT (gray) and OTI-CD69KO cells (white) within the transferred cell population. Histograms from representative animals show gated populations of transferred OTI-CD69KO (white) or OTI-WT (gray). Duplicate experiments gave similar results.
Figure 4-8:

```
Figure 4-8:

Percentage of transferred cells

BAL
Lung
MLN
Spleen
Blood

10dpi
20dpi
30dpi

DKO
WT

CD103
CD69

CD103
CD69

DKO
WT

32% 0%
15% 0%

44% 0%
32% 0%

5% 0%
0% 0%

28% 0%
9% 0%

76% 7%
87% 1%

67% 0%
70% 0%

63% 0%
62% 15%

54% 2%
65% 1%

50% 10%
48% 0%

31% 1%
28% 1%

44% 1%
25% 1%

33% 1%
19% 1%

39% 4%
33% 4%

5% 12%
44% 25%

5% 12%
44% 25%

5% 12%
44% 25%

5% 12%
44% 25%

```
Figure 4-8. Dual CD69/CD103KO induces inefficient migration of virus-specific CD8 T cells into the lungs.

Equal numbers (total $10^6$) of F5-WT and F5-DKO cells were transferred to B6 mice two days before E61-13-H17 infection. The transferred F5 cells were analyzed using CD45.1 and CD45.2 congenic markers. The bar graphs indicate the frequencies of F5-WT (gray) and F5-DKO (white) cells within the transferred cell population. Overlaid histograms indicate representative animals from each group of 3 mice. The percentages of cells that are positive for each marker are displayed for F5-WT (above the line) or F5-DKO (below the line) cells. Statistical significance was determined using an unpaired students T test. Mean±SD from 3 mice per group are shown (*P<0.05 **P<0.01 and ***P<0.001).
Figure 4-9:
Figure 4-9. The imaris software is used to count the transferred CD8 T cells.

(A) Equal numbers of CD45.1 F5-WT \(10^4\) and GFP+ F5-CD69KO \(10^4\) cells were transferred to 2.3 mice two days before E61-13-H17 infection. The transferred OTI cells were analyzed using confocal microscopy using CD45.1 and GFP 17dpi. Each color indicates F5-WT (Blue), F5-CD69KO (Green), CD31 (Yellow) and Ep-CAM (Red). The Ep-CAM antibody was used to mark the outer boundary of the airway. (B) The numbers of transferred F5 cells were counted using Imaris imaging program.
Figure 4-10:

A  

CD45.1  
F5-WT  
GFP+  
F5-CD69KO  

-2d  0  +10d  +25d  
E61-13-H17  

GFP+2.3  

B  10dpi  

Ep-CAM  CD31  F5-CD69KO  F5-WT  

C  25dpi  

E61-13-H17
Figure 4-10. Dual transfers were used to show that CD69 deficiency reduces the numbers of virus-specific CD8 T cells in the lungs.

(A) Experimental protocol and timeline. Equal numbers of CD45.1 F5-WT (10⁴) and GFP+F5-CD69KO (10⁴) cells were transferred to GFP+2.3 mice two days before E61-13-H17 infection. The transferred F5 cells were analyzed using confocal microscopy using CD45.1 and GFP (B) 10 and (C) 25dpi. Each color indicates F5-WT (Blue), F5-CD69KO (Green), CD31 (yellow) and Ep-CAM (Red). The Ep-CAM antibody was used to mark the outer boundary of the airway. The numbers of transferred cells were counted inside and outside the white line. 4 animals gave similar results.
Figure 4-11:
Figure 4-11. Confocal microscopy was used to analyze the distribution of dual CD69/CD103KO cell in the lungs.

Equal numbers of CD45.1 F5-WT ($10^4$) and GFP+F5-DKO ($10^4$) cells were transferred to GFP+2.3 mice two days before E61-13-H17 infection. The transferred F5 cells were analyzed using confocal microscopy using CD45.1 and GFP 10 and 25dpi. Each color indicates F5-WT (Blue), F5-DKO (Green), CD31 (yellow) and Ep-CAM (Red). The Ep-CAM antibody was used to mark the outer boundary of the airway. Images show representative areas from each group (A) 10dpi and (B) 25dpi. a & c show around the airways, but b & d display the parenchyma. The numbers of transferred cells were counted inside and outside the white line. Multiple animals gave similar results.
Figure 4-12:

A

10dpi

F5 WT

F5 CD69KO

F5 DKO

Ep-CAM  F5 cells  CD31
Figure 4-12:

B

F5 WT

F5 CD69KO

F5 DKO

25dpi

Ep-CAM | F5 cells | CD31 | Coloc

113
% of F5 cells associated with blood vessels

WT  CD69KO  DKO
Figure 4-12. Confocal microscopy shows that many CD69KO cells are close to the blood vessels during late antigen presentation.

2.3 mice were received either CD45.1 F5-WT (10⁴), or GFP+F5-CD69KO (10⁴), or GFP+F5-DKO cells two days before E61-13-H17 infection. The transferred F5 cells were analyzed in the lungs using confocal microscopy (A) 10dpi and (B) 25dpi. GFP (green), CD31 (blue) and Ep-CAM (Red). The Ep-CAM staining was used to mark the outer boundary of the airways. (B) Colocalization analysis and isosurfacing were performed using the Imaris imaging software 25dpi. Magenta indicates contacts between the F5 cells (green) and the blood vessels (blue). a-c show the airways for F5-WT, F5-CD69KO and F5-DKO cells, respectively.

(C) Cumulative data from multiple fields. Red and black indicate data from different animals analyzed 25dpi with E61-13-H17. The cell numbers are shown in table 4-1. Statistical significance was determined using an unpaired students T test. Mean±SD from multiple images of 4 mice per group are shown (*P<0.05 **P<0.01 and ***P<0.001). Duplicate experiments gave similar results.
Table 4-1:

A. Numbers of contacts with vessels

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>WT</th>
<th>% Contact</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2/48</td>
<td>32/107</td>
</tr>
<tr>
<td></td>
<td>12/63</td>
<td>5/51</td>
</tr>
<tr>
<td>1</td>
<td>15.7±5.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6/71</td>
<td>5/74</td>
</tr>
<tr>
<td></td>
<td>4/65</td>
<td>6/87</td>
</tr>
<tr>
<td></td>
<td>5/62</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7.3±0.3</td>
<td></td>
</tr>
</tbody>
</table>

B. Numbers of contacts with vessels

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>CD69KO</th>
<th>% Contact</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26/54</td>
<td>9/45</td>
</tr>
<tr>
<td></td>
<td>6/38</td>
<td>23/62</td>
</tr>
<tr>
<td></td>
<td>15/59</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>29.3±5.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33/55</td>
<td>36/73</td>
</tr>
<tr>
<td></td>
<td>30/126</td>
<td>13/71</td>
</tr>
<tr>
<td></td>
<td>13/44</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>36.2±7.9</td>
<td></td>
</tr>
</tbody>
</table>

C. Numbers of contacts with vessels

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>DKO</th>
<th>% Contact</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20/90</td>
<td>16/93</td>
</tr>
<tr>
<td></td>
<td>42/133</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>23.7±4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33/115</td>
<td>42/120</td>
</tr>
<tr>
<td>2</td>
<td>31.9±3.2</td>
<td></td>
</tr>
</tbody>
</table>
Table 4-1. The numbers of virus-specific effector CTLs colocalized with the blood vessels.

Either (A) CD45.1+ F5-WT (10^4), or (B) GFP+ F5-CD69KO, or (C) GFP+ F5-DKO cells were transferred to the separate GFP+2.3 recipient mice one day before E61-13-H17 infection. The transferred F5 cells were analyzed using GFP+ and CD45.1 congenic marker 25dpi. The numbers of cells that were touching blood vessels are shown as a fraction of the total cells in the field.
Figure 4-13:

A

10dpi | 20dpi
---|---
Respiratory | Oral

# of OVA + cells (x 10^5)

BAL

Lung

Spleen

B

10dpi | 20dpi
---|---
WSN-OVAI | LM-OVA

Lung

Spleen

CD103

CD69
Figure 4-13. The route of infection changes the distribution of virus-specific CD8 T cells in mucosal tissues.

C57BL/6 mice were infected with either WSN-OVA (i.n.) or LM-OVA (oral feeding). MHCI tetramers were used to analyze influenza virus-specific CD8 T cells in different tissues 10 and 20dpi.

(A) The numbers of OVA-specific CD8 T cells in the BAL, Lungs and spleens were compared between WSN-OVA and LM-OVA infected mice.

(B) The OVA-specific CD8 T cells from the WSN-OVA or LM-OVA infected mice were analyzed for CD69 and CD103 expression. Statistical significance was determined using an unpaired students T test. Mean±SD from multiple images of 4 mice per group are shown (*P<0.05 **P<0.01 and ***P<0.001). Duplicate experiments gave similar results.
Figure 4-14:

A

B

C

CD69

CD103

IEL

LP

PP

No recall

9dpi recall (LM-OVA)

15dpi recall (LM-OVA)

NP31

PA

NP31

PA

NP31

PA

IEL

LP

PP

IEL

LP

PP

IEL

LP

PP
Figure 4-14. Bystander virus-specific memory CTLs are recruited in the small intestine during local infection with LM-OVA.

(A) Experimental protocol and timeline. C57BL/6 mice were infected with the HKx31 virus. B6 mice were infected with LM-OVA (oral) 6 months after influenza virus infection (i.n.). MHCI tetramers were used to analyze influenza virus-specific CD8 T cells. (B) The numbers of NP- and PA-specific memory CTLs were counted in different regions of the small bowel 9 and 15dpi. (C) CD69 and CD103 expression on NP- and PA-specific memory CD8 T cells after LM-OVA infection. Duplicate experiments gave similar results.
CHAPTER V:

DISCUSSION AND CONCLUDING REMARKS
The objective of this thesis was to investigate how virus-specific Trm cells protect the lungs during the recovery from a local viral infection. Previously, members of our laboratory used parabiosis studies to show that virus-specific CTLs which express CD69 and CD103 in the lung airways are non-migratory cells (Figure 5-1) \cite{145}. Studies of humans also show that a large numbers of Trm cells can be found in the non-inflammed lungs, which express CD69, CD103 and VLA-1 \cite{115}. These lung Trm cells have the ability to produce cytokines including TNF\(\alpha\), IFN\(\gamma\), IL-4, and IL-17 after \textit{in vitro} stimulation using heat killed influenza virus-pulsed APCs \cite{115}. A previous study has shown that VLA-1+ influenza virus-specific CD8 T cells in the lungs provide immediate protective immunity against secondary challenge \cite{80}, however the role of CD103 on Trm cells is largely unknown.

\textit{Trm cells provide immediate heterosubtypic immunity.}

Virus-specific memory CD8 T cells are heterogeneous populations that can be divided into distinct subsets including Tcm and Tem. The Tem cells preferentially circulate nonlymphoid tissues, while Tcm cells circulate between the blood and secondary lymphoid tissues \cite{113}. The numbers of circulating Tem cells decline over time, since they progressively lose the ability to migrate into the peripheral tissues \cite{202}. Resident populations of Trm cells have been found in many different tissues including the skin, gut, salivary gland, lung and brain \cite{80,114-116,118,119}. Some studies found that local antigen stimulation was required for induction and maintenance of CD103 expression on Trm cells in the brain and lungs \cite{115,116,171}. In contrast, others suggest that local antigen stimulation is not necessary for chronic CD103 expression on Trm cells in the skin and salivary gland \cite{118,119}. The best evidence that Trm can promote immunity
comes from skin models which were used to show that Trm cells contribute to protection against reinfection with HSV by accelerating viral clearance \[^{119}\].

Many studies have shown that activated virus-specific CD8 T cells which express CD69 and CD103 also become greatly enriched in the airways after respiratory virus infections \[^{73;145;146;179}\]. These virus-specific Trm cells persist in the lungs for several months and are believed to mediate an accelerated response to reinfection by making IFN\(\gamma\) and other proinflammatory cytokines that recruit other immune cells into the local tissues. Although these cells are not lytic, they can acquire cytolytic activity after proliferation in response to antigen \[^{146;203}\]. Most resting Trm cells in the airways do not constitutively express perforin and granzyme B, but after \textit{in vitro} peptide stimulation they are capable of making cytotoxic molecules \[^{204}\].

\textit{CD103 enhances virus-specific CTL function.}

The reason that airway resident Trm cells lack cytotoxic activity may be related to the loss of CD11a expression \[^{72;73}\], which can be cleaved by metalloproteinase-9 (MMP-9) expressed by macrophages \[^{74}\]. LFA-1 is the adhesion molecule that promotes cytolytic activity by stabilizing the interactions between CTLs and target cells, resulting in granule polarization \[^{72;205}\]. Since CD103 is an adhesion molecules which promotes interactions with epithelial cells \[^{206}\], it is possible that CD103 provides an alternative mechanism for virus-specific CTL to kill infected cells in the airways. Evidence to support this idea comes from transplanted islet allografts which are not rejected in host mice that lack CD103 expression, but transfer of CD103+ CD8 T cells into the mice promotes rejection of the allografts \[^{207}\]. In addition, another study demonstrates that the engagements of CD103 and E-cadherin played a role in the destruction of lung tumor cells by CTLs through granzyme B polarization and exocytosis \textit{in vitro}, when
CD11a/ICAM-1 interactions were blocked \[^{208}\]. These studies suggest that CD103 expression on CTL can promote lytic activity toward epithelial cells that express E-cadherin.

Lung epithelial cells that express E-cadherin are major targets for influenza virus replication. Since CD103 is an adhesion molecule that plays a role in CTL migration to mucosal tissues \[^{206}\], I investigated whether virus-specific CTL migration after influenza virus infection. Interactions with E-cadherin are believed to enhance the adhesion properties of virus-specific CTLs during interactions with epithelial cells \[^{209}\]. My \textit{in vivo} competition studies (Figure 3-7) demonstrate that CD103 deficiency does not impact virus-specific CTL migration during acute viral infection, but reduces the ability of virus-specific CTLs to stay in the lungs during the recovery stage of the infection. Other studies also show that CD103 expression on virus-specific CTLs has a role in motility. A recent study shows that E-cadherin \textit{in vitro} culture influences the movements of CD103+ cells by increasing cellular protrusions and filopodia \[^{210}\], suggesting that their interactions play roles in cell motility and morphology. I found smaller numbers of CTL in the lungs when CD103 not expressed. The biggest difference was late stage of the infection which was consistent with a role in retention not migration.

\textit{TGFβ regulates the numbers of Teff cells during viral infection.}

My data show that TGFβ is required for CD103 expression on virus-specific CTLs (Figure 3-5). TGFβ is a pleiotrophic cytokine which controls a variety of cellular processes including cell death \[^{106}\]. A recent study showed that TGFβ selectively induces apoptosis of KLRG1+ virus-specific Teff cells in LCMV infected mice during early clonal expansion using dnTGFβRII mice \[^{107}\]. I used the same animals to examine how TGFβ signals influences CTL responses in the lungs and found much larger
numbers of virus-specific CTLs which expressed KLRG1 in the lungs and spleen when TGFβ signals were blocked (Figure 3-1). In addition, these KLRG1+ cells were capable of making proinflammatory cytokines, including IFNγ and TNFα following in vitro stimulation with synthetic peptides.

Since IFNγ and TNFα can cause severe immunopathology \cite{152,153}, I used histological analysis to examine whether blockade of TGFβ signals induced severe lung pathology in the influenza virus infected lungs. My data showed that there were extensive areas of inflamed tissue in the lungs including large numbers of infiltrating leukocytes in the dnTGFβRII+ mice during the peak of CTL response. However, the wild type animals showed more extracellular exudates and evidence of hemorrhage than the dnTGFβRII+ mice (Figure 3-4). Surprisingly, there were no dead animals in either group of mice. There are a couple of possibilities which may explain why the infection did not kill any animals in the absence of TGFβ signals. First, the animals were infected with HKx31 which is a mildly pathogenic strain. Second, larger numbers of virus-specific CTL may have accelerated viral clearance in the lungs of the dnTGFβRII+ mice. To confirm whether influenza viruses are cleared faster in the dnTGFβRII+ animals, we will measure the kinetics of viral clearance after infection with HKx31 virus using a plaque assay. In addition we will analyze the pathology at earlier time points after infection with HKx31 and WSN-OVA, which is a more pathogenic strain.

KLRG1 is another cell surface molecule can interact with E-cadherin on epithelial cells. Since the engagements of KLRG1 with E-cadherin inhibit downstream NFAT pathway, which reduces IL-2, TNFα and Fas ligand (CD94L) \cite{168}, this pathway could be third mechanism that stops the dnTGFβRII+ animals from dying. Although the blockade of TGFβ signals increased the numbers of KLRG1+ virus-specific CTLs in the lungs,
these cells did not express CD103 which means that KLRG1 and CD103 did not compete with each other to interact with E-cadherin. Repeated interactions between KLRG1 and E-cadherin may reduce the effector function of virus-specific CTLs.

Our data show that the CTLs which expressed CD103 in the lungs of the WT mice were located in the airways during the recovery phase of the infection. These data indicated that TGFβ signals selectively regulate the survival of different subsets of virus-specific CTLs during influenza virus infection, and also change distribution of Trm cells which express CD103. TGFβ can use multiple signaling pathways to alter cell differentiation and apoptosis. At this time we do not know whether one or two TGFβ signaling pathways regulate the CTL response.

Antigen-induced signals increase the numbers of Trm cells in the lungs.

CD69 is transiently induced on CD8 T cells by activation of the TcR signaling pathway [183] or by exposure to type I interferon [184;185]. Since large percentages of endogenous virus-specific CTLs express CD69 in the lung airways during the recovery from the influenza virus infection [145], I investigated how antigen persistence changed the numbers and phenotypes of virus-specific CTL in the lungs during the late stage of the infection using recombinant influenza viruses. WSN-OVAI and X31-OVA both express the SIINFEKL epitope [136], but differ in the ability to support antigen persistence (Figure 4-1). When I followed the phenotypic markers on the endogenous CD8 T cells, antigen persistence increased the numbers of recent activated antigen-specific CTLs which express CD69 in the airways and the lungs (Figure 4-2). In addition blocking SIINFEKL:MHCI complex by injecting 25D1.16 antibody showed reduced numbers of OVA-specific CTLs which express CD69 and CD103 in the lungs (Figure 4-4A and 4-4B). In contrast, there were decreased numbers of OVA-specific CTLs in the airways, but the
percentages of CD69+ cells were unchanged. Micro array data prepared by another member of our laboratory did not reveal prolonged expression of type I IFN mRNA in the lungs 30dpi. However, we did not examine expression levels of type III IFN which may induce CD69 expression. My data show that antigen presentation is required for CD69 expression on virus-specific CTLs in the lungs parenchyma, but may not be essential in the airways, during the late stages of the infection.

**CD69 signals regulate immune responses of virus-specific CTLs in the lungs.**

Large amounts of TGFβ can be produced by a variety of cells in the lungs including epithelial cells, fibroblasts, stromal cells, macrophages, T cells, and some DCs [106;106;160;161]. The TGFβ is secreted as an inactive form called LAP which can be activated by several different enzymes including TSP-1 protease, integrin αvβ6 and some influenza virus NA [163;164]. Interestingly, it has been suggested that CD69 has a regulatory role by inducing expression of the LAP TGFβ form. Previous studies show evidence that cross-linking CD69 using monoclonal antibody induces activation of ERK signaling pathway which is further involved in TGFβ expression in CD4 T cells [211;212], but whether CD69 can induce TGFβ expression in CD8 T cells is not known.

CD69-deficient mice also develop autoimmune diseases, including arthritis and autoimmune myocarditis as well as contact dermatitis and allergic asthma, which suggest that CD69 has an inhibitory role in the pathogenesis of some diseases. A recent study supports the idea that CD69 engagements activate Jak3/Stat5 pathway which induces IL-2 and TGFβ production, resulting in inhibition of Th17 differentiation [213;214]. Similarly, CD69 deficiency in mice can influence inflammatory responses by enhancing Th0 into Th17 differentiation [214]. Therefore, CD69 signals regulate the immune balance through TGFβ that plays a role in differentiation naïve T cells into either regulatory T
CD69 plays a role in T cell migration.

S1P is a ligand for S1PR1 and high concentration of S1P is maintained in the blood and lymphatic vessels. Several studies suggest that CD69 plays a role in T cell migration. The S1PR1 is required for T cell egress the secondary lymphoid organs [189]. CD69 slows the rate of T cell exit from the peripheral lymph node by down regulation S1PR1 following T cell activation [187, 188]. The transmembrane and membrane proximal domains of CD69 directly bind to helix 4 of S1PR1, which induces a conformational change that may provide a higher affinity for S1P to down regulates CD69-S1P1 complex [216]. My confocal microscopy studies support the idea that some CD69-deficient Trm cells are closely colocalized with the blood vessels during the recovery from the influenza virus infection (Figure 4-12B). These data imply that CD69KO cells maintain high levels of S1PR1 on the surface and are therefore attracted to the blood vessels by an S1P gradient (Figure 5-2). Our studies suggest that inflammation-induced CD69 expression on virus-specific CTLs override S1P gradient. When CD69 is not expressed, inflammation may be also sufficient. However, inflammation and CD69 are absent, the S1P gradient becomes dominant. To investigate this possibility, I will use FTY720 which can bind to S1P receptors to block cell surface expression.

In this thesis I investigated whether CD69 and CD103 are necessary for Trm cell to populate the lungs. My data show that TGFβ kills KLRG1+ CTLs, while Trm cells express CD103. In vivo competition studies show that CD69 alters CTL migration, while CD103 is required for retention of Trm cells in the lungs. These data indicate that TGFβ protects the lungs from immune damage and also plays a role in protective immunity during the infection with a new strain of influenza virus.
Figure 5-1:
Figure 5-1. Parabiosis has been used to follow CD8 T cell migration in the blood and the spleen.

Parabiosis is a surgical technique that can be used to join the blood supply of two animals. In the diagram, one mouse was infected with HKx31 virus, while the other mouse was not infected. One month later the infected mouse was surgically conjoined with naïve mouse and CD45.1/2 congenic markers were used to follow CD8 T cell migration 15 days after surgery. The data showed that some (1) Tem and (2) Tcm cells migrate to the lungs of the partner animal, but they do not express CD69 and CD103. In contrast some (3) Trm cells stay in the lungs of immune mouse long after viral clearance, where they maintain stable CD69 and CD103 expression.
Figure 5-2:

**Inflammation**

**Blood Vessel**

**Lymphatic Vessel**

**CXCR3 ligands**

**CD69KO CTL**

**CD62Llow CCR7-**

**WT CTL**

**CD62Llow CCR7-**

**Naïve T**

**CD62L+ CCR7+**

**S1P1**

**S1P**

**CD69**
Figure 5-2. CD69 may regulate virus-specific CTL migration to the peripheral tissues.

Blood vessels contain high concentrations of sphingosine-1-phosphate (S1P). (1) Naïve CD8 T cells circulate between the blood and secondary lymphoid organs using the S1PR1. (2) Activated CTLs enter the peripheral tissues in response to inflammation. When inflammation subsides, Tem cells lose CD69 expression and return to the lymphatic vessels in response to S1P. (3) Data suggest that this pathway does not apply to mucosal tissues, where Trm cells do not lose CD69 expression. When CD69 is not expressed, some Trm cells may artificially collect around the blood vessels which also contain high concentration of S1P.
LIST OF REFERENCES


38. Gack MU, Albrecht RA, Urano T et al. Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. Cell Host Microbe 2009;5:439-449.


72. Ely KH, Cookenham T, Roberts AD, Woodland DL. Memory T cell populations in the lung airways are maintained by continual recruitment. J.Immunol. 2006;176:537-543.


129. Strutt TM, McKinstry KK, Dibble JP et al. Memory CD4+ T cells induce innate

130. Larsson M, Messmer D, Somersan S et al. Requirement of mature dendritic
cells for efficient activation of influenza A-specific memory CD8+ T cells.

131. Murata K, Inami M, Hasegawa A et al. CD69-null mice protected from arthritis

132. Schon MP, Arya A, Murphy EA et al. Mucosal T lymphocyte numbers are
1999;162:6641-6649.

133. Filippi CM, Juedes AE, Oldham JE et al. Transforming growth factor-beta
suppresses the activation of CD8+ T-cells when naive but promotes their
survival and function once antigen experienced: a two-faced impact on

134. Kalajzic I, Kalajzic Z, Kaliterna M et al. Use of type I collagen green
fluorescent protein transgenes to identify subpopulations of cells at different

135. Lindsten K, Menendez-Benito V, Masucci MG, Dantuma NP. A transgenic
902.

136. Mintern JD, Bedoui S, Davey GM et al. Transience of MHC Class I-restricted
2009;106:6724-6729.

137. Daly K, Nguyen P, Woodland DL, Blackman MA. Immunodominance of major
histocompatibility complex class I-restricted influenza virus epitopes can be

138. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry.

139. Flynn KJ, Belz GT, Altman JD et al. Virus-specific CD8+ T cells in primary

140. Belz GT, Xie W, Altman JD, Doherty PC. A previously unrecognized H-2D(b)-
restricted peptide prominent in the primary influenza A virus-specific CD8(+) T-cell response is much less apparent following secondary challenge. J.Virol.


