DEVELOPMENT OF ANTI-VIRAL $CD8^+$ T CELL MEMORY IN THE RESPIRATORY ENVIRONMENT

by

HILLARY LEE SHANE

(Under the direction of Kimberly Klonowski)

ABSTRACT

Mucosal surfaces represent the major portal by which pathogens enter the body, yet there is limited understanding of how $CD8^+$ T cell responses develop and are maintained at these sites. The majority of knowledge on $CD8^+$ T cell responses and memory formation has been amassed in models of acute, systemic infections, even though it is appreciated that mucosal sites consist of immunological environments unique from sites in which responses to systemic infections develop. Moreover, a firm understanding of how memory is generated in mucosal sites is important for the development of vaccines, which may employ a systemic or mucosal route of immunization, such as those directed against influenza virus. Vaccines that target protective $CD8^+$ T cell responses are of particular interest for influenza, as memory $CD8^+$ T cells can limit severe disease and can offer protection against multiple influenza subtypes. This study shows that the respiratory environment can directly influence $CD8^+$ cell responses, resulting in localized changes in $CD8^+$ T cell memory formation as well as broadly inhibiting the formation of long-lived memory cells. We show that the mucosal cytokine thymic stromal lymphopoietin (TSLP) is produced early following mucosal

influenza infection, and acts on $CD8^+$ effector cells in a direct and non-redundant way, promoting the proliferation of these cells at the site of infection. This early response influences memory development resulting in more of an effector memory population of cells. I go on to show that by comparing vesicular stomatitis virus infection delivered by the intranasal or intravenous route, that the respiratory environment results in memory $CD8^+$ T cell population that is skewed from the archetypical memory developmental programs defined in systemic models of infection, resulting in numerically deficient memory. Together this work suggests that the respiratory environment can uniquely transform $CD8^+$ T responses towards a more short-lived population of cells, and that protective vaccination strategy will require thoughtful modifications to bypass the restrictions conferred by the respiratory environment and promote memory development.

INDEX WORDS: CD8+ T cells, Memory, Cytokines, Respiratory, Influenza

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Respiratory infections are, and long have been, a major cause of illness and death for many species, including humans. According to the World Health Organization (WHO) respiratory infection is currently the 3rd leading cause of human death worldwide, and is the leading cause of death due to infectious disease (1). Respiratory pathogens are often highly infectious, being transmitted through aerosolized droplets, eliminating the need for direct contact for transmission. Aerosolization increases their ability to spread, thus transmission is often not limited to a single species, and zoonotic reservoirs can result in human infection, as well as provide pools in which virus can replicate and mutate. These defining characteristics of many respiratory pathogens are associated with the ability of a pathogen to cause pandemic infections, making respiratory viruses especially important to vaccinate against to prevent disease outbreaks and ease the economic burden caused by pandemics (2). Additionally, due to the anatomy of the respiratory tract, infection via this route is highly permissive, making this a major portal of pathogen entry into the body (3).

One of the most prevalent respiratory viruses in humans is the influenza virus, with seasonal epidemics resulting in 3-5 million severe infections and 250,000 to 500,000 deaths annually, and these numbers jump dramatically during pandemics (4). Influenza viruses (Influenza A, B, and C) are negative sense single stranded RNA viruses,

belonging to the *Orthomyxoviridae* family. Influenza A viruses, which have historically caused most human infections, are divided further into subtype based on the expression of the viral coat proteins hemagglutinin (HA) and neuraminidase (NA). To date, there are 17 different types of HA and 11 different types of NA defined (5), which together are used to define the circulating strains of virus. For example, H1N1 viruses and H3N2 viruses are strains that have co-circulated since the late 1970s (6) causing seasonal epidemics. As such, H1 and H3 antigens have been the predominant target in seasonal vaccine preparations.

Influenza viruses have a particularly high pandemic potential, due to their ability to replicate in, mutate in, and be transmitted between animal reservoirs (7). Influenza has resulted in at least 3 major pandemics in recorded history, the most notable being the "Spanish Flu" of 1918, which is estimated to have resulted in the global death of 50 million individuals between the years of 1918-1919 (8). It was not until the early 1930s when influenza viruses were first isolated from infected swine (9), and not until 1933 that a human influenza virus was isolated (10). In 1938 Jonas Salk and Thomas Francis developed the first influenza vaccine in the United States. This vaccine consisted of a formalin killed virus which upon injection initiated a protective antibody response (11). Although almost 70 years have passed since the formulation of the first vaccine, the modern influenza vaccine has not changed dramatically. Furthermore, this vaccine does not elicit the high-level of protection that some other vaccines are able to provide, with many vaccinated, exposed individuals still becoming ill each year (12). The majority of modern influenza vaccines are (formalin killed) trivalent/quadrivalent subunit vaccines comprised of surface proteins from prominent circulating influenza viruses. Due to

antigenic drift and shift, whereby the coat proteins of the virus either mutate or completely change, respectively, current vaccines only provided protection for several years, at best, against a specific subtype, and are completely ineffective against new emerging subtypes (13). Due to the lengthy production time of the vaccine, manufacturing must begin at least 6 months prior to the influenza "season". Thus, the make-up of the vaccine is decided yearly, based on predictions made by an expert committee hosted by the WHO of what the circulating strains of the upcoming influenza season will be (12). While the dominant circulating strains (seasonal) can be predicted with reasonable accuracy, the emergence of a new viral strain is often not predicted, resulting in a vaccine with near zero efficacies against this new strain. This was evident in 2009 with the emergence of a novel H1N1, resulting in pandemic infection in as little as 4 months. The rise of this "new" influenza virus led to approximately a 6 month delay between recognition and vaccine availability, after the peak of the outbreak had occurred (12).

While the influenza vaccine has certainly evolved in terms of manufacturing (purification of antigenic proteins and delivery methods), the basis for immunological protection provided by the vaccine is essentially the same as the first vaccine: protective neutralizing antibodies. Meanwhile, our understanding of the immune system has changed dramatically since the 1940s. In 1960 it was discovered that non-serum portions of the blood could destroy cells *in vitro* (14), a discovery that stirred much controversy at the time. This discovery that cellular components of the immune system were not only responsible for the production of antibodies, but could recognize and destroy infected cells was not widely accepted until the early 1970s (15, 16). By 1974, in work that

resulted in the Nobel Prize, Zinkernagel and Doherty described MHC-I restriction of cytotoxic T cells (17, 18), a discovery that had broad implications in furthering our understanding of $CD8^+$ T cell biology. Still, it was not until the late 1980s when the mechanism of how $CD8^+$ T cells recognize antigens presented by MHC I became understood (19), and not until 1996 that the entire MHC/antigen/TCR complex was fully elucidated (20). The early 2000s to the present have been a major period of discovery for T cell biology, particularly in the ability to tract $CD8^+$ T cells following infection and elucidate their role in facilitating immune memory.

Due to the aforementioned shortcomings of the current influenza vaccines, there is hope that a universal influenza vaccine can be developed that elicits protection through memory $CD8^+$ T cell responses. By virtue of the nature of antigen presentation to $CD8^+$ T cells via the MHC class I pathway, $CD8^+$ T cells can recognize internal viral proteins, some of which are highly conserved, even between viral subtypes (21). This attribute confers memory $CD8^+$ T cells with the ability to protect against different subtypes of influenza viruses, a phenomenon termed heterosubtypic immunity. Heterosubtypic immunity against influenza viruses has been well defined in murine models of influenza infection (22-25), and is the basis for a universal $CD8^+$ T cell vaccine. However, in mice heterosubtypic immunity is lost several months following infection (24, 26). While evidence suggests that there may be long-lasting cellular immunity against influenza in humans (27, 28), the mechanisms under which protective memory responses are developed have not been defined. Thus, the understanding of the development and maintenance of protective $CD8^+$ T cell responses to respiratory pathogens is an essential step in the development of a broad long-lasting $CD8^+$ T cell based vaccine.

The majority of work which has defined the developmental pathways for $CD8^+$ T cell memory has been done in models of acute, systemic infection. However, current research suggests that memory development is quite heterogeneous and may be impacted by the route which an infection is acquired, or a vaccine is administered. The focus of this dissertation work is to determine how, under the conditions studied, the respiratory environment impacts the development of and maintenance of $CD8^+$ T cell memory. We hypothesized that following respiratory infection both respiratory specific cytokines and cells would influence developing memory $CD8^+$ T cells, in a distinct way from what is observed following systemic infection. The findings of this work contribute to the overall understanding of respiratory-specific $CD8^+$ T cell memory development, and presents factors that will be important to consider in future $CD8^+$ T cell based vaccine design.

1.2 The Immune Response to Influenza Infection

Innate Mechanisms of Respiratory Anti-viral Control

Like other mucosal barrier sites, the resting lung is engaged in a constant balancing act regarding immunity and tolerance. It is estimated that we breathe in 10,000 liters of air per day, with each breath containing a plethora of allergens, environmental pollutants, and pathogens. Inappropriate response to non-harmful antigens could lead to prolonged inflammation and pulmonary disease, yet a lack of response could results in continuous or persistent infection. To facilitate appropriate responses, multiple layers of innate protection exist in the respiratory tract to both prevent the entry of harmful pathogens into the body, and to preclude any inappropriate initiation of immune responses. The most basic of these layers is the mucosal barrier itself. The lining of the upper respiratory tract is composed of ciliated epithelial cells and mucus-secreting goblet

cells which together function as a “mucociliary escalator” facilitating expulsion of these innocuous agents, pathogenic agents, as well as some commensal organisms, from the respiratory tract without activation of adaptive immune responses. Beyond providing a physical barrier, the mucus contains anti-microbial peptides and oxidizing enzymes that give the mucus antimicrobial activity (29). The lower respiratory tract does not contain mucous, and is characterized by numerous “pockets” where gas exchange occurs, termed alveoli. These small cavities make up more surface area in the lungs than the larger airways combined, by approximately three-fold (3), increasing the susceptibility of these sites to infection dramatically. The cells lining the alveoli are specialized epithelial cells known as type I and type II alveolar epithelial cells, which form the structural architecture of the alveoli and secrete pulmonary surfactants, respectively (30). Pulmonary surfactant is comprised of approximately 90% phospholipids and 10% proteins, and its major function is to lower the surface tension of the alveoli, preventing collapse (3). However, the protein component of surfactant has been shown to play a role in the immune response, by binding surface proteins of microbes increasing aggregation and opsonization (30). These innate barrier mechanisms not only appropriate protection against inappropriate immune responses, but also prevent pathogen infections of the respiratory tract.

If an infectious agent breaches these innate barriers, it must cross only a single layer of epithelial cells to reach the body. Many respiratory pathogens, including influenza viruses, infect the epithelial cells directly, entering the cells via receptors expressed on their luminal surfaces.. Influenza viruses gain entry to host epithelial cells via their interactions with luminally expressed *N*-acetylneuraminic acid, or sialic acid,

linked to galactose by α2,3 and α2,6 linkages with HA molecules expressed on the surface of the viral coat (31). These interactions result in receptor mediated endocytosis. Virus then enters the cell through an endosome, and later viral membrane fuses with the endosomal membrane releasing its contents in to the cytoplasm of the cell (31). It is during this time of viral entry into the cell when the active arm of the innate immune system will be initiated, responding to virus-specific molecular patterns. Infected epithelial cells detect virus using pattern recognition receptors (PRRs), which are able to broadly identify infectious agents based on common antigenic determinants. During influenza infection the major PRRs triggered are endosomal Toll-like receptors (TLRs) 3 and 7 (32, 33), the cytoplasmic Retinoic Acid-Inducible Gene-I-like receptor (RIG-I)(32) , and the nucleotide oligomerization domain-like receptors (NLR) pathways leading to activation of the NLRP3 inflammasome (34). TLR3 and TLR7 recognize viral genetic material, RIG-I recognizes replicating virus in the cytoplasm, while NLRP3 activation is the result of sensing cellular damage and viral RNA (35). Engagement of these PRRs and sensory pathways leads to downstream signaling events that result in the production of type I interferons (IFNs), proinflammatory cytokines, as well as chemokines which will bring other innate cells from the blood into the site of infection. IFN-α and IFN-β are produced following influenza infection and induce an anti-viral state by stimulating a family of hundreds of interferon stimulated genes (ISG-S), inducing the apoptosis of infected cells, limiting viral replication and making surrounding cells resistant to invasion (36).

Chemokines produced during this early response result in the migration of innate immune cells into the lungs and lung airways. The migration of innate immune cells into

the lungs early after influenza infection is dominated by neutrophils and natural killer (NK) cells and occurs through 3-6 days following infection (37). While neutrophils are prominent in number, the role that they play in response to infection is not fully understood, with loss of neutrophil recruitment to the site of infection resulting in no ill effect (38), or having a negative effect in the case of more pathogenic viral infection (39). NK cells, however, are capable of directly recognizing and killing influenza infected cells (40). As viral titers in the lung begin to decrease before the arrival of $CD8^+$ T cells it is likely that NK cells are playing a role in viral control (41). However, akin to neutrophils, the role of NK cell protection against severe disease is somewhat controversial, where they play either a protective role (42, 43), or may contribute to immunopathology when viral tires are high (44). Beyond being able to directly kill influenza-infected cells, NK cells are responsible for the early production (~3 days post infection) of IFN-γ in the lung (45).

In addition to infected epithelial cells initiating responses in the lung, the respiratory tract is home to resident innate immune cells, largely composed of dendritic cells (DCs) and macrophages, which express PRRs and can become activated following influenza infection. Lung resident DCs are comprised of 3 major populations; airway associated $CD103^+$ DCs, parenchyma resident $CD11b^+$ DCs and parenchymal plasmacytoid DCs (pDCs) (46). Lung resident DCs can be directly activated by influenza viruses (47), resulting in the up-regulation of molecules involved with antigen presentation (MHC I and MHC II) and co-stimulation (CD80 and CD86). The major function of $CD103^+$ and $CD11b^+$ DCs is to migrate to the lung draining mediastinal lymph node (MdLN) where they will present antigens acquired at the site of infection to

activate naïve T cells either directly (48, 49), or by "passing" antigen to lymph node (LN) resident (CD8α+) DCs (50). There is some evidence that DCs can become infected with influenza virus (where virus enters the cell yet does not actively replicate), but the main mode of antigen acquisition and presentation to $CD8^+$ T cells is through cross presentation, where DCs pick up antigen from the environment (and dying cells), and processes it so that it enters the MHC I peptide presentation pathway (51). The mechanisms of cross presentation are not completely understood, but it is clear that migratory DCs activated during influenza infection are capable of cross presentation, and are important for the activation of naïve T cells (52). DCs in the lung also play a major large role in cytokine secretion early following influenza infection. pDCs are potent producers of Type I IFNs early after influenza infection (53) and $CD11b^+$ DCs produce pro-inflammatory chemokines, including CCL2, CCL3, CCL4, CCL5, and CCL12 (MCP-1, MIP-1α, MIP-1β, RANTES, and MCP-5) (54) leading to the influx of innate and adaptive immune cells into the lung. Within the alveoli, alveolar macrophages have been shown to be essential for protection against H1N1 influenza in swine (55), yet influenza infection can lead to their depletion, resulting in more severe secondary bacterial infections, a major cause of human disease following influenza infection (56).

Together, these innate immune responses play an important role in early control of viral infection in the lung and ultimately result in the induction of adaptive immune responses. However, their role in the immune response does not stop here, as innate cells and their products remain at the site of infection and influence adaptive immune responses in the lung. This topic will be discussed further in the context of the adaptive immune responses and memory formation.

Adaptive Mechanisms of Respiratory Anti-viral Control

While innate responses act to limit infection and viral replication (35), full viral clearance is dependent on productive adaptive immune responses (57-59). Adaptive immune responses are separated into two arms, humoral immunity and cellular immunity. While there is no doubt that humoral immunity plays an important role in the secondary response to influenza infection, the participation of B cells in primary responses is limited, due to the time that it takes to undergo isotype switching and affinity maturation. However, B cells and the antibodies that they produce have been shown to positively influence late viral clearance (60, 61), due to their ability to produced virus-specific IgM (62). The cellular arm of the adaptive immune response is comprised of $CD4^+$ T cells and $CD8^+$ T cells. The functions of $CD4^+$ T cells in immune responses to influenza are far less delineated than those of $CD8^+$ T cells. Traditionally their role is thought to consist of providing help to B cells in producing robust and high-quality antibody responses (58). Interestingly, $CD4^+$ T cells may also directly eliminate influenza-infected epithelial cells due the ability of the epithelial cells to induce expression of MHC II (63). However the overall contribution of this direct killing to viral clearance is minimal, as loss of $CD4^+$ T cells during primary infection neither results in increased viral titers nor more severe morbidity (64). $CD8^+$ T cells have the most significant impact on primary influenza infection, as loss of these cells results in delayed viral clearance and increased mortality (57), and can result in protection even in the absence of antibody responses (61). Furthermore, the events of primary $CD8^+$ T cell induction and subsequent effector responses will set the stage for formation of memory CD8+ T cells, which will provide heterosubtypic immunity and together are the major focus of this work. For these reasons,

this section will focus on respiratory anti-viral $CD8^+$ T cell responses and their role in protection during and following influenza infection.

Activation of a $CD8^+$T cell requires a naïve $CD8^+$ T cell to establish contact with a DC expressing its cognate antigen in the context of MHC I and receive co-stimulatory signals, namely CD80 and CD86 (which are up-regulated either through direct activation at the site of infection or via licensing by $CD4^+$ T cells). During influenza infection, activated, antigen-laden respiratory DCs migrate to the MdLN to interact with surveying naïve $CD8^+$ T cells. The majority of these migratory DCs fall into two subsets, airway-associated $CD103^+$ DCs and lung parenchyma $CDllb^{hi}$ DCs (65), where $CD103^+$ DCs play the largest role in priming naive T cells (66, 67). These DCs begin to migrate from the lung towards the MdLN as early as 6 hours post infection (68), although peak levels of $CD8^+$ T cell priming by these DCs occurs at approximately 3 days post-infection (dpi) (50). Although the majority of these studies were performed in mouse models, analogous DC subsets have been identified in humans (69, 70), indicating that the role they play in T cell priming following influenza infection may be conserved between species.

Recognition of cognate antigen/MHC, along with the co-stimulatory signals provided by the activated DC, will trigger naïve T cell activation. Full activation will require a 3^{rd} signal provided by cytokine signaling. In the case of $CD8^+$ T cell activation, this 3^{rd} signal is commonly provided by IL-12 or Type I IFNs (IFN-α and/or IFN-β) (71). Following activation $CD8^+$ T cells will begin to gain effector functions and rapidly clonally expand. This expansion is an essential step in the immune response as the precursor frequency of epitope-specific $CD8^+$ T cells is approximately 100-200 cells/mouse (72, 73). Following activation they will go through at least 7, but up to 15

divisions in a week's time (74), resulting in a population of effector cells, which calculations have estimated to be up to 500,000 fold more than naïve precursors (75). Activation of naïve $CD8^+$ T cells in the lung draining lymph node is characterized by many phenotypic markers, including the early upregulation of CD69, CD25, CD44 and CD11a, the downregulation of CD62L, and the acquired ability to produce IFN-γ (76). Following the activation and proliferation period, $CD8^+$ cells will leave the LN via efferent lymphatic vessel, enter the blood stream, and migrate to the site of infection where they will elicit their effector functions. In the case of the lung, this means that $CD8^+$ T cells will exit into the lung parenchyma and airways where they will be able to directly kill infected epithelial cells and control viral infection.

Cells can enter the lung via two circulatory systems: the bronchial system, which provides oxygenated blood to the lung tissue, and the pulmonary circulation, which includes vessels that bring deoxygenated blood to alveoli and subsequently drain oxygenated blood back to the heart (77). The lung epithelium surrounding the alveolar airway spaces share a fused basal lamina with the adjacent capillary endothelium to allow gas exchange and could facilitate direct blood to airway traffic. Because pulmonary vessels are small in diameter and thin walled, blood pressure in these vessels is relatively low, thus allowing lymphocytes to traverse the endothelium independent of the multistep paradigm described for lymphocyte migration through larger vessels, which are dependent on selectins, integrins, and chemokines (78). However, migration of cells into the lung parenchyma and into the larger airways occurs via extravasation from the blood stream in a typical method, involving loose adhesion and rolling on the endothelium via selectins followed by the activation of integrins by chemokines, resulting in firm

adhesion and subsequently egress into the underlying tissue. While access into distinct anatomical sites within other mucosal tissues such as the skin and gut is highly correlated with expression of tissue-specific homing receptors (79-81), analogous molecules have not yet been uniquely identified for lung homing $CD8^+$ T cells. Nonetheless, some chemotactic signals are associated with T_{eff} migration into inflamed lung tissues including CXCR3 (82) and CXCR6 (83). Within the lung tissue effector T cells move about via the expression of integrin α1β1 or α2β1 or VLA-1 and VLA-4 (84), which bind to collagen. Expression of these integrins may be especially important in locating CD8+ T cells in the collagen-rich areas which make up in the intravascular space (85). In the lung, CD8+ T cells will continue to migrate into as well as proliferate within the respiratory tract, resulting in peak numbers of effector cells present at approximately 10 dpi. Effector cell proliferation within the lung is thought to be driven by the transpresentation of IL-15 by pulmonary dendritic cells (86). Importantly, this in situ proliferation contributes to the overall levels of protection afforded by $CD8^+$ T cells (87).

In the lung the role that $CD8^+$ T cells play in the elimination of infection is clear and direct, with the loss of $CD8^+$ T cells during primary infection resulting in delayed viral clearance and increased mortality (57). $CD8^+$ T cells will kill influenza infected cells largely in a perforin dependent manner (88), while more "classical" granzymes, GrzA and GrzB, do not play a major role, as loss of these proteins does not result in more severe disease or a lack of the ability to clear virus (89). There is evidence however, that other granzymes such as GrzK may play a role in the elimination of infected cells in this particular viral infection (89). $CD8^+$ T cells are also able to induce the apoptosis of infected cells through Fas-FasL dependent pathways and tumor necrosis factor (TNF)-

related apoptosis inducing ligand (TRAIL) (88, 90). In addition to the direct killing of cells in the lungs and airways, effector $CD8^+$ T cells are a significant source of IFN-γ , TNF, and CCL3 (MIP1-α) (91). Interestingly, $CD8^+$ T cells in the lung (and not lymphoid tissue) are capable of producing IL-10 (92), which may be important for prevention of excessive inflammation and loss of essential tissue functions. Therefore, during a primary immune response to influenza $CD8^+$ T cells play a pivotal role in the clearance of virus infected cells, and may also play an important role in the return to homeostasis.

Influenza Specific Memory CD8+ T Cells and Recall Responses

The adaptive immune system is defined by its ability to mount antigen-specific responses and generate long-lived memory cells. These memory cells provide protection from secondary infection, by responding more rapidly and effectively when encountering a previously seen antigen. The majority of influenza-specific effector $CD8^+$ T cells that develop during infection are specific for one of two influenza proteins, the nucleoprotein (NP) or the viral polymerase (PA)(72). Using this knowledge, we can track and phenotype influenza-specific $CD8^+$ T cells in mice using MHC I tetramers, loaded with an immunodominant influenza epitope (93). NP-specific $CD8^+$ T cells respond the best following secondary challenge, due to differential presentation of antigen (94) and increased antigen availability (95). Thus, the majority of work has focused on NP-specific $CD8^+$ T cell responses.

Upon resolution of influenza infection the majority of effector cells die via apoptosis; this is defined as the contraction phase of the $CD8^+$ T cell response. However, a subset of these cells will become memory cells, and long-lived NP-specific $CD8^+$ T cells can be found in lymphoid organs such as the spleen and LNs, as well as in the

respiratory tract (96). Within the respiratory tract, memory $CD8^+$ T cells exist within two basic compartments, the airways and the lung parenchyma. Airway $CD8^+$ T cells can exist outside of the body, within the lumen of the respiratory tract (yet under the protective layer of the pulmonary surfactant (3)), or they can exist much like they do in the intestinal epithelium, between cells of the epithelial later, as intraepithelial cells. Cells within the airways, and very likely some intraepithelial cells, can be isolated by performing a bronchoalveolar lavage (BAL), while the remaining lymphocytes in the parenchyma are isolated through a process involving the enzymatic digestion of collagen. Additionally the localization and characterization of these cell populations can be defined by microscopic analysis of lung tissue sections, although phenotyping of these cells by this method is currently limited.

Memory $CD8^+$ T cells that reside within, and adjacent to, the lung airways play a major role in the protection against severe disease following heterosubtypic viral challenge (24, 26, 96). Somewhat surprisingly then, is the fact that these cells appear to have poor cytolytic function (97). This is thought to be due to the loss of expression of CD11a upon entry into the airways (98). Current evidence suggest that these cells instead are important for early cytokine production and the recruitment of non-specific $CD8^+$ T cells to the site of infection (37). This occurs prior to the reactivation, and subsequent migration, of memory cells in the lymphoid organs, providing an early mechanism for defense. In addition to the loss of CD11a, these airway $CD8^+$ memory T cells are phenotypically distinct from other memory cells, in several ways. One of the most striking differences is that unlike influenza specific memory cells in lymphoid tissues, which retain numerically stable pools of memory over time, the memory cells in the lung

airways steadily decline, eventually plateauing at a number which is insufficient for protection (24, 96). The mechanism of this decline of cells is still unknown, but may be due to the loss of expression of cytokine receptors associated with the long term survival and turnover of these cells, a topic which will be discussed in greater detail in later sections.

It is thought that memory $CD8^+$ T cells in the lung airways, at least for some period of time, are partially maintained by the continual recruitment to the airways. In support of this, Slutter et al. showed that CXCR3 is required for the continual recruitment of cells into the airways, and that loss of CXCR3 expression results in the accelerated loss of antigen-specific $CD8^+$ T cells specifically from the airways (99). Tracking the entry of memory CD8+ T cells from the circulation is also possible by monitoring CD11a expression which is lost ~40 hours after $CD8^+$ T cell emigration into the airways (100). Indeed, when memory $CD8^+$ T cells are extracted from the airways (up until at least 13 months post infection), portions of the antigen-specific $CD8^+$ T cells express high levels of CD11a, indicating that they recently arrived at the site. Finally, while evidence suggests that a circulating population of cells is actively recruited into the lung airways during steady state conditions (99, 100), it is clear that these recruits are not sufficient (either in number or function) to provide protection against heterosubtypic influenza challenge, as protection wanes while recruitment continues. Perhaps the limited migration and supplementation of competent memory cells from within the lung parenchyma may augment this pool and maintain heterosubtypic immunity, at least temporarily. In 2004, Ray et al. showed that influenza specific $CD8^+$ T cells persisted in the highly collagenized area between the airways and the blood vessels, and that this retention was

dependent on the expression of VLA-1 (85). VLA-1 binds to type IV and type I collagen (101, 102) which are important structural components of the lung interstitium, specifically between the bronchi and the vasculature, and the basement membranes of both the pulmonary vasculature and the epithelium of the airway, respectively (103, 104). The collagen-rich environment of the lung may provide a framework or scaffold in which $CD8^+$ memory cells can persist close to the site of antigen acquisition, yet not actually within the epithelial layer of the lung where they may be subject to the harsh environment of the airways.

1.3 $CD8^+$ T Cell Mediated Protection from Influenza Infection

A role for $CD8^+$ T cell mediated protection from influenza virus has been defined for quite some time. As early as 1977, it was recognized that $CD8^+$ T cells could directly kill cells infected with influenza virus (105), and in 1978 it was established that adoptive transfer of $CD8^+$ T cells could result in a shorter duration of infection (22). However, a direct function for memory $CD8^+$ T cells in protection during secondary infection was harder to experimentally define. While $CD8^+$ T cells are not expected to provide sterilizing immunity, as they can only recognize and destroy infected cells, it is anticipated that that $CD8^+$ T cells can play a protective role against severe disease against highly pathogenic pandemic strains of influenza (106). Also, despite the apparent instability of airway memory $CD8^+$ T cells, evidence exists which supports the role that $CD8^+$ T cells can play in protecting against severe disease, thus targeting this population may provide an alternative means of vaccination.

Identifying a certain cell type as playing the critical role in protection from infection is difficult in human populations, for multiple reasons. These reasons include,

but are not limited to, genetic differences, differences in infection histories of the patients, limitations on tissue samples (often limited to blood, or biopsy samples), and, depending on the disease being studied, inability to know when infections were acquired. Despite these inherent difficulties in human studies, the evidence gained from such studies creates a framework for developing a more comprehensive and precise understanding of the function of particular cells in animal models.

There are several lines of evidence derived from human studies that point to a protective role for $CD8^+$ T cells in influenza infection, even in the absence of neutralizing antibodies. The earliest evidence in humans suggesting that $CD8^+$ T cells may play a role in the immune response to influenza came in 1983, following an experiment where a group of volunteers were infected with attenuated influenza virus. Decreased viral shedding in these individuals was correlated with increases in $CD8^+$ T cells, in the absence of neutralizing antibodies (107). During the 2009 H1N1 pandemic researchers were able to monitor cohorts of individuals, and found those who had pre-existing influenza specific CD8+ T cells developed no or milder symptoms after H1N1 infection, once again in the absence of strain-specific antibodies (28). Furthermore, in humans $CD8^+$ T cells populate the airways where they hold protective qualities. For instance, post mortem microscopic analysis of the human lung determined one-third of the total $CD8^+$ T cell population (108), or over 10 billion total cells (109), exhibited characteristics that indicated they were derived from previous respiratory infection, and therefore may be an established memory cell population providing a front line of defense. However, due to the nature of this study, it is not known when these cells were established in the respiratory tract and how long-lived they are.

Together, these studies, along with data obtained from mouse models of influenza infection, indicate that $CD8^+$ T cells can play a protective role against the development of severe disease following influenza infection. However, the loss of protection over time is concerning and not ideal for vaccine development. Therefore, the study of memory $CD8^+$ T cell development and their maintenance in the respiratory tract is of upmost importance for developing efficient and protective vaccines. The next sections will discuss our current knowledge of memory $CD8^+$ T cell development, largely derived from systemic models of infection, and how respiratory specific factors may change the norm of memory cell development.

1.4 Development and Maintenance of Memory $CD8^+$ T cells

As effective $CD8^+$ T cell vaccination approaches depend on the production of a protective pool of memory cells there is considerable interest in understanding what programs certain cells to survive through contraction and transition into long-lived memory cells. Accordingly, there has been a great deal of research aimed at understanding what signals positively lead to the formation of a stable memory cell pool. Despite years of study, a definitive model for memory development has not been defined. However, it is clear that a single naïve T cell has the capability to develop into both an effector T cell and a memory T cell, and go on to develop diverse fates (110-112). These findings were particularly important, as they indicated that perhaps environmental factors influence the development of memory. Also, memory populations are far from homologous (113, 114), differing from one another in terms of anatomical location as well as effector functions. Furthermore, through molecular and cellular studies, it is clear that cells which are destined to become memory cells are identifiable early in the effector

phase of the immune response (115, 116), at least in acute, viral, systemic infection models.

Defining Memory Subsets

The $CD8^+$ memory cell compartment consists of distinct populations of cells with different migratory preferences and phenotypes that can further vary depending upon anatomical location in the body (Table 1.1). These memory populations also display differing roles in recall responses upon secondary infection (37, 117). Initially, memory T cells were broadly categorized into two populations based on homing preferences, circulating between secondary lymphoid organs as central memory T cells (T_{CM}) or less discreetly throughout the periphery, including non-lymphoid tissues, defined as effector memory T cells (T_{EM}) (118). These memory pools are distinguished from one another by their differential expression of the lymph node homing molecules L-selectin (CD62L) and CCR7, with T_{CM} expressing high levels of these molecules for lymph node entry and retention (119) and T_{EM} cells expressing low levels. While this simplified T_{CM}/T_{EM} paradigm predominated memory T classification for several years, subsequent studies using parabiotic mice (120) and adoptive transfer systems (121) demonstrated that at least one additional pool of memory exists with tissue-specific residency and little migratory potential. Additional studies confirmed the existence of these tissue-locked memory cells at portals of pathogen entry and led to the T resident memory cells (T_{RM}) nomenclature. This diversity in $CD8^+$ T cell memory is thought to be acquired as a result of different levels of co-stimulation, inflammation, or T cell help, which not only vary throughout the course of a single infection but are also impacted by infection route.

Early Signals Influencing $CD8^+$ T Cell Memory Development

As previously mentioned, full activation of $CD8^+$ T cell requires three signals: detection of cognate peptide/MHCI complex, co-stimulation, and a cytokine signal (122). The combination of these three signals, which may vary in intensity and type, results not only in clonal expansion and acquisition of effector function, but also influences long-term cellular fate (123). In many cases, the overall memory potential of the antigen-specific $CD8^+$ T cell is driven by lineage-associated transcription factors and acquired epigenetic changes (124) which can be experimentally monitored. These programming signals are influenced by the type of (priming) APC, antigen availability, and inflammatory properties of the pathogen which can vary based on the individual pathogen and the route which infection is acquired.

Another important factor which can be highly variable during infection is the presence of particular cytokines, which influence both memory cell potential (125, 126), and the specific pool of memory cells that develops (127, 128). The potential for an effector T cell to become a memory T cell has been defined based on the expression of CD127 and KLRG1 (116, 125). Effector $CD8^+$ T cells largely fall into one of three categories: terminally differentiated short-lived effector cells (SLECs, $KLRG1^{hi}/CD127^{lo}$), early effector cells (EECs, $KLRG1^{lo}/CD127^{lo}$) or memory precursor effector cells (MPECs, $KLRG1^{lo}$, $CD127^{hi}$). It is the latter population which develops into long-lived, bona fide memory cells of various phenotypes, including T_{CM}, T_{EM}, and T_{RM}. MPECs can form early in the lymph node, or can arise from EECs in peripheral tissues, as EECs have the potential to differentiate into both SLECs and MPECs (129). The inflammatory cytokine IL-12 promotes the development of terminally differentiated

SLECs in a dose dependent manner via induction of the transcription factor T-bet (125). Interestingly, graded induction of IL-12 is observed after systemic infection with two different pathogens: *L. monocytogene*s (LM) induces a high concentration of IL-12, whereas vesicular stomatitis virus (VSV) induces much lower IL-12 levels. High concentrations of IL-12 during LM infection promote a skewed development favoring SLECs while VSV infection (lower IL-12) favors EEC development (129). As bona fide memory cells arise from KLRG1^{lo} precursors, high levels of IL-12 would likely negatively impact memory development, and indeed this has been shown to be the case (125). The transcription factor Eomesodermin (Eomes) has been shown to positively influence memory development (130), yet is repressed by high levels of IL-12 (131). Type 1 IFNs, which also provide signals for CD8+ T cell activation, can influence memory development. Like IL-12, high levels of IFN-α favors SLEC development (132). Therefore, at the time of activation CD8+ T cells may need to see the "just right" amount of their signal 3 stimulation to gain the potential to differentiate into long lived memory cells. It is quite possible that graded expression of these signal 3 cytokines may preferentially influence the development of certain subsets of memory, although this has not been experimentally determined.

Role of common gamma chain cytokines in memory development and maintenance

The common gamma chain (γc) cytokines, a family of cytokines that share γc as a component of their receptor, classically play a major role in memory CD8^{+} T cell development and survival. Members of this cytokine family, IL-2, IL-4, IL-7, IL-15 and IL-21 all modulate T cell responses to infection and therefore impact memory cell development, yet the role of IL-7, and IL-15 in this process is remarkable (127, 133). It is

important to note, however, that both the presence of the cytokine and cytokine receptor are regulated through the response to infection, allowing signaling to be temporally regulated. Naïve $CD8^+$ T cells express both CD127 and CD122, the specific receptors for IL-7 and IL-15, respectively. CD127 is rapidly down-regulated following T cell activation. However, as previously noted, CD127 expression on a population of cells during the effector phase of the response can identify cells destined to become memory cells (116). Interestingly, CD127 expression is not sufficient to generate memory (134), nor is IL-7 a requirement (135) suggesting that IL-7Ra expression simply correlates with cells with memory differentiation or functions via an unknown mechanism. IL-15 has been shown to be important for the persistence of memory $CD8^+$ T cells in models of systemic infection, with mice deficient in IL-15 or IL-15 signaling having normal peak effector $CD8^+$ T cell responses, yet losing these cells during contraction resulting in numerically deficient memory populations (136, 137). In terms of cytokines important for parsing T_{mem} into defined subsets, the common gamma chain cytokines IL-2 and IL-15 have been shown to play a role in $CD8^+$ T cell differentiation into T_{CM} and T_{EM} cells. T_{CM} cells can be identified as a distinct population arising from MPECs as early as 5 dpi, and are formed through IL-15 signaling (when IL-2 is limited), whereas IL-2 signaling leads to T_{EM} phenotypes (123).

The ability to cells to respond to the γc cytokines depends not only on expression of the specific cytokine receptor (which will vary between naïve, effector, and memory cells) but also cytokine availability and relative concentration within a given tissue. Any potential anatomical separation of cells with a particular cytokine provides a preferential ability to affect distinct populations of memory cells. Although not a γc cytokine, the

closely related cytokine thymic stromal lymphopoietin (TSLP) may also play important roles in memory $CD8^+$ T cell development and maintenance. The heterodimeric receptor for TSLP consists of CD127 and a unique receptor (TSLP-R), which shares sequence homology with the γc (138, 139). This cytokine is produced by epithelial cells, and is found largely at mucosal sites including the respiratory tract (140), perhaps participating in the formation or maintenance of respiratory specific memory cells. The role that TSLP plays in $CD8^+$ T cell memory development will be discussed in Chapter 2.

1.5 Respiratory Factors Influencing Memory $CD8^+$ T cells

As mentioned in previous sections, there are populations of memory cells that arise following respiratory infection that have altered phenotypes (Table 1) and decreased longevity (96) as compared to those that develop following systemic infection. Furthermore, respiratory infection results in lower overall pools of memory cells, as well as the development of cells which are maintained independently of the classic memory cytokine IL-15 (141). This evidence indicates that the formation of memory following respiratory infection may be developmentally distinct from the paradigms which were established based on systemic models of infection. This section will describe mucosal and respiratory specific factors which likely influence the development and maintenance of $CD8^+$ T cells derived from infections at this site.

Priming of respiratory infection derived $CD8^+$ T cells

Following infection in the respiratory tract several DC cell populations participate in $CD8^+$ T cell priming: respiratory resident $CD103^+$ DCs and $CD11b^+$ DCs, as well as LN resident $CD8\alpha^+$ DCs. These DC populations are not activated, nor induced to migrate

into the lymph nodes following acute systemic infections (142), and therefore may be able to influence $CD8^+$ T cells responding to respiratory infections in a distinct fashion.

The different migratory subsets found in the lung draining LNs following influenza infection differentially activate effector CD8+ T cells. $CD103^+$ DCs are important for the priming of naïve $CD8^+$ T cell responses in influenza infection, and the loss of this subset of DCs results in poor $CD8^+$ T cell responses and defects in viral clearance (52, 143). However, the priming initiated by these cells may negatively affect memory development. $CD103^+$ DCs have been shown to be requisite for complete effector differentiation, defined by expression of standard effector markers and their ability to enter inflamed tissues ($CD25^{hi}$, T-bet^{hi} and Blimp-1^{hi} and $CD62L^{lo}$ $CCR5^{hi}$). In contrast, $CD11b^{hi}$ DCs are more likely to prime $CD8^+$ T cells which largely remain in the lymph nodes, expressing molecules associated with the development of T_{CM} ($CD62L^{hi}$, T-bet^{lo}, Blimp-1^{lo} $CD25^{lo}$, and $CD127^{hi}$ (144). Therefore, in respiratory infection, the necessary protection that is provided by effector cells that migrate to the site of infection (activated by $CD103^+$ DCs) may come at a cost to overall memory formation. However, this has not been directly tested nor has it been assessed whether these $CD103^+$ DCs preferentially give rise to specific subsets of memory $CD8^+$ T cells

CD8+ T cell memory development in the inflamed lung

The pioneer T_{eff} cells immigrating to the lung arrive ~5-6 days after initial respiratory infection. Prior to their arrival, innate immune cells have accumulated, keeping viral titers low, and as a result, some local tissue damage has occurred via cytolysis of infected epithelial cells, affecting barrier function. The inflammatory effects of this local immune response in the lung are still very present at the time of T cell entry,

and can influence CD8+ T cells in terms of proliferation, effector function, and perhaps memory formation. However, since anti-influenza effector cells migrate to the lung asynchronously over several days (peaking at ~10 days post viral infection), all T cells do not encounter equivalent levels of inflammation which will likely affect the fate of individual effector cell clones.

The first $CD8^+$ effector cells to arrive at the site of infection will encounter the greatest level of inflammation, as infectious virus is still present (at least until ~8 days post influenza infection) and innate effectors such as NK cells are producing local IFN-γ (45). Inflammatory monocyte-derived DCs arrive in the inflamed lung at the same time as initial T_{eff} and function as lung APCs, amplifying the inflammatory milieu and locally expanding the emigrating effector cells (145). Additionally, $CD8^+$ T cell proliferation continues in the lung, a process requisite for viral control after influenza infection (87). This additional expansion, however, is not without a cost. Increased levels of cellular division is not only associated with increased levels of apoptosis within the highly dividing populations (146), the aforementioned cytokines also promote terminal differentiation of the T cells and the formation of $KLRG1^+$ SLECs (126, 147). Therefore, this early inflammatory environment skews cells away from becoming memory cells, yet may paradoxically pave the way for resolution from infection and inflammation so that later immigrants may develop into memory cells.

As influenza virus replicates primarily in epithelial tissue, the localization of $CD8^+$ T cells adjacent to antigen may expose these cells to unique cytokines available in and near the epithelium such as TGF-β. TGF-β production can be transiently activated by influenza virus infection (148, 149) and plays a role in both the contraction of effector T

cells (150) and the establishment of T_{RM} (151, 152). Interestingly, following influenza infection a large majority of antigen-specific $CD8^+$ T cells begin to express the α1β1 integrin VLA-1 (85). T_{effs} localized cells to the collagen rich areas near the airways and basement membranes that are VLA^+ have a survival advantage over those that do not express VLA at the peak of the $CD8^+$ T cell response (85). The localization and retention of cells within the lung parenchyma, as well as the survival advantage may make VLA-1 expression a unique marker for cells destined to become lung $CD8^+$ memory cells.

As previously mentioned, $CD8^+$ T effector cells themselves produce cytokines in the lung, including IL-2, IFN-γ and TNF-α which enhance the overall inflammatory response (37). Interestingly, while $CD8^+$ T cells activated in lymph nodes rapidly gain the ability to produce the inflammatory cytokine IFN-γ, entry into the lung tissue imparts IL-10 production (92, 153). IL-10 production by $CD8^+$ T cells is dependent on the inflammatory lung environment (154), indicating that an enhanced activation status resulting from high levels of inflammation induces the $CD8^+$ T cells to produce regulatory cytokines. IL-10 is also produced at high levels by regulatory T cells (T_{regs}) activated in the lung following influenza infection (155). The production of regulatory cytokines by T_{regs} and $CD8^+$ T cells is important to initiate "dampening" the immune responses in the lung to prevent excessive damage and loss of function of this essential organ. Importantly, the production of IL-10 can directly impact the development of memory cells by inducing MPEC populations in a STAT3 dependent manner (156), however, it is unclear whether IL-10 has any direct consequences on the development of memory populations

Gamma Chain Cytokines and Respiratory CD8$^+$ T cell Memory

Aside from differences in the type of APC which initiates priming following respiratory infection, another major difference encountered by respiratory-derived CD8$^+$ T cells is the presence of distinct cytokines present in the inflammatory and resting respiratory tract, as well as their ability to respond to cytokines based on the expression (or lack thereof) of specific cytokine receptors. As noted in the previous section γc cytokines are important for both the development and maintenance of CD8$^+$ T cell memory, thus, it is clear that these differences may have major implications in the development and maintenance of CD8$^+$ T cell memory in the respiratory tract.

One of the paradigms established in systemic infection is that the γc cytokine IL-15 is required for the maintenance of memory CD8$^+$ T cell populations. However, our laboratory established respiratory CD8$^+$ memory T cells develop and are maintained normally in the absence of IL-15 (141). Intriguingly, the receptor for IL-15, CD122, is down-regulated on populations of memory cells which exist in peripheral sites (Table 1.1), suggesting that this IL-15 independence may be a broad attribute of non-systemically derived CD8+ T cells, or a subset of these cells. In the lung airways, CD127 is also expressed at lower levels, than other sites, perhaps being enzymatically cleaved from the cells within the lung (157). Therefore, two of the established cytokines needed for the survival and maintenance of CD8$^+$ T cells following systemic infection, have an inferior ability to confer signals to respiratory-CD8$^+$ memory T cells due to decreased receptor expression. As previously mentioned, CD8$^+$ T cells in the airways are lost over time, perhaps due to the inability to respond to these signals. Yet, small populations of these cells are maintained, and the loss of these cells is not immediate (declining steadily

over months). Therefore it is possible that these cells are maintained, at least to some extent, by a different cytokine. We hypothesized that the γc-related cytokine TSLP could provide this signal to $CD8^+$ T cells in the respiratory tract, positively influencing their maintenance in the lung where other cytokines (IL-7 and IL-15) were not likely functioning. The results from this work will be discussed further in Chapter 2.

1.6 Summary and Structure of Dissertation

Understanding the development and maintenance of $CD8^+$ T cell memory is fundamental for developing protective $CD8^+$ T cell based vaccines. Yet, there remains an inadequate understanding how mucosal memory $CD8^+$ T cells develop. While standards for developing "ideal" memory have been defined in systemic models of infection, there is reason to believe that these standards cannot be broadly applied to memory development in all tissue locations. Therefore, in the context of developing protective $CD8^+$ T cell based vaccines against respiratory pathogens, it is important to consider the respiratory environment and how it may shape respiratory derived $CD8^+$ T cell responses. In Chapter 2 of this dissertation we hypothesize that the cytokine TSLP may influence the survival and maintenance of influenza specific $CD8^+$ cells in the lungs and lung airways. We will describe a role for TSLP in promoting direct effector cell proliferation, which in turn results in memory programming at the site of infection. In Chapter 3 we hypothesize that priming of $CD8^+$ T cells by the respiratory route results in a memory developmental pathway that is distinct from systemically derived $CD8^+$ T cells. We test this hypothesis using a VSV model of infection delivered by the intranasal (IN) or intravenous (IV route). We demonstrate that respiratory infection results in a numerically inferior memory population, despite enhanced effector $CD8^+$ T cells response. By comparing CD8+ T cell

differentiation of respiratory anti-viral $CD8^+$ T cell memory formation in direct contrast to a systemic model, we are able to describe unique developmental features of $CD8^+$ T cell memory arising from priming in the respiratory environment. The final chapter of this dissertation will discuss the implications of our findings, and discuss future directions for this work.

Table 1.1: Factors associated with the positioning and survival of defined pools of memory CD8+ T cells in specific anatomical sites[1]

	CD127	CD122	PD-1	CD103	CXCR3	IFITM3	CD69	CD27	VLA-1
T_{EM}	+++	+++	−	−		−/+	−	+	
T_{CM}	+++	+++	−	−		−/+	−	++	
T_{RM} Lung	−/+	+	++	−/+	+++	+++	+++	++/+++	+++
T_{RM} Gut	+/++	+		+++			+++	+	++*
T_{RM} Skin	+	+		+++	+++		+++		+*
T_{RM} Brain	++	+	++	+++		+++	+++		−

−absent, +low levels, ++moderate levels, +++high levels, blank = no data for this tissue.

**Indicates data is from human studies, all other data in table obtained from mouse models.*

[1]Table as originally published in Shane HL and Klonowski KD (2014) Every breath you take: the impact of environment on resident memory CD8 T cells in the lung. *Front. Immunol.* **5**:320. doi: 10.3389/fimmu.2014.00320

CHAPTER 2

A DIRECT AND NON-REDUNDANT ROLE FOR THYMIC STROMAL LYMPHOPOIETIN ON ANTI-VIRAL CD8 T CELL RESPONSES IN THE RESPIRATORY MUCOSA[1]

[1] Shane, H.S. and Klonowski, K.D. 2014. *The Journal of Immunology*. 192(5) 2261-70.
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2.1 Abstract

Mucosally produced thymic stromal lymphopoietin (TSLP) regulates Th2 responses by signaling to DCs and CD4 T cells. Activated CD8 T cells express the TSLP receptor (TSLP-R), yet a direct role for TSLP in CD8 T cell immunity in the mucosa has not been described. Since TSLP shares signaling components with IL-7, a cytokine important for the development and survival of memory CD8 T cells in systemic infection models, we hypothesized that TSLP spatially and non-redundantly supports the development of these cells in the respiratory tract. Here, we demonstrate that influenza infection induces the early expression of TSLP by lung epithelial cells with multiple consequences. The global loss of TSLP responsiveness in TSLP-$R^{-/-}$ mice enhanced morbidity and delayed viral clearance. Using a competitive adoptive transfer system, we demonstrate that selective loss of TSLP-R signaling on anti-viral CD8 T cells decreases their accumulation specifically in the respiratory tract as early as day 8 post infection, primarily due to a proliferation deficiency. Importantly, the subsequent persistence of memory cells derived from this pool was also qualitatively and quantitatively affected. In this regard, the local support of anti-viral CD8 T cells by TSLP is well suited to the mucosa, where responses must be tempered to prevent excessive inflammation. Together these data suggest that TSLP uniquely participates in local immunity in the respiratory tract and modulation of TSLP levels may promote long-term CD8 T cell immunity in the mucosa when other pro-survival signals are limiting.

2.2 Introduction

Mucosal surfaces including the lung airways and the gastrointestinal tract are major portals of antigen entry due to their large surface areas, intimate interactions with the environment, and barriers often composed of only a single layer of epithelial cells. The constant bombardment of these entry points with a variety of external stimuli, coupled with vital tissue functions that are compromised by excessive immune responses, warrants a uniquely regulated immunological microenvironment. Consequently, the mucosal immune system has adapted to respond rapidly to detrimental pathogens while maintaining tolerance against repeated non-pathogenic antigen stimulation in order to prevent the development of inflammatory diseases. These properties have led us and other investigators to study mucosal immune responses as unique immunological entities that when compared to systemic infection models may have different requirements for generating protective immunity and memory.

CD8 T cells are requisite for the clearance of many respiratory viral pathogens, including influenza viruses (57, 158). To date, however, the majority of our knowledge regarding the biology of anti-viral CD8 T cell responses has been limited to models of acute, systemic infections where the tightly regulated balancing act between protection and maintenance of tissue function is not as essential. In these models, the common gamma chain (γc) cytokines play a predominant role in the anti-viral CD8 T cell response, both in the effector and memory phases (127, 159). Specifically, IL-2, IL-21, IL-7, and IL-15 are known to have an influence on anti-viral CD8 T cell responses, with IL-2 and IL-21 influencing early responses to infection (160-163) and IL-7 and IL-15

traditionally implicated in the formation and survival of memory CD8 T cells (127, 137, 164). However, emerging evidence suggests that many environmental factors, including the γc cytokines, relevant for optimal CD8 T cell responses in systemic anti-viral immunity are either differentially regulated or disposable in mucosal systems (141, 157, 165). Indeed, data from our own laboratory has shown that memory CD8 T cells originating from a respiratory influenza infection develop and are maintained independently of IL-15, unlike those anti-viral CD8 T cells derived from a systemic viral infection (137, 141). As mucosally delivered vaccines become more popular, both in concept and clinical practice, it is becoming increasingly important to understand the impact that mucosally derived factors have on the development of effective CD8 T cell responses and subsequent memory formation. One factor that is largely isolated to mucosal tissues and has the potential to influence local CD8 T cell responses is the cytokine thymic stromal lymphopoietin (TSLP).

TSLP is a γc-like cytokine which signals through a high affinity heterodimeric receptor composed of IL-7Rα (CD127) and the specific TSLP receptor (TSLP-R) (138, 139). The TSLP-R is expressed on variety of hematopoietic cell types of the innate and adaptive immune system including mast cells, dendritic cells (DCs), B cells and T cells (166-168), as well as non-hematopoietic cells such as intestinal epithelial cells (169). Relevant to our studies, TSLP is produced constitutively by cells that constitute mucosal tissues, both in the airways and the intestinal tract (170-172) and is often elevated at these sites under inflammatory conditions such as chronic allergy and asthma (171, 173). While epithelial cells appear to be the predominant source of TSLP in the resting mucosa, other cell types including keratinocytes, mast cells, smooth muscle cells, and DCs have

been shown to express TSLP when exposed to a wide variety of stimuli, including TLR and NOD2 ligands, environmental stimulants, proinflammatory and Th2 cytokines, and viruses (140). Because TSLP production is enriched at mucosal surfaces, particularly following inflammatory or viral stimuli, TSLP signaling may uniquely modulate immune responses in these sites.

The majority of research on TSLP has focused on the cytokine's effect on CD4 T cells, the development of Th2 immune responses, and asthma, leaving TSLP's influence on the CD8 T cell response to infection less well explored. The TSLP-R is expressed on naïve murine CD8 T cells at low levels (168) and is undetectable on naïve human CD8 T cells (174), limiting the ability of TSLP to act directly on these cells. However, the TSLP-R is transiently upregulated following TCR stimulation in both mice and humans (168, 174), enhancing the potential for TSLP to act directly on activated CD8 T cells. Indeed, provision of TSLP to CD8 T cells activated by αCD3/αCD28 *in vitro* induces STAT5 phosphorylation, the upregulation of Bcl-2, and increased survival, although to a much lesser extent than providing IL-7 (168). Importantly, cells destined to become memory CD8 T cells preferentially express CD127 (116). As the receptors for TSLP and IL-7 both share CD127 and some downstream signaling components (175), it is possible that the two cytokines may have some overlapping and/or non-redundant functions related to memory cell survival. In summary, these data suggest that TSLP has the capability to act directly on CD8 T cells; however to date, investigators have yet to define a direct role for TSLP on antigen-specific CD8 T cell responses independent of the effects of the cytokine on secondary players (i.e. DC or CD4 T cells) participating in the immune response.

In this study we sought to determine whether mucosally derived TSLP acts directly on CD8 T cells after influenza infection and influences their response to infection and/or subsequent development into specific memory cell pools in a way distinct from other cytokines. We show that TSLP is produced locally following influenza virus infection and positively regulates the anti-viral response. Importantly, TSLP acts directly on antigen-specific CD8 T cells in the respiratory tract in a manner that increases their proliferation and persistence into later stages of the immune response. To our knowledge, our study implicates a newly defined role for TSLP acting on antigen-specific CD8 T cells responding to an infection and adds to the emerging story designating unique roles for cytokines in the context of mucosal immune responses.

2.3 Materials and Methods

Mice and viruses

C57BL/6 mice were purchased from Charles River (Wilmington, MA) through the National Cancer Institute program and TSLP-$R^{-/-}$ mice (176) were generously provided by Dr. Steve Ziegler (Benaroya Research Institute, Seattle, WA). C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice were generously provided by Dr. Leo Lefrancois (University of Connecticut, Farmington, CT) and maintained on a CD45.1 $Rag^{-/-}$ background. These mice were bred in house with CD45.2 TSLP-$R^{-/-}$ mice to produce CD45.2 and CD45.1/CD45.2 TSLP-$R^{-/-}$ OT-I mice on a $Rag^{-/-}$ background. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Georgia. The influenza virus A/HK-x31(x31, H3N2) was generously provided by Dr. S. Mark Tompkins (University of Georgia, Athens, GA) while the recombinant x31-OVA expressing the CD8 H2-K^{b} restricted SIINFEKL epitope was

generously provided by Dr. Peter Dougherty (St. Jude Children's Research Hospital, Memphis, TN).

Influenza Infections

For *in vitro* experiments, mouse lung epithelial (MLE)-15 cells (177) were grown in 12 well plates in H.I.T.E.S Medium (178) supplemented with 4% FBS (growth media) and either mock or x31 infected with a multiplicity of infection of 50% (.5 MOI) for 1 hour at 37°C in growth media. Following infection, cells were washed with PBS then cultured in growth media until cells were harvested. For *in vivo* experiments, age and sex matched anesthetized animals were infected intranasally (i.n.) with 10^3 pfu x31 or x31-OVA in 50µl PBS. Mock infected animals received 50µl PBS i.n..

Quantitative RT-PCR

Cells or whole tissues were collected in RNA*later* (Qiagen, Valencia, CA) or PrepProtect (Miltenyi Biotec, Auburn, CA) and stored at -80°C until processing. RNA was purified from the samples using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Reverse transcriptions were performed using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA). Quantitative PCR assays were prepared using the ABI TaqMan Gene Expression Master Mix from ABI 7500 Real Time PCR System and TSLP–FAM (Mm01157588_m1) and 18s-VIC (#4319413E) assays in a multiplex reaction assessed on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Thermal cycling conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of denaturation (95°C for 15 s) and annealing (60°C for 60 s). Samples were analyzed in triplicate, normalized against 18s, and expressed relative to mock-infected animals. The results are expressed as relative quantity over a mock

infected control samples determined by the ΔΔ cycle threshold method with analysis performed on the 7500 System SDS Software v1.3.1.

Plaque Assays

Plaque assays were performed as previously described (179). Briefly, whole lungs isolated from infected mice were isolated, weighed, and homogenized using a Tissue Lyser (Qiagen, Hilden, Germany). Serial dilutions of 10% homogenate were made in dilution media (1×MEM, 1 µg/mL TPCK-treated trypsin) and incubated for 1 hour atop confluent monolayers of Madin-Darby canine kidney cells (MDCKs) grown in 12 well plates for 1 hour at 37°C. Following infection, cell layers were washed with PBS and overlaid with MEM containing 1.2% Avicel microcrystalline cellulose (FMC BioPolymer, Philadelphia, PA), 0.04 M HEPES, 0.02 mM L-glutamine, 0.15% $NaHCO_3$ (w/v), and 1 µg/mL TPCK-treated trypsin. After 72 hours at 37°C, the overlay was removed, and the cells were washed with PBS, fixed by incubation with cold methanol/acetone (60:40%), and stained with crystal violet. Plaques were counted and plaque-forming units per mg of lung tissue determined.

Tissue Preparation

Single cell suspensions from tissues were obtained as previously described (141). Briefly, cells from the lung airways were obtained by means of bracheoaveolar lavage (BAL) in which the trachea was intubated and 1 ml of PBS was introduced and recovered from the lung airway four times. Following BAL collection, cells were isolated from the lung parenchyma after first perfusing the lungs with ~10 mL of PBS/heparin. The perfused lungs were excised, minced and incubated with 1.25 mM EDTA at 37°C for 30 minutes followed by a one hour incubation of with 150 units/mL collagenase (Gibco, life

technologies, Grand Island, NY). After passage through cell strainers, lymphocytes were resuspended in 44% Percoll, underlaid with 67% Percoll, centrifuged and the cellular interface collected. Lymph nodes and the splenic tissues were mechanically disrupted then passed through a cell strainer. Erythrocytes were depleted from the spleens using Tris-buffered ammonium chloride. Cell numbers were determined using a Z2 Coulter Particle Counter (Beckman Coulter, Fullerton, CA).

Flow Cytometry

The influenza nuclear protein (NP) MHC class I [H-2D^b/ASNENMETM] tetramer was generated at the National Institute of Allergy and Infectious Diseases Tetramer Facility (Emory University, Atlanta, GA). Staining was carried out at room temperature for 1 hour in conjunction with other surface staining mAbs: PerCP/Cy5.5-conjugated αCD8α or αCD44, FITC-conjugated αCD11a or αCD122, PE-conjugated αCD127 or αCD43, APC/Cy7-conjugated αCD62L or αCD8α, and PE/Cy7-conjugated αKLRG1 or αCD27 (all from eBioscience, San Diego, CA). When tetramer was not used, cells were surface stained for 20 min at 4°C. Data was acquired using an LSRII with FacsDiva software (BD Biosciences, San Jose, CA) and analysis of data was performed using FlowJo software (Tree Star INC, Ashland, OR). All samples were gated on single cells prior to subsequent gating and analysis.

CD4 T cell IFN-γ production assay

Following isolation, lymphocytes were incubated at 37°C for 5 hours with or without the x31 derived Haemagglutinin (HA) (195-209) (YVQASGRVTVSTRRS) peptide (ANASPEC INC, Fremont, CA) in the presence of GolgiStop (BD Pharmingen, San Diego, CA). 1x10^6 naïve splenocytes were added to the lymphocyte populations

isolated from the BAL as an antigen presenting population. Following the stimulation period the cells were extracellularly stained with αCD8, αCD4 and αCD44 antibodies (eBioscience, San Diego, CA) for 20 min at 4°C, fixed in 2% paraformaldehyde overnight, permeabilized using Perm/Wash Buffer (BD Biosciences, San Diego, CA) and intracellularly stained using FITC conjugated αIFN-γ (BD Pharmingen, San Diego, CA) for 30 min at 4°C. Samples were analyzed by flow cytometry as described above.

Proliferation/ Death Assays

At 6 days post infection mice were injected intraperitoneally (i.p.) with 1 mg BrdU solution (BD Pharmingen, San Jose, CA). 24 hours post injection, mice were sacrificed and tissues were collected. Isolated lymphocytes were first surface stained as previously described and subsequently stained intracellularly using αBrdU mAb conjugated to APC (BD Pharmingen, San Jose, CA). To assay cell death, lymphocytes isolated at the indicated times were first surface stained with the appropriated identifying antibodies then incubated with Annexin V-PE and 7-Aminoactinomycin D (7-AAD) Viability Staining Solution (eBioscience, San Diego, CA) and analyzed via flow cytometry.

Competitive Adoptive Transfers

Splenocytes isolated from CD45.1 OT-I mice and CD45.2 or CD45.1/CD45.2 TSLP-$R^{-/-}$ OT-I mice were counted, resuspended in PBS at 1,000 cells/100μL and injected at a 1:1 ratio intravenously (i.v.) into congenically distinct (CD45.1/CD45.2 or CD45.2) recipient mice. 24 hours post transfer mice were infected i.n. with x31-OVA. Donor cells were detected by flow cytometry using mAb from eBioscience for the appropriate anti-CD45 molecule (PE/Cy7-conjugated αCD45.1, APC-conjugated

αCD45.2), along with stains for phenotyping of the donor populations (APC/Cy7-conjugated αCD8α or αCD62L, PE-conjugated αVα2, FITC-conjugated αCD44 and PerCP/Cy5.5-conjugated αCD127.

Statistics

Statistical analysis was carried out using Prism 5 software (GraphPad Software). Significance was determined when the p-value was $p<0.05$ and is indicated, along with the type of analysis used, in the figure legends.

2.4 Results

In vitro and in vivo induction of TSLP mRNA following influenza infection

Following inhalation, influenza virions preferentially infect respiratory epithelial cells which alert the immune system to infection via activation of TLRs 3 and 7 and RIG-I pattern recognition receptors (32, 180). Studies have demonstrated that stimulation of TLR3 using dsRNA can induce the expression of TSLP in human airway (166) and bronchial epithelial cells (181). Moreover, TSLP expression was also enhanced following infections with the respiratory pathogens Rhinovirus and Respiratory syncytial virus in human and rat airway epithelial cells, respectively (182). Recently, it has also been shown that infection with the highly pathogenic strain of influenza A virus, Puerto Rico/8 (PR8) can induce the production of TSLP mRNA in the lungs and trachea of mice (183), although data is conflicting as to whether or not this affects the anti-influenza CD8 T cell response, either directly or indirectly, and to what extent (183, 184).

In this study we assessed the role that TSLP plays on the immune response to the influenza virus A/HK-x31 (x31; H3N2). This virus closely mimics seasonal influenza infections and attenuated vaccines, as it is much less pathogenic in mice, even at high

doses. We first used an *in vitro* culture system to determine whether x31 infection could elicit TSLP mRNA expression since TSLP protein expression has been difficult to reliably detect using ELISA based methods. The mouse lung epithelial cell line, MLE-15, was infected with x31 or mock infected with PBS. Following infection, the cells were maintained in culture for the indicated times until they were harvested and RT-qPCR for murine TSLP mRNA was performed. Infected samples were directly compared to mock infected controls incubated in the same culture conditions for corresponding amounts of time. TSLP mRNA was induced in MLE-15 cells after influenza infection as early as 12 hours post infection and expression remained elevated until as late as 72 hours post infection (Figure 2.1, top left) at which point the experiment was terminated due to increasing levels of epithelial cell death. While this data demonstrates that x31 infection can elicit the production of TSLP by lung epithelial cells *in vitro*, it was unclear whether analogous infection of mice *in vivo* could also induce TSLP expression.

To test whether TSLP is produced following x31 infection *in vivo,* mice were infected intranasally (i.n.) with the virus or mock infected with PBS. At the indicated times post infection bracheoaveolar lavage (BAL) fluid, lungs, the lung draining mediastinal lymph nodes (MdLN), and spleens from infected and mock infected animals were harvested and TSLP mRNA levels were quantified via RT-qPCR. Over the course of infection, TSLP levels in the spleen (Figure 2.1, top right) were low and did not increase above the reference levels observed after a mock infection. In contrast, TSLP mRNA production was increased in the lung by 24 hrs post x31 infection (Figure 2.1, bottom left). These levels remained elevated until at least day 10 post infection (p.i.), which corresponds to the peak of the anti-influenza CD8 T cell response in the

respiratory tract. TSLP levels in the lung subsequently returned to baseline by day 15 p.i. when the majority of anti-influenza CD8 T cells are contracting and some are transitioning to memory. TSLP had similar fold increases in expression in the MdLN as the lung following infection with x31, peaking at 2 days p.i. (Figure 2.1, bottom right), indicating that TSLP is present at the site of T cell priming and may act on these influenza-specific CD8 T cells during their initial activation. As the timing of TSLP expression in the draining lymph nodes correlates well with the arrival of migratory DCs to this site (68), and TLR-activated DCs have been shown to produce TSLP (185, 186), it is possible that this cell population confers TSLP expression at the site of T cell priming. However, it should be noted that constitutive TSLP mRNA levels in the lung were approximately 10 times higher than those observed in the MdLN, which had little to no TSLP expression in mock infected animals (data not shown). Therefore, although fold induction was similar between lung and MdLN at the peak of expression (approximately 4 fold), the levels of TSLP mRNA were actually much higher in the lung both before and after influenza infection. These data indicate that while some migratory lymphoid cells (perhaps DCs) are able to induce TSLP expression in sites within close proximity to the respiratory tract, the lung is the main TSLP source after influenza infection. TSLP mRNA was not detected in the cells obtained from the BAL (data not shown), further confirming that non-lymphoid cells predominately contribute to TSLP production *in situ*. Together, these data demonstrate that respiratory infection with the x31 influenza virus evokes the local production of TSLP which is likely the result of the viral infection of the epithelial cells themselves as opposed to the highly inflammatory environment associated with more immunopathogenic influenza viruses. Moreover, the location and kinetics of

TSLP expression following influenza infection suggest that TSLP can act on CD8 T cells during their initial priming event in the MdLN and later upon their arrival as effector cells at the infection site.

Global loss of TSLP-R signaling increases morbidity in mice after influenza infection

Once it was established that local TSLP expression was induced by influenza infection, we wished to determine whether the global loss of TSLP signaling would impact the overall anti-influenza response. To that end, age and sex matched TSLP-$R^{-/-}$ mice and wild type (WT) C57BL/6 controls were infected with a sub-lethal dose of x31 and overall morbidity, viral burdens, and adaptive immune responses were measured. As indicated in Figure 2.2A, infection with the mouse adapted x31 strain of influenza results in overall low morbidity in WT mice, which lose negligible weight over the course of infection. In contrast, TSLP-$R^{-/-}$ mice lost significantly more weight than WT controls beginning as early as day 2 p.i. and continuing until about a week p.i., with starting body weight recovered by approximately 8 days p.i. (Figure 2.2A). The early and sustained weight loss in TSLP-R null mice may be the result of the inability of these animals to repair and maintain epithelial cell tight junctions (187) which were initially disrupted after influenza infection (188). In addition to the role that TSLP may be playing in enhancing the integrity of the epithelial barrier, the increased morbidity seen in the TSLP-$R^{-/-}$ mice could result from increased viral titers, immunopathology, or an inability to heal as well as WT mice (169).

To test whether TSLP-$R^{-/-}$ mice have a deficiency in their ability to clear influenza infection as effectively as their WT counterparts, both groups of mice were infected i.n. with x31, lungs were harvested at the indicated times following infection, and viral titers

assessed by plaque assay (Figure 2.2B). Early after infection and at the peak of viral replication (days 2-3), we did not observe any difference in viral titers between WT and TSLP-$R^{-/-}$ mice. However, at day 4 and particularly by day 6 post infection, TSLP-$R^{-/-}$ mice harbored higher viral titers in their lungs. TSLP-$R^{-/-}$ mice were unable to fully clear the virus by day 8 post infection whereas WT mice had completely resolved the infection (Figure 2.2B). Data from later time points show that TSLP-$R^{-/-}$ mice eventually clear virus with a 2 day delay over WT or by day 10 p.i. (data not shown). Together these data indicate that TSLP-$R^{-/-}$ mice were not more susceptible to influenza infection, as early viral titers and peak viral titers were similar, but instead had a defect in their ability to clear the virus as rapidly as WT mice.

The prolonged viral burden in the TSLP-$R^{-/-}$ mice could be the result of a defective or delayed adaptive immune response. We first analyzed the CD4 T cell response in WT vs TSLP-$R^{-/-}$ mice, as TSLP is well known to influence CD4 T cell polarization both directly (189, 190) and through interactions with DCs (191). CD4 T cells isolated from the BAL, lung, and spleen of WT and TSLP-$R^{-/-}$ mice 10 days p.i. were equally competent in their ability to produce IFN-γ after stimulation with the x31 influenza CD4 HA epitope (Figure 2.2C). Limited data suggests that TSLP can modulate antibody responses (192) however we did not expect the anti-influenza IgG2a titers, which correlate with influenza virus clearance (193), to be significant early enough to impact viral clearance prior to day 10 p.i.. Indeed, compared to x31 immune animals there were no detectable levels of these antibodies in either WT or TSLP-$R^{-/-}$ sera 9 days post infection (data not shown).

As CD8 T cells are requisite for efficient and complete clearance of influenza virus (57, 158), their localization and activity in response to influenza infection coincides with the timing of respiratory TSLP expression (194)(Figure 2.1), and TSLP-R is expressed on activated CD8 T cells (168, 174), we reasoned that TSLP may be acting on CD8 T cells at the site of infection. To evaluate the role that TSLP may play on anti-influenza CD8 T cell responses we used a MHC Class I tetramer loaded with the immunodominant epitope of the influenza nucleoprotein (NP), H-2D^{b} / ASNENMETM. Using this reagent we assessed the frequency of influenza-specific CD8 T cells present in both the lymphoid tissues (spleen and lymph nodes) and peripheral effector sites (lung and BAL) at effector and memory phases of the anti-influenza response in WT and TSLP-R$^{-/-}$ mice (Figure 2.3). We did not observe any difference in the overall frequencies of NP-specific CD8 T cells isolated from the assayed lymphoid and non-lymphoid tissues at the peak of infection (10 days p.i) or during time frames consistent with the development of early (32 days p.i.) or late (115 days p.i.) memory CD8 T cells. Additionally, there were no significant differences in the total number of NP-specific CD8 T cells over the period assayed (data not shown). We also failed to observe any difference between the two groups regarding the phenotype of NP-specific cells recovered after infection in terms of CD127, KLRG1, CD62L, CD122, CD27 and CD43 expression (data not shown).

TSLP can act directly on CD8 T cells at the site of infection, influencing their proliferative and developmental fate

While we did not observe any difference in the frequency of influenza-specific CD8 T cells recovered between WT and TSLP-R$^{-/-}$ mice, it was still quite possible that

TSLP functionally participated in the normal CD8 T cell response to respiratory infection. Our inability to detect any CD8 T cell deficiencies in the TSLP-$R^{-/-}$ mouse could be due to cytokine redundancy in which those cytokines eliciting similar functions (like IL-7) could functionally compensate and mask the consequences of loss of TSLP alone. Furthermore, non-redundant roles of cytokines can be difficult to discern *in vivo* where disparities in the extent of infection and inflammation between individual animals and/or strains may conceal subtle differences in CD8 T cell responses. Thus, we modified our experimental system to better determine the role that TSLP plays on the anti-influenza CD8 T cell response directly, exclusively, and with greater sensitivity.

In order to elucidate the direct and individual contribution of TSLP to the anti-influenza CD8 T cell response, we utilized a competitive adoptive transfer system. In this system, the response of TSLP-R deficient and sufficient CD8 T cells of identical specificity is assayed within the same host. By design, this experimental system will reveal the functional consequences of the individual loss of TSLP signaling, even when compensatory pathways are present, as fully competent WT antigen-specific CD8 cells could have a selective advantage over TSLP-signaling deficient cells. Moreover, any secondary effects resulting from loss of TSLP signaling are excluded from the analysis. We therefore incorporated the competitive adoptive transfer scheme outlined in Figure 2.4A , in which we adoptively transferred 1,000 congenically mismatched TSLP-$R^{-/-}$ OT-I cells and TSLP-$R^{+/+}$ OT-I cells (WT OT-I) into congenically unique recipients. Using this method, we isolated the effects of TSLP signaling deficiency to the T cells themselves, independent of the indirect effects of TSLP on CD8 T cells via DCs or CD4 T cells.

To test the hypothesis that the direct loss of TSLP signaling on CD8 T cells impacts their response to respiratory viral infection, we i.n. infected our recipient mice harboring the OT-I chimeras with x31-OVA and monitored the frequencies of the specific OT-I subsets over time using the appropriate combination of CD45 antibodies. Comparisons of the ratio of TSLP-$R^{-/-}$ to WT OT-I cells at the proliferative peak of the anti-OVA CD8 T cell response (day 8 p.i.) demonstrated that WT OT-I cells preferentially accumulated proximal to the site of infection (BAL, Lung and MdLN) compared to TSLP-$R^{-/-}$ OT-I cells (Figure 2.4B) while there was little difference in the accumulation of either genotype of antigen-specific effector CD8 T cells at sites distal to the infection (spleen and inguinal lymph nodes (ILN)). Importantly, the inability of TSLP-R deficient OT-I cells to accumulate in the respiratory tract was maintained and exacerbated into the development of memory as observed at day 50 p.i., and this trend also continued until day 125 p.i., although donor populations became more difficult to find (data not shown). These data suggest that the early defect in the TSLP-R deficient CD8 T cell response in the respiratory tract is maintained and numerically affects the resultant population of memory CD8 T cells. The result is particularly important, as maintenance of CD8 T cells in the respiratory tract is requisite for prolonged CD8 T cell-based heterosubtypic immunity to influenza infection (24, 195).

To determine whether TSLP signaling qualitatively influences the development of memory cells, we analyzed the transferred OT-I cells for the expression of CD127 and CD62L. CD127 is used as a marker to delineate the precursors of bona fide memory cells (116) and IL-7 signaling via CD127 is important for their long term survival after systemic infection (164, 196). Overall, no significant differences were observed in the

CD127 expression between WT or TSLP-$R^{-/-}$ OT-I CD8 T cells (Figure 2.5A). However, differences were seen in the levels of CD127 on a per cell basis (as measured by median fluorescent intensity) between the OT-I cells derived from lymphoid (Spl and LNs) vs peripheral (lung and BAL) sites, with the latter showing decreased levels of CD127 expression. Interestingly, we observed a difference in the expression of CD62L between the OT-I groups 50 days p.i. (Figure 2.5B). The OT-I memory cells deficient in TSLP signaling harbored a greater proportion of $CD62L^{+}$ cells in the BAL, lung, MdLN and the spleen compared to their wild type counterparts (Figure 2.5B). The selectin CD62L confers lymph node homing potential, and is used to distinguish populations of memory cells as being either central memory cells ($CD62L^{+}$) or effector memory cells ($CD62L^{-}$). In this context, TSLP expression may influence the development of memory cells, with either early or sustained TSLP signaling throughout the transition to memory promoting an effector memory phenotype typical of influenza-specific CD8 T cells derived from mucosal sites. Together, these experiments demonstrate that following respiratory infection with influenza virus, TSLP signals directly to TSLP-R competent antigen-specific CD8 T cells in the respiratory tract. As a result, the number of antigen-specific cells at the site of infection is increased at the peak of the CD8 T cell response which carries over into the resulting CD8 memory cell pool. Moreover, TSLP may concomitantly modify the phenotype of the memory populations as assayed by differential CD62L expression.

TSLP can affect the magnitude of the CD8 T cell pool at the site of infection in several ways: by modulating migration into the tissue, promoting *in situ* proliferation, and/or enhanced survival of the cells. While it has been reported that TSLP-$R^{-/-}$ cells

express lower levels of the inflammatory chemokine receptor CXCR3 (184), TSLP-R$^{-/-}$ OT-I cells were able to accumulate in the respiratory tract (Figure 2.4), indicating to us that loss of TSLP did not impact migration to the mucosa. Rochman and Leonard showed that TSLP positively affected the survival of CD8 T cells under homeostatic conditions via the upregulation of Bcl-2 (168). Conversely, Akamatsu et al. showed that TSLP enhanced the proliferation of *ex vivo* stimulated human CMV-specific CD8 T cells (174). In our adoptive transfer system, infection of recipient animals harboring WT OT-I CD8 T cells with x31-OVA results in a dramatic increase of these antigen-specific CD8 T cells starting at day 7 p.i. and peaking sharply at day 8 p.i., before decreasing, once again quite dramatically, by day 9 p.i. (Figure 2.6A). This curve indicates a period of rapid proliferation prior to the peak of OT-I CD8 T cell response that is followed by rapid death of these cells. Therefore, in order to gain a better understanding of how TSLP signaling influences antigen-specific CD8 T cells in the respiratory tract, we assayed both proliferation and survival of the adoptively transferred populations of cells at physiologically relevant times surrounding the peak of the response. In order to assay cellular proliferation, recipient mice were injected i.p. with the thymidine analogue BrdU at 6 days p.i.. Twenty-four hours later (7 days p.i.) lymphocytes were isolated from the indicated tissues and surface stained for their identifying congenic markers and intracellular BrdU. Although varying levels of proliferation were observed among the recipient mice, consistent differences were observed between the level of proliferation of OT-I cells derived from the WT and TSLP-R$^{-/-}$ backgrounds within a single recipient mouse. Within the respiratory tract, TSLP-R$^{-/-}$ OT-I cells proliferated approximately 10% less than their WT OT-I counterparts (Figure 2.6B, C). These results were significant

specifically at the site of infection (BAL and Lung), where mucosally produced TSLP could directly act on respondent antigen-specific effector CD8 T cells. At day 7 p.i. we also observed that the cell cycle marker Ki67 was expressed in a lower frequency of TSLP-$R^{-/-}$ OT-I cells compared to WT OT-I cells (data not shown), further indicating that TSLP signaling on CD8 T cells directly leads to increased levels of antigen specific CD8 T cell proliferation. Surprisingly, and in contrast to published findings that TSLP regulates the survival of activated CD8 T cells both *in vitro* and *in vivo* (168), we did not observe any differences in the rate of cell death between WT and TSLP-$R^{-/-}$ OT-I cells as measured by staining with 7-AAD and Annexin V at days 9 and 10 p.i. (Figure 2.6D). These data indicate, to our knowledge, a previously undefined role for TSLP, where the cytokine produced in the respiratory mucosa acts directly on responding antigen-specific CD8 T cells to increase their local proliferation and establishment as a pool of memory cell precursors at this site.

2.5 Discussion

Mucosal surfaces harbor unique and specialized immunological niches which are tightly regulated to promote immunity while causing minimal immunopathology. Mucosal environments employ many regulatory mechanisms, both constitutively and when faced with inflammatory stimuli, to maintain their vital tissue function. Cytokines classified as anti-inflammatory (TGF-β and IL-10) or Th2 biasing (IL-4, TSLP, and the alarmin IL-33) are integral in maintaining mucosal tissue integrity. Dysregulation of many of these cytokines results in the development of inflammatory bowel or allergic airway diseases (197, 198) highlighting the importance of these cytokines in immune homeostasis at barrier sites. Relevant to our work, studies have

demonstrated that respiratory viral infections can enhance levels of IL-33 (199) and TSLP (182) and disruption of these cytokine networks results in poor immunological outcomes in response to these pathogens (200). Whereas in many cases this is directly related to defective barrier function (191), direct modulation of immune effectors by these cytokines could also impact immunity.

With growing interest in developing mucosal vaccines, particularly those targeting CD8 T cells (201, 202), there is a need to gain a deeper understanding of how cytokines influence the development and maintenance of memory CD8 T cells at these sites. It is known that over the course of the CD8 T cell response to infection, diametric signals exist to positively direct anti-viral CD8 T cells towards a memory vs short-lived effector cell fate. One way to discriminate memory cell potential is through the expression of IL-7Rα which imparts a survival advantage to this pool of cells after IL-7 encounter (164). However, evidence suggests that redundant mechanisms also exist to regulate memory cell fate (135) and that different signaling pathways may regulate memory CD8 T cell development in systemic vs mucosal infection (141).

Our original hypothesis was that mucosally-derived TSLP, which shares common signaling pathways with IL-7 and binds to a receptor also containing IL-7Rα, participates as an alternative and non-redundant pathway for memory CD8 T cell development in mucosal sites. However, we observed that TSLP participates much earlier in the anti-influenza CD8 T cell response by promoting the local proliferation of antigen-specific effector CD8 T cells (Figure 2.6B) which not only temporally increases their number but also those memory cells derived from this pool (Figure 2.4B). This proliferative role of TSLP was only apparent in the competitive adoptive transfer system where competition

between TSLP-R deficient and WT CD8 T cells for identical resources revealed this distinct and non-redundant function for TSLP. The proliferative role for TSLP was also only observed in the BAL and lung (Figure 2.6B), highlighting the relevance of this cytokine in driving CD8 T cell division *in situ*. Whether low-level constitutive TSLP expression in the respiratory tract is responsible for any periodic homeostatic proliferation of Tmem over time is still unknown. Interestingly, however, the proliferative function of TSLP in the context of influenza infection is independent of alterations in survival (Figure 2.6D) unlike previous studies assessing the role of TSLP in homeostatic conditions (168). These data would suggest that perhaps TSLP signaling has contextual effects on CD8 T cell responses dependent on location, signaling thresholds, and cytokine/ receptor expression levels, many of which are different under inflammatory and homeostatic conditions.

An important aspect of our study was that TSLP affected local effector cell proliferation and ablation of TSLP-R signaling did not completely block the development and maintenance of memory CD8 T cells, likely due to intact cytokine networks. IL-2 and IL-7 support the early division and survival outside of the respiratory tract and are available to effector CD8 T cells early post activation (159).While IL-7 in particular is superior compared to TSLP in providing survival and/or proliferation signals to CD8 T cells (168, 184), naïve T cells trafficking between secondary lymphoid tissues compete for IL-7 survival signals (203). As a consequence of this competition, peripheral memory cells express higher levels of CD127 on a per cell basis compared to their naïve counterparts (116). Mucosal (vs systemic) memory CD8 T cells express less CD127 (Figure 2.5B)(157) , but do not compete with naïve cells for TSLP in the respiratory tract,

such that even limited CD127 expression could fully support TSLP signaling *in situ*. Likewise, it is unclear whether cytokine compartmentalization results in a greater dependence on TSLP once effector CD8 T cells enter the respiratory tract. While post-translational regulation of TSLP is unknown, IL-7 bioavailability is carefully regulated *in vivo* via selective binding to heparin sulfate moieties on basement membranes whose composition may differ in the lung (113). Lack of reliable assessment of IL-7 and TSLP protein levels by standard methods have prevented testing the hypothesis that cytokine bioavailability limits respiratory CD8 effector T cell proliferation. However, the expression of TSLP mRNA clearly support a spatial and temporal focus which could influence anti-influenza effector CD8 T cells which seed the respiratory tract and differentiate into memory cells *in situ*. Moreover, the inferior TSLP-driven proliferative signal in the respiratory tract could account for the limited survival of airway resident effector/ memory cells observed in influenza infection models (24).

Interestingly, loss of TSLP signaling resulted in the increased expression of CD62L on the antigen-specific OT-I cells in the respiratory tract compared to the WT OT-I cells at 50 days p.i. (Figure 2.5B). Traditionally, CD62L imposes lymph node homing on $CD62L^+$ T central memory (T_{CM}) cells whereas $CD62L^-$ T effector memory (T_{EM}) cells accumulate at peripheral sites (204). Beyond differences in tissue localization, the longevity of T_{CM} is greater than T_{EM} cells, which are thought to be more terminally differentiated yet superior at maintaining protection at barrier sites (205). As sustained proliferation of transitioning effector CD8 T cells maintains low levels of CD62L expression (206) and results in terminal differentiation (207), our data would suggest that the TSLP-driven proliferative burst at the site of infection can drive the formation of Tem

cells. In support of this theory, blocking the migration of effector CD8 T cells to the lungs abrogated the development of short-lived effector cells (208) perhaps due in part to their inability to access and proliferate in response to TSLP.

In summary, we believe that TSLP plays an important role in anti-viral immune responses in the lung both by maintaining barrier function and modulating the proliferation of effector CD8 T cells *in situ*. In regards to the latter, our data support a model in which TSLP directly regulates respiratory anti-viral CD8 T cells by acting as a rheostat to balance protective immunity and limit immunopathology. Under inflammatory conditions, including influenza infection, respiratory epithelial cells temporally increase local TSLP levels (Figure 2.1) (140, 183). Consequently, activated CD8 T cells immigrating into the respiratory tract are juxtaposed to TSLP, which supports a limited, additional proliferative burst to these effector cells *in situ* (Figure 2.6B), quantitatively enhancing the peak number of anti-influenza CD8 effector T cells seeding the respiratory tract as well as memory CD8 T cells derived from this pool (Figure 2.4B). As data, including our own (Figure 2.5A), suggest that activated and memory CD8 T cells in respiratory mucosal tissue are less responsive to the proliferative affects of gc chain cytokines, such as IL-7 and IL-15 (141, 157), TSLP may provide the dominant proliferative signal available to these cells early *in situ*. However, our data suggest that the modest proliferation supported by TSLP may affect the long-term destiny of the respiratory anti-influenza CD8 memory T cells in that the numerical advantage may be at the expense of a shorter-lived, more terminally differentiated fate.

2.6 Acknowledgements

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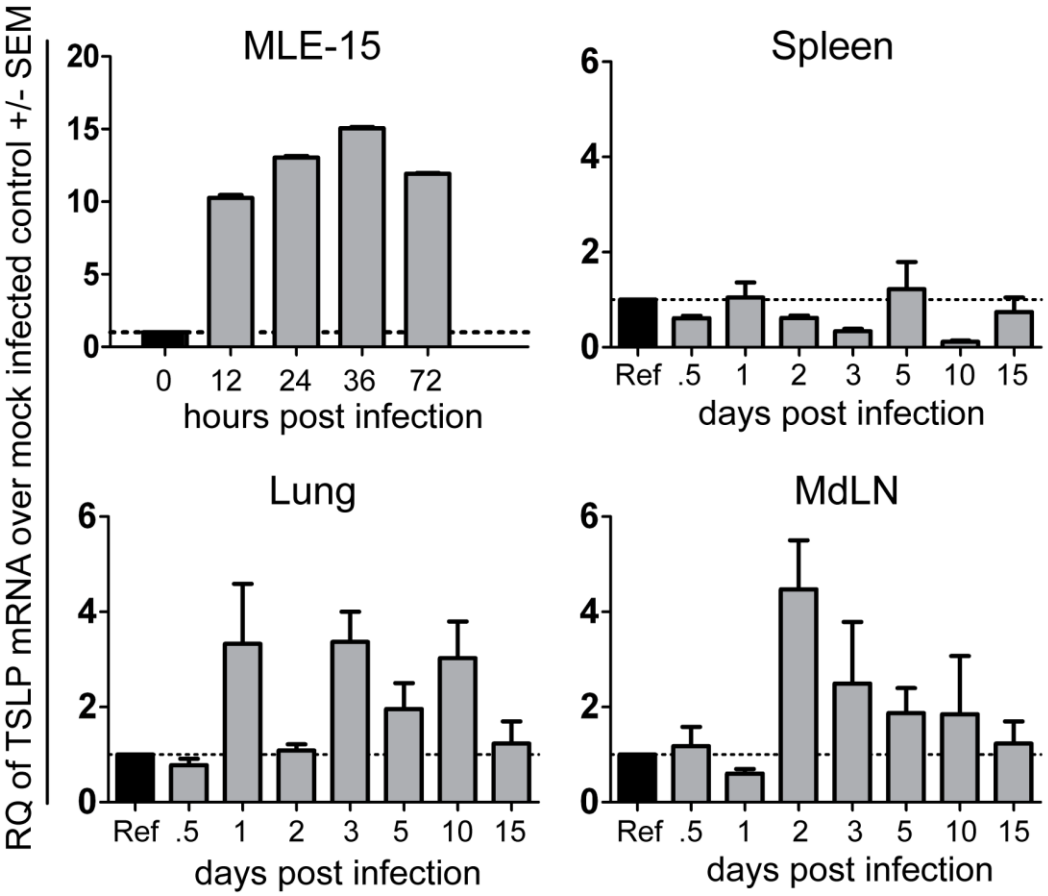

RQ of TSLP mRNA over mock infected control +/- SEM
MLE-15
20
15
10
5
0
0 12 24 36 72
hours post infection
Spleen
6
4
2
0
Ref .5 1 2 3 5 10 15
days post infection
Lung
6
4
2
0
Ref .5 1 2 3 5 10 15
days post infection
MdLN
6
4
2
0
Ref .5 1 2 3 5 10 15
days post infection

FIGURE 2.1. Influenza infection induces the expression of TSLP mRNA. The murine lung epithelial cell line (MLE-15) was infected with 0.5 MOI of x31 and cultured for the indicated times post infection before cells were harvested, RNA was extracted, and RT-qPCR was performed. For *in vivo* analysis, C57BL/6 mice were infected with 1,000 pfu x31 or mock infected with PBS i.n.. Tissues were collected at the indicated times post infection and TSLP mRNA was quantified using RT-qPCR. Data was normalized using an endogenous control and is displayed as relative quantification over mock-infected controls (1 or Ref) for each indicated time point, as determined by the ΔΔct method. Values are shown as mean RQ +/- SEM (n=3 samples/group). Data is representative of two independent experiments.

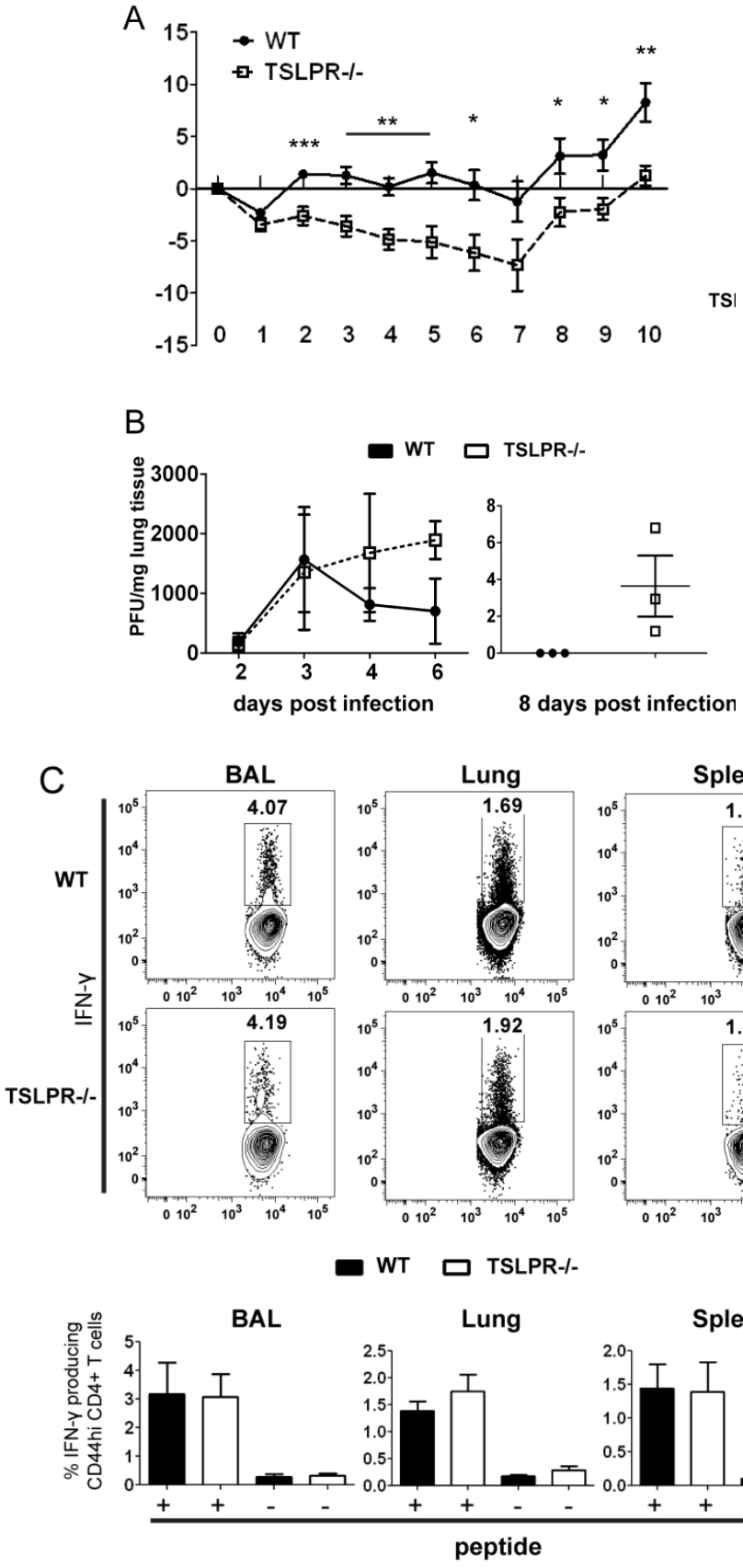

A
WT
TSLPR-/-
B
WT
TSLPR-/-
PFU/mg lung tissue
days post infection
8 days post infection
C
BAL
Lung
Spleen
WT
TSLPR-/-
IFN-γ
4.07
1.69
1.31
4.19
1.92
1.63
% IFN-γ producing CD44hi CD4+ T cells
peptide

FIGURE 2.2. TSLP-R-/- mice develop more severe disease than WT mice. (**A**) WT B6 mice (n=10) and TSLP-$R^{-/-}$ mice (n=9) were infected i.n. with 1,000 pfu of x31 and weighed daily. Data is shown as mean percent change in body weight over time +/- SEM. Overall significance in weight change between WT and TSLP-$R^{-/-}$ mice was assessed using a two-way ANOVA test, p=0.0017. Significance between groups at individual time points was assessed using a two-tailed student t-test (*p=<0.05, **=p<0.01, ***=p<0.001). Data is representative of two independent experiments. (**B**) WT and TSLP-$R^{-/-}$ mice were infected i.n. with 1,000 pfu x31. Viral titers were measured by plaque assay at days 2, 3, 4, 6, (left) and 8 (right) post infection and graphed as pfu/mg of lung tissue +/- SEM (n=3mice/group/day). Data is representative of two independent experiments. **(C)** Lymphocytes isolated from the BAL, lung and spleen of WT and TSLP-$R^{-/-}$ mice (n=3/group) at 10 days post x31 infection were stimulated *ex-vivo* in the presence or absence of a class II-restricted HA peptide, and CD4 T cells were assessed for IFN-γ production. The top panels show representative IFN-γ staining in $CD4^{+}$ ($CD44^{hi}$) lymphocytes. The bottom panel shows the quantification of this data. Data is representative of two independent experiments.

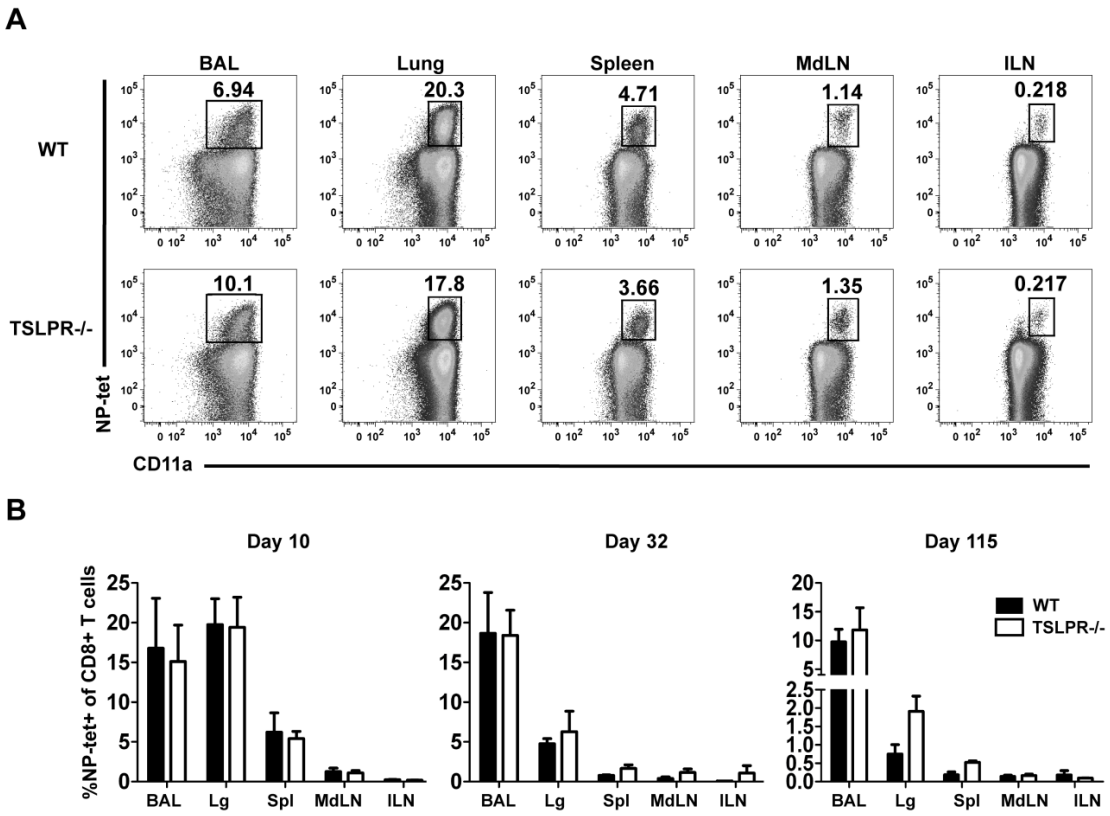

A
BAL
Lung
Spleen
MdLN
ILN
WT
TSLPR-/-
6.94
20.3
4.71
1.14
0.218
10.1
17.8
3.66
1.35
0.217
NP-tet
CD11a
B
Day 10
Day 32
Day 115
%NP-tet+ of CD8+ T cells
WT
TSLPR-/-
BAL
Lg
Spl
MdLN
ILN

FIGURE 2.3. TSLP-R$^{-/-}$ mice harbor similar frequencies of influenza-specific CD8 T cells as WT mice. Lymphocytes were isolated from the indicated tissues of x31 infected animals and analyzed by flow cytometry for tetramer reactivity at 10, 35, and 115 days p.i. (**A**) Representative dot plots from the BAL, Lung, Spleen, MdLN and non-draining inguinal LN (ILN) of WT and TSLP-R$^{-/-}$ mice at day 10 p.i.. Cells were first gated on total CD8^{+} lymphocytes and analyzed for CD11a expression and tetramer reactivity. (**B**) Average percent NP-tetramer^{+} of CD8 T cells isolated from indicated tissues at days 10, 32, and 115 p.i. in WT and TSLP-R$^{-/-}$ animals. Data is shown as the mean percent of tetramer positive cells of the CD8 T cells +/- SEM (n=3 mice/group), and is representative of two independent experiments. Data was analyzed for significance using a two-tailed students t-test; no significant differences were found.

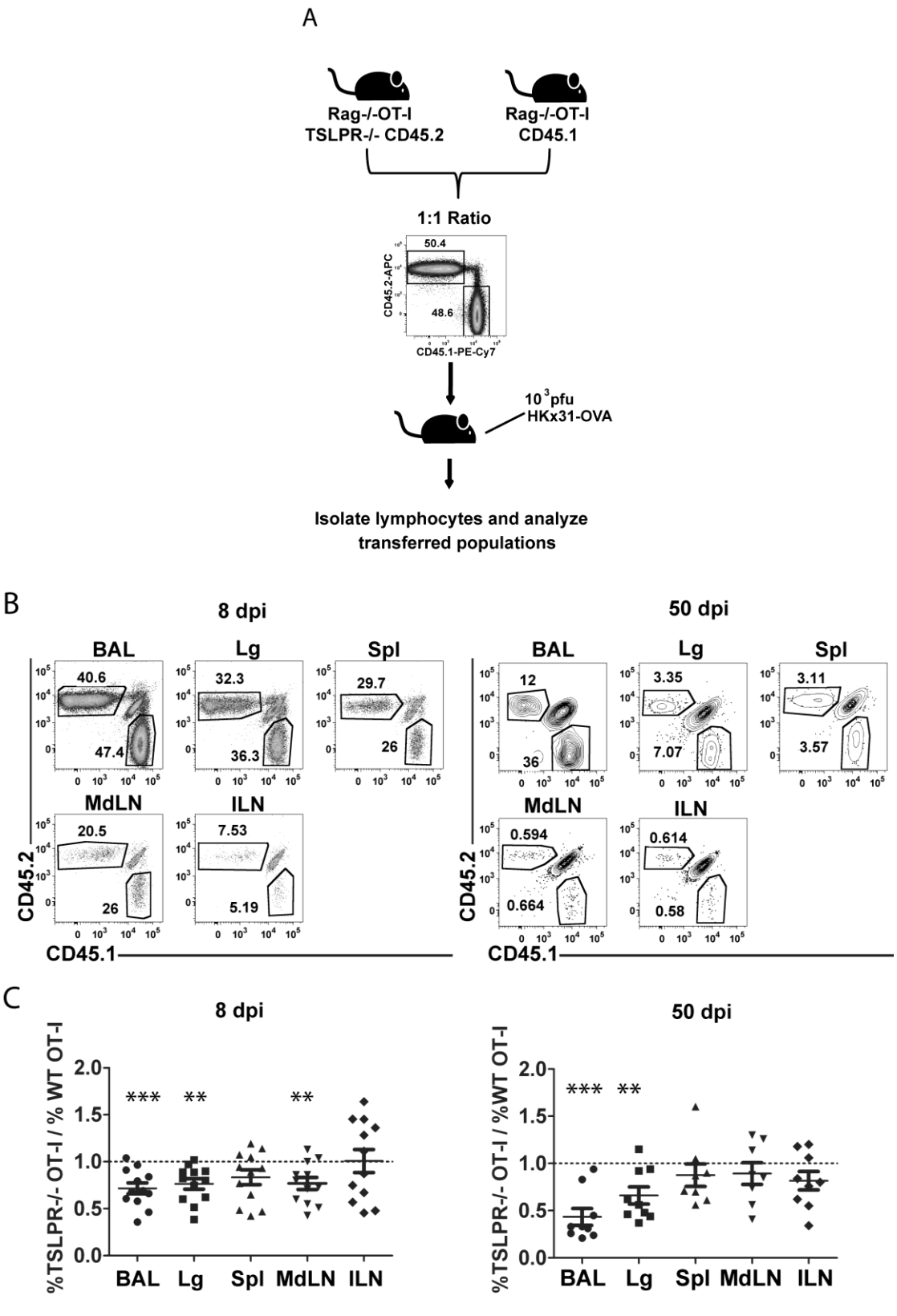
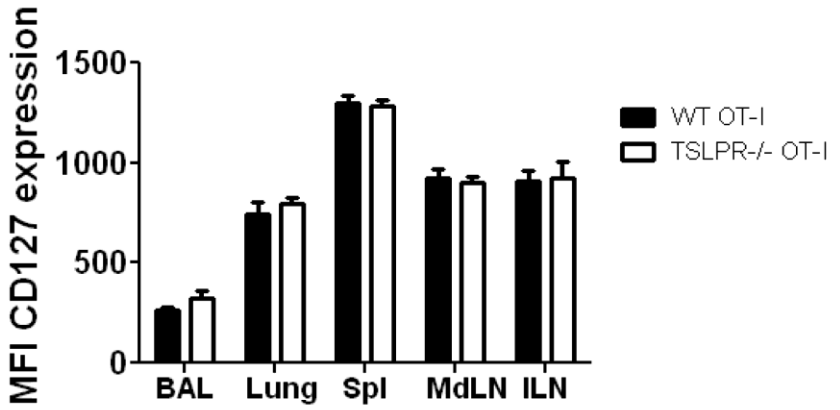

A
Rag-/-OT-I
TSLPR-/- CD45.2
Rag-/-OT-I
CD45.1
1:1 Ratio
50.4
48.6
CD45.2-APC
CD45.1-PE-Cy7
10 3 pfu
HKx31-OVA
Isolate lymphocytes and analyze
transferred populations
B
8 dpi
50 dpi
BAL
Lg
Spl
MdLN
ILN
40.6
47.4
32.3
36.3
29.7
26
20.5
26
7.53
5.19
12
36
3.35
7.07
3.11
3.57
0.594
0.664
0.614
0.58
CD45.2
CD45.1
C
8 dpi
50 dpi
%TSLPR-/- OT-I / % WT OT-I
%TSLPR-/- OT-I / %WT OT-I
***
**
**
***
**
BAL
Lg
Spl
MdLN
ILN

FIGURE 2.4. TSLP-R$^{-/-}$ OT-I cells are less prevalent at the site of infection than WT OT-I cells following influenza infection. (**A**) Competitive adoptive transfer scheme; Congenically mismatched WT OT-I and TSLP-R$^{-/-}$ OT-I cells (1,000 each) were adoptively transferred via tail vein injection into congenically distinct WT mice that were infected 24 hours later i.n. with 1,000 pfu x31-OVA . (**B**) Representative flow from the indicated tissues at days 8 and 50 p.i. Single cell lymphocyte populations were first gated on double positive Vα2^{+}, CD44^{hi} cells. (**C**) Frequencies of WT and TSLP-R$^{-/-}$ OT-I cells at days 8 and 50 following infection as assessed by flow cytometry. Data is shown as pooled samples from 3 identical experiments and expressed as a ratio of TSLP-R$^{-/-}$ OT-I cells / WT OT-I cells. Significance was determined using one sample t-test against a theoretical mean of 1 (**=$p<0.01$, ***=$p<0.001$).

A


MFI CD127 expression
1500
1000
500
0
BAL
Lung
Spl
MdLN
ILN
WT OT-I
TSLPR-/- OT-I

B

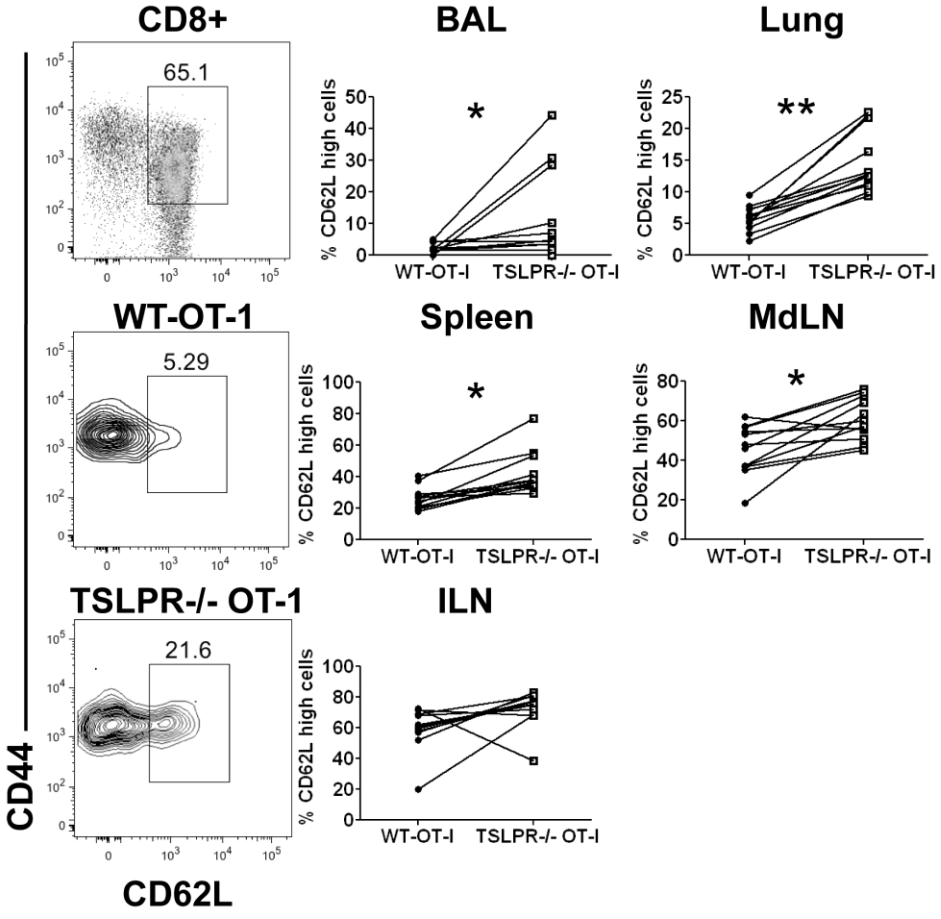

CD8+
BAL
Lung
65.1
% CD62L high cells
*
**
WT-OT-I
TSLPR-/- OT-I
WT-OT-1
Spleen
MdLN
5.29
TSLPR-/- OT-1
ILN
21.6
CD44
CD62L

FIGURE 2.5. TSLP-R$^{-/-}$ OT-I cells express higher levels of CD62L than WT-OT-I cells. (**A**) Median fluorescent intensity was determined on the transferred populations of cells for staining against CD127 at day 50 p.i. Data is representative of three independent experiments (n=6). (**B**) CD62L expression was analyzed on the populations of adoptively transferred cells at day 50 p.i.. Data is pooled from three independent experiments and displayed as frequency of CD62L high cells among total WT and TSLP-R$^{-/-}$ OT-I donors (with these populations within individual recipients connected by line). Data is shown as pooled samples from 3 identical experiments. Statistical significance was determined using a two-tailed student t-test comparing the mean frequency of CD62L high OT-I cells between groups (*=$p<0.05$, **$p<0.01$).

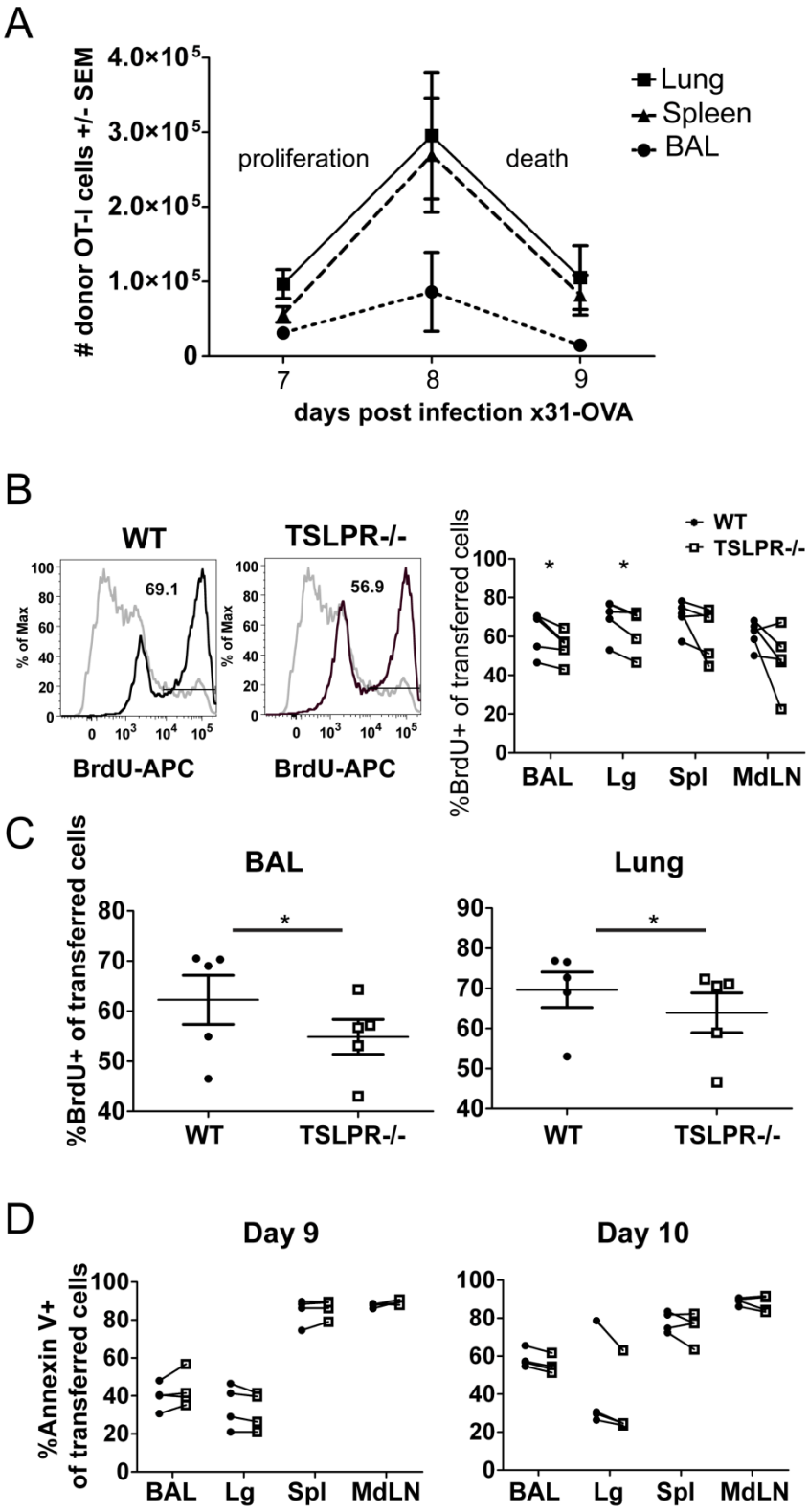

A
donor OT-I cells +/- SEM
4.0×10^5
3.0×10^5
2.0×10^5
1.0×10^5
0
proliferation
death
Lung
Spleen
BAL
7
8
9
days post infection x31-OVA
B
WT
TSLPR-/-
69.1
56.9
% of Max
BrdU-APC
%BrdU+ of transferred cells
BAL
Lg
Spl
MdLN
C
BAL
Lung
%BrdU+ of transferred cells
WT
TSLPR-/-
D
Day 9
Day 10
%Annexin V+ of transferred cells
BAL
Lg
Spl
MdLN

FIGURE 2.6. TSLP-R$^{-/-}$ OT-I cells proliferate less following influenza infection than WT-OT-I cells. (**A**) 2,000 CD45.1 OT-I cells were transferred into CD45.2 recipient mice and infected i.n. with HKx31-OVA 24 hours later. Number of donor CD8 T cells in the BAL, Lung and Spleen were quantified using flow cytometry and displayed as mean +/- SEM (n= 4 mice per group). **B** and **C**, Congenically distinct TSLP-R$^{-/-}$ OT-I and WT OT-I cells (1,000 each) were transferred into recipient mice that were infected i.n. with x31-OVA 24 hours following transfer. 100ug of BrdU was administered i.p. at 6 dpi (24 hours prior to sacrifice) and BrdU incorporation was assessed at 7 days post infection by intracellular staining followed by flow cytometric analysis. **(B)** The left panel depicts representative BrdU staining in either the WT or TSLP-R$^{-/-}$ OT-I pools activated in the same animal. BrdU incorporation for the individual OT-I pools is quantified for all tissues analyzed on the right. Data is shown as % BrdU positive cells where each set of connected points represents the transferred populations of OT-I cells found within the same recipient mouse. Differences in the level of BrdU incorporation between the WT and TSLP-R$^{-/-}$ OT-Is are depicted for the respiratory tract only **(C)**. Significance in (**B, C**) was tested for using a paired student's t-test (*=$p<0.05$). Data shown is representative of 3 experimental repeats. (**D**) Cell death was measured by antibody staining for Annexin-V and 7-AAD in the indicated tissues at 9 and 10 days p.i., each set of connected dots represents the transferred OT-I populations within the recipient mouse. Data shown is representative of two experimental repeats; significance was tested for using a paired student's t test and no significant differences were found.

CHAPTER 3

THE RESPIRATORY ENVIRONMENT DIVERTS THE DEVELOPMENT OF ARCHETYPICAL ANTI-VIRAL MEMORY CD8+ T CELLS[1]

[1] Shane, H.S., Verbist, K.C., and Klonowski, K.D. To be submitted to *Mucosal Immunology*

3.1 Abstract:

Our understanding of memory $CD8^+$ T cells has been largely derived from acute, systemic infection models. However, memory $CD8^+$ T cells generated from mucosal infection exhibit unique properties and, in respiratory infections, are poorly maintained long-term. To better understand how infection route modifies memory differentiation, we compared murine $CD8^+$ T cell responses to an identical VSV challenge generated intranasally (IN) or intravenously (IV). IN infection resulted in greater peak expansion of VSV-specific $CD8^+$ T cells. However, this numerical advantage was rapidly lost during the contraction phase of the immune response, resulting in memory $CD8^+$ T cell numerical deficiencies when compared to IV VSV infection. Interestingly, the anti-viral $CD8^+$ T cells generated in response to IN VSV exhibited a biased and sustained proportion of early effector cells ($CD127^{lo}KLRG1^{lo}$) akin to the $CD8^+$ developmental program favored after IN influenza infection, suggesting that respiratory infection favors an incomplete memory differentiation program. Correspondingly, IN VSV infection resulted in lower CD122 expression and EOMES levels by VSV-specific $CD8^+$ T cells, further indicative of an inferior transition to bona-fide memory. These results were independent of sustained antigen, but may be due to early priming induced by distinct dendritic cell subsets activated following IN *vs*. IV infection. Together these data suggest that the environments encountered in distinct immunization routes are sufficient to modulate both the quality and quantity of anti-viral effector and memory $CD8^+$ T cells in response to an identical pathogen and should be considered in $CD8^+$ T cell-based vaccine design.

3.2 Introduction:

The mammalian respiratory tract is an organ in which gas exchange occurs at high efficiency. This is achieved by the vast surface area of the conducting airways and the extreme proximity of the airways (and thus the external environment) to the rich, underlying vascular bed. These properties of the lung, however, leave the host in a compromised state for protection against pathogen entry if innate barriers are breached. This susceptibility of the lung to infection is reflected in infectious disease statistics, where lower respiratory tract infections are the 3rd most common cause of human death worldwide, and the most common cause of death from an infectious disease (1). Of these pathogens, viruses account for the largest number of disease cases due to their prominence and high rates of mutation and infectivity. One such respiratory virus, influenza, is particularly prevalent in human populations, and harbors properties that make it prone to result in pandemics (7).

Current influenza vaccines target a protective antibody response (6). However, these vaccines are less effective in application due to mutation and evolution of the targeted hemagglutinin antigens. Evidence in mouse and human models not only implicate CD8$^+$ T cells as requisite for viral clearance but also protective against heterologous challenge with novel influenza strains (24, 209). While to date no approved vaccine has been developed to specifically generate memory CD8$^+$ T cells, it is quite possible that the superiority of the live attenuated (versus the subunit) influenza vaccine may in part be the result of concomitant induction of an anti-viral antibody *and* a specific CD8$^+$ T cell response. Indeed, human studies have detected influenza-specific CD8$^+$ T

cell memory responses following administration of this vaccine for at least 60 days post infection (dpi) (210). While the contribution of CD8 T cell memory to vaccine efficacy has not been tested comprehensively or longitudinally, $CD8^+$ memory T cells generated in mouse models to this vaccine survive poorly (211). Thus, there is a need to better understand the development of memory $CD8^+$ T cells (T_{mem}) following respiratory infection for targeting $CD8^+$ T cells both directly or in concert with neutralizing antibodies.

Over the last few decades, several laboratories have delineated pathways important in $CD8^+$ T_{mem} development and defined the attributes and molecules which support robust T cell memory long-term. This gold standard for $CD8^+$ T cell memory has been defined in murine models of acute viral infection whereby the pathogen of interest was delivered via the intravenous (IV) route (116, 136). However, it is becoming increasingly clear that the formation of T_{mem} is a dynamic process, with memory potential influenced by a variety of factors including cytokines (129), the type of antigen presenting cells involved (144) , the strength and duration of antigen exposure (95), all of which are unique and highly specialized in the respiratory mucosa. Indeed, our laboratory and others have demonstrated that mucosally-derived anti-viral $CD8^+$ T cells acquire properties incongruent with memory formation as defined from the systemic infection models (141, 212). For example, by simply altering the route of viral acquisition, from IV to intranasal (IN), $CD8^+$ T_{mem} are not only less abundant overall, but develop and are maintained independent of the cytokine IL-15 , a deficiency that results in T_{mem} decay after systemic infection (136, 137, 141). Thus, as $CD8^+$ T_{mem} generation does not appear to be a “one model fits all” scenario, it is important to understand how and why the

mucosal memory T_{mem} program is offset from the benchmark T_{mem} derived in systemic model systems to improve vaccine formulation.

In this study we sought to determine the role of respiratory environment on the development of $CD8^+$ $T_{mem.}$. We used vesicular stomatitis virus (VSV) here as our model pathogen since it has been widely used in $CD8^+$ T_{mem} studies and, unlike influenza virus, it can be transmitted naturally through multiple routes in both mice and its natural host (cattle), including the respiratory tract (213). We previously validated this model of respiratory infection in vivo as T_{mem} derived from IN VSV infection numerically and phenotypically resemble anti-influenza $CD8^+$ T_{mem} (141). Using this system, we have now found that although respiratory VSV infection results in higher $CD8^+$ effector T cell responses early after infection, this numerical advantage is lost rapidly though the contraction phase of the $CD8^+$ T cell response, resulting in a quantitatively reduced T_{mem} pool. This loss is likely facilitated by phenotypic differences in memory cells early after infection, including lowered expression of Eomes and CD122 as well as altered effector phenotypes based on the expression of CD127 and KLRG1. Furthermore, IN infection leads to the accumulation of distinct populations of dendritic cells (DCs) in respiratory tract draining lymph nodes (LNs), providing a possible mechanism for these altered phenotypes and developmental deficiencies in memory.

3.3 Materials and Methods:

Mice and Viruses

C57BL/6 mice were purchased from Charles River (Wilmington, MA) through the National Cancer Institute program and bred in house. C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice were generously provided by Dr. Leo Lefrancois (University of Connecticut,

Farmington, CT) and maintained on a CD45.1 $Rag^{-/-}$ background. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Georgia. For VSV infections, age and sex matched mice were infected with 10^4 pfu (plaque forming units) of VSV-Indiana serotype or VSV-Indiana-OVA (provided generously by Dr. Leo Lefrancois) either intranasally in 50 µl of PBS or intravenously through the tail vein in 200 µl of PBS.. Stocks were maintained and isolated by growth in BHK cells while viral titers were determined by plaque assay. The influenza virus A/HK-x31(x31, H3N2) was generously provided by Dr. S. Mark Tompkins (University of Georgia, Athens, GA). For influenza experiments, age and sex matched animals were infected intranasally (IN) with 10^3 pfu x31 in 50 µl PBS.

Tissue Preparation

Single cell suspensions from tissues were obtained as previously described (141). Briefly, cells were isolated from the lung parenchyma after first perfusing the lungs with ~10 mL PBS/heparin. The perfused lungs were excised, minced and incubated with 1.25 mM EDTA at 37°C for 30 minutes followed by a 1 h incubation with 150 units/mL collagenase (Life Technologies, Grand Island, NY). After passage through 40 µM cell strainers, lymphocytes were resuspended in 44% Percoll, underlaid with 67% Percoll, centrifuged and the cellular interface collected. Lymph nodes and the splenic tissues were mechanically disrupted then passed through a cell strainer. Erythrocytes were depleted from the spleen samples using Tris-buffered ammonium chloride (TAC). Blood samples were obtained either by retro-orbital eye bleeding or by cardiac puncture at time of sacrifice. Erythrocytes were depleted from blood samples by two serial treatments, 10 min each at 37° C, with TAC. Cell numbers were determined using a Z2 Coulter Particle

Counter (Beckman Coulter, Fullerton, CA). For lymphocyte numbers obtained from blood samples 250 µl of blood was counted and expressed as lymphocytes/mL.

Flow cytometry

The VSV nucleoprotein (N) MHC class I [H-2K^b/RGYVYQGL] tetramer and the influenza nuclear protein (NP) MHC class I [H-2D^b/ASNENMETM] tetramer (conjugated to APC) were generated at the National Institute of Allergy and Infectious Diseases Tetramer Facility (Emory University, Atlanta, GA). Staining was carried out at room temperature for 1 hr in conjunction with other surface staining. Antibodies used for staining were as follows: αCD127-PE (A7R34) , αCD8a (53-6.7), αCD11b-FITC (M1/70), αCD19-PerCP-Cy5.5 (1D3), αCD8a-violetFluor 450 (2.43) (Purchased from Tonbo Biosciences, San Diego, CA), αEomes-PE (Dan11mag), αT-bet PerCP (eBio4B10), αKLRG-1-PE-Cy7 (2F1), αCD103-PE (2E7), αNK1.1-PerCP-Cy5.5 (PK136), αCD3e-PerCP-Cy5.5 (145-2C11), αCD11c-PE-Cy7 (N418), αCD80-APC (16-10A1), αMHC II-APC-eFluor 780 (M5/114.15.2) (purchased from eBioscience, San Diego, CA) and αCD44-FITC (IM7) (Purchased from BD Biosciences, San Jose, CA). When tetramer was not used, cells were surface stained for 20 min at 4°C. For analysis of intracellular proteins (T-bet and Eomes) cells were fixed and permeabilized using eBioscience Fix Perm and intracellularly stained according to the manufacturer (eBioscience, San Diego, CA). Following staining, cells were fixed in 2% paraformaldehyde and flow cytometric analysis was performed using a BD LSR II and data were acquired with FacsDiva software (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software version 9.6.2 from Tree Star Inc. In all analyses, cells were first gated on single cells, followed by lymphocytes (or all cells for dendritic cell

phenotyping) as determined by forward (FSC-A) and side scatter (SSC-A). Subsequent gating strategies are notes in the figure legends.

CFSE labeling/OT-I transfers

Ovalbumin-specific lymphocytes isolated from the spleens of Rag-/- OT-I mice were enumerated and labeled with carboxyfluorescein succinimidyl ester (CFSE) by incubating 10^7 cells/ml of HBSS with 1ul of 5mM CFSE/ml of HBSS for 10 min at 37° C in the dark. 2 x10^6 CD45.1 labeled OT-1 cells were injected via the tail vein into CD45.2 recipient mice 12 days follow infection with 10^4 pfu VSV or VSV-OVA administered IN or IV, or mock infected with PBS. Five days following transfer lymphocytes were isolated from the spleen, ILN, MdLN and lung as previously described. Cells were surface stained to identify $CD8^+$ T cells and congenic markers and proliferation was determined by assessing the population of $CD8^+$ $CD45.1^+$ cells in which CFSE was diluted.

Statistics

Statistical analysis was carried out using GraphPad Prism, version 5 or 6. Significance was determined when the p-value was $p<0.05$ and is indicated, along with the analysis selected, in the figures and figure legends.

3.4 Results:

Intranasal infection, compared to systemic, generates numerically deficient $CD8^+$ T_{mem} derived from a larger effector cell pool

Infection at mucosal surfaces such as the lung often produce $CD8^+$ T_{mem} responses which are limited, both in number and lifespan, compared to systemic challenges (96, 141). This is believed to be due, at least in part, to the heightened level of immune

regulation at these surfaces that limits inadvertent immunopathology, which could lead of loss of essential tissue function. However, the mechanisms linked to the development of substandard T_{mem} have been poorly understood, mainly due to inequitable comparisons made using two different pathogens with distinct tissue tropisms and inflammatory signals. Therefore, we sought to modify route of infection alone to address how the respiratory environment affects the development $CD8^+$ T_{mem}.

To first validate that the respiratory route of infection modifies the development of $CD8^+$ T_{mem}, mice were infected either IN or IV with a sub-lethal dose of VSV. This virus produces a replicating viral infection via multiple routes (214) and thus provides a model to compare the emergence of $CD8^+$ T_{mem} arising from systemic and respiratory infection. Antigen-specific $CD8^+$ T cell responses were assessed at 35 days post infection (dpi) using MHC-I tetramers against the immunodominant epitope of the VSV-nucleoprotein (N-tet^+ cells). As previously reported, IN viral delivery of VSV resulted in a lower frequency of VSV N-tet^+ $CD8^+$ T_{mem} when compared to systemic infection using the same viral dose (Figure 3.1A, left) (141). In addition to the observation of lower frequencies, IN infection also resulted in a numerically deficient $CD8^+$ T_{mem} pool, in sites both proximal (lung) and distal (spleen) to the respiratory tract (Figure 3.1A, right). Thus, in a system where an identical pathogen is used, intranasal infection resulted in a quantitatively incomplete memory cell pool.

The size of the memory $CD8^+$ T cell pool is often correlated with the overall size of the corresponding effector population (215). Given that the lung has multiple barriers that may preclude infection (as compared to injecting a pathogen into the blood stream), and that the mucosa of the lung may promote immune tolerance (91), we speculated that

the effector $CD8^+$ T cell (T_{eff}) response may be numerically suppressed following respiratory infection. To test this, we assessed the early effector $CD8^+$ T cell responses to IV and IN VSV infection to determine whether the deficiency in memory was a direct result of a poor T_{eff} response.

Surprisingly, the quantitative deficiency of IN derived N-tet^+ $CD8^+$ T_{mem} was in direct contrast to what was observed at the peak of the antigen-specific $CD8^+$ T cell response, where the IN derived $CD8^+$ T cells were numerically more prominent (Figure 3.1B-F). Throughout the early-peak $CD8^+$ T cell response to infection (ranging from 7-9 dpi) IN infection resulted in overall higher levels of $CD8^+$ T cell activation in the blood, as assessed by CD44 expression, as well as higher levels of VSV-specific N-tet^+ $CD8^+$ T cells (Figure 3.1B, left and right panels, respectively). A higher $CD8^+$ T cell effector response was also confirmed across multiple tissues at 8 dpi, including respiratory and respiratory-proximal sites, the lung and the MdLN, as well as peripheral sites, such as the spleen and inguinal lymph nodes (ILNs) based on CD44 expression (Figure 3.1C-D) and antigen-specific N-tet^+ $CD8^+$ T cell responses (Figure 3.1E-F). These data show that the reduced T_{mem} response was not due to a reduced T_{eff} cell response following respiratory infection.

Intranasally derived VSV-specific CD8+ T cells display reduced conversion to memory during the contraction phase of the immune response

Intranasal VSV infection results in a higher peak effector $CD8^+$ T cell response, yet a quantitatively decreased memory pool. This indicates that respiratory derived $CD8^+$ T cells either transition to memory poorly, and at a lower frequency than their systemically-derived counterparts, or they are not maintained following development. To

test the former of these two possible scenarios we monitored the antigen-specific responses throughout the contraction phase of the $CD8^+$ T cell response (8-15 dpi), to determine if memory development was impaired following respiratory infection with VSV.

Comparison of the kinetics of contraction between IN and IV VSV-infected mice indicated that antigen-specific $CD8^+$ T cells that develop following IN infection are lost more rapidly during the contraction phase in all analyzed tissues, between 8 and 15 dpi (Figure 3.2). VSV-specific $CD8^+$ T cell levels normalize between both of the routes of infection by 12 dpi (indicating a more rapid loss, as can be seen by the slopes of the lines), and are deficient in most of the tissues of IN infected animals by 15 dpi. The only exception was the MdLN, which continued to harbor higher numbers of VSV-specific CD8 T cells after IN infection. This in part was not surprising as this location would be a dominant site of priming after IN infection whereas priming sites would be diffuse after IV infection. Interestingly, in the IV infected group, the total number of N-tet^+CD8^+ T cells is stable between 12-15 dpi, where numbers of cells continue to decrease between 12-15 dpi in the IN infected animals, even in the MdLN (Figure 3.2C). Together, the rapid and sustained loss of antigen-specific $CD8^+$ T cells during the contraction phase after IN VSV infection indicated that perhaps $CD8^+$ T cells derived from this infection route possessed an inferior ability to transition into long-lived memory cells, a characteristic which is essential for survival of the cell through contraction.

Respiratory infection generates anti-viral CD8 T cells which lack the phenotypic characteristics of classical memory cells derived from systemic infection

One of the key transcription factors identified in promoting memory cell development is eomesodermin (Eomes) (216). Eomes-deficient $CD8^+$ T cells undergo primary clonal expansion but are defective in long-term survival (217). As respiratory derived N-tet^+CD8^+ T cells expanded normally, yet developed substandard memory responses, we tested the hypothesis that N-tet^+ $CD8^+$ T_{eff} derived from a respiratory infection fail to initiate Eomes expression and the subsequent memory cell programming conferred by this transcription factor. As early as 6 dpi IN-derived N-tet^+CD8^+ T cells isolated from the lung and spleen expressed less Eomes than those Ag-specific cells derived from systemic infection (Figure 3.3A). This deficiency in Eomes expression was exacerbated and reduced to levels of 25-50% of T_{mem} derived following systemic infection by 35 dpi (Figure 3.3B). These data indicated that a known initiator of memory cell programming was considerably less in T_{mem} cells which are also numerically reduced after respiratory infection.

One of the downstream targets of Eomes is CD122 (130) which is classically expressed at high levels on memory cells and confers IL-15 reactivity which is necessary for the maintenance of memory cells following systemic infection (136, 137). In contrast, IL-15 is dispensable for the development of memory following respiratory infections (141). As Eomes expression was higher on N-tet^+ $CD8^+$ T cells following IV infection compared to IN infection, we assessed whether the IV memory cell program is responsible for the differences in IL-15 dependency between infection routes. Indeed, the level of CD122 expression on the memory cells that developed following IN VSV

infection was greater than those derived from IV infection. The proportion of N-tet^{+} CD8^{+} T_{mem} cells that express CD122 following IN infection was reduced in both the spleen and the lung, compared to IV VSV infection (Figure 3.4C). This loss in CD122^{hi} cells is even more apparent when overall numbers of CD122^{hi} cells were quantified (Figure 3.4D). The reduction in Eomes and CD122 expression, along with the more significant contraction of CD8+T_{effs} indicate that early signals following IN infection result in a numerically deficient CD8+ T_{mem} pool.

Anti-viral effector CD8 T cells derived from respiratory infection do not fully transition to memory precursor cells

At the proliferative peak of the anti-viral CD8 T cell response, T_{mem} can be identified within the effector cell pool using the IL-7 receptor alpha chain (CD127) and killer-cell lectin like receptor G1 (KLRG1) (115, 116, 125). These markers have been used extensively in CD8^{+} T cell memory studies and can predict which cells will survive the contraction phase, especially in the context of systemic viral infections (115, 125). Memory precursor cells or MPECs are CD127^{hi}KLRG1^{lo} and will dominate the antigen-specific CD8 T cell pool over time based on their enhanced survival conferred by IL-7 (116, 164). These MPECs differentiate from ancestral clones, referred to as early effector cells (EEC, CD127^{lo}KRLG-1^{lo}). EECs have the greatest developmental plasticity, with the potential to develop into any of other phenotypes (129), but are generally thought not to persist into memory due to their lack of CD127 expression. Short-lived effector cells or SLECs (CD127^{lo}KLRG1^{hi}) constitute the majority of the early anti-viral CD8+ T cell responses during systemic infection (125) yet are terminally differentiated and lost during CD8^{+} T cell contraction (116). Since respiratory-derived Ag-specific CD8^{+} T cells

become activated, but do not appear to phenotypically or quantitatively match their systemically derived counterparts, we sought to determine whether memory cell differentiation was stalled following IN infection, aborting the development of MPECs and affecting T_{mem} development.

To this end, we monitored the emergence and persistence of the aforementioned effector $CD8^+$ T cell phenotypes in the blood following IN and IV VSV infection using CD127 and KLRG1 expression. Early after IV infection (Figure 3.4A), both SLEC and EEC N-tet^+ cells predominated until ~11 dpi when MPECs surpassed these subsets as the dominate phenotype. The survival advantage of these MPECs is very apparent by 50 dpi where this subset prevails. In contrast, N-tet^+ effector cells derived from an IN infection (Figure 3.4B) harbor predominately EECs with sustained persistence compared to IV infection. Moreover, MPECs do not emerge as the dominant subset until ~15 dpi. Direct comparison of the composition of the effector pools between the two routes of infection at 12 dpi highlight the conclusion that the prolonged frequency of EECs observed after IN infection is largely at the expense of the generation of SLECs and MPECs (Figure 3.4C). Furthermore, the pattern of effector $CD8^+$ T cell distribution following respiratory VSV infection is similar to that observed following influenza infection, where EECs can be observed for a sustained period of time in the blood (Figure 3.4D). These data, combined with the earlier emergence of MPECs after IV infection, has consequences for T_{mem} development as MPECs have a survival advantage due to expression of CD127 (116), which could explain the numerical difference of T_{mem} observed between IV and IN infection. The inability of the enhanced numbers of EECs to transition to MPECs as efficiently after IN infection could mechanistically explain the steep and persistent

decline of $CD8^+$ T cells during contraction resulting in numerically reduced CD8+ T_{mem} populations.

We next sought to confirm whether the anti-viral T_{eff} /developing T_{mem} in the tissues were equally impacted by route of infection. Thus we monitored the kinetics of the appearance and contribution of the MPECs and other $T_{eff\ subsets}$ to the overall pool in the lung, spleen and MdLN at 8, 12 and 15 dpi (Figure 3.5, Table 3.1). At 8 dpi, SLECs dominate the IV derived N-tet^+ $CD8^+$ T cell response, totaling up to 50% of the overall antigen-specific T_{eff} pool. Importantly, and similar to the blood, significantly higher EECs are observed in all tissues examined following IN infection (~50% or more of the VSV-specific CD8 T cells). Notably, this is not due to a loss of SLEC development following respiratory infection, as the size of the overall SLEC compartment is similar, or quantitatively greater, in IN compared to IV infection (Figure 3.5B, 5C and Table 3.2). Through the contraction phase of the $CD8^+$ T cell response, EECs continue to make up a significant portion of the IN-derived N-tet^+ $CD8^+$ T cells, resulting in a smaller frequency of these cells having an MPEC phenotype (Figure 3.5C). Furthermore, while the overall numbers of MPECs remain somewhat stable through the contraction following IV infection, there is a loss of this population following IN infection (Figure 3.5D). These data would suggest that MPECs arise earlier after systemic IV infection whereas antigen-specific T_{eff} derived from IN infection develop EEC more frequently and spend an extended period of time as this early stage of development. Furthermore, this prolonged EEC representation after both respiratory VSV and influenza infection suggests a common developmental pathway exists for respiratory-derived $CD8^+$ T cells where

sustained effector cell generation may come at the expense of the development of memory.

Prolonged antigen exposure does not bias the development of Te_{ff} subsets

The sustained prevalence of cells expressing neither CD127 or KLRG1 (EECs) could be due to multiple factors, one of which could be the continued activation and generation of new CD8 T_{eff} bearing the EEC phenotype. Indeed, following respiratory infection with viral pathogens, including influenza and VSV, antigen was shown to persist after the clearance of replicating virus, up to ~30 dpi (218, 219). Thus, we examined whether the sustained contribution of EEC to the overall $T_{eff\ pool}$ and failure to fully launch the T_{mem} program was solely the consequence of prolonged antigen exposure and sustained T cell activation after respiratory infection. We choose to functionally assess antigen persistence by adoptively transferring CFSE labeled naïve OVA-specific $CD8^+$ T cells into cohorts of mice following IV and IN VSV-OVA infection at 12 dpi and monitoring the proliferation of these antigen-specific cells. This period of measurement was chosen based on the differential development of EEC derived after either respiratory infection of either parental VSV (Figure 3.3) or recombinant VSV expressing OVA (Supplemental Figure 3.1). Five days after transfer into either IN infected, IV infected or control infected animals proliferation of the OT-I cells was assessed by CFSE dilution (Figure 3.6). Overall, the percentages of OT-I cells that divided were similar between infection route in most tissues, and significantly above infection matched controls (VSV not expressing OVA) (Figure 3.6). However, increased levels of proliferation were observed in the MdLN following respiratory VSV infection. Since the transferred OT-I cells in this experiment were identical, this would suggest that the OT-I cells in the IN

cohort may either interact with antigen earlier or for an extended period of time. In either case, the data obtained in this experiment indicates that following both IN and IV infections, $CD8^+$ T cells continue to contact antigen through the contraction phase of the response eliminating the possibility that sustained antigen alone is driving the development and maintenance of EECs after IN infection.

Respiratory-derived $CD103^+$ dendritic cells uniquely participate in lymph node priming after intranasal infection

Our data thus far indicate that early events following respiratory (*vs.* systemic) infection results in a different developmental program, ultimately resulting in numerically deficient $CD8^+$ T cell responses. By using an identical pathogen (VSV) in our studies, we have eliminated pathogen-specific pattern recognition receptor bias and the events underlying disparate PRR signaling pathways. However, mucosal sites harbor many unique pools of resident and migratory dendritic cells (DCs) which could influence downstream $CD8^+$ T cell responses. In the case of the priming after respiratory infection, naïve $CD8^+$ T must contact antigen in draining LNs and thus requires the involvement of a network of highly specialized DCs with migratory and cross-presentation capabilities. Pulmonary migratory DCs classically fall into two subsets, $CD103^+$ DCs and $CD11b^+$ DCs, both important for antigen presentation in draining LNs (48). LN resident DCs ($CD8\alpha^+$) have also been shown to be important for naïve T cell priming following influenza infection, acquiring antigen from other DCs in the LN (50). Recently, $CD103^+$ DCs have been shown to strongly activate naïve $CD8^+$ T cells, driving effector functions (144). Therefore, we hypothesized that the preferential activation by respiratory $CD103^+$

DCs following IN infection may facilitate the observed increases of effector frequency of these cells, while resulting in a delayed and substandard transition to memory.

To first assess if $CD103^+$ respiratory DCs were activated and migrating to LNs following respiratory VSV infection, populations of DCs were defined in secondary lymphoid organs at 3 days following IV and IN infection. DCs were defined as lineage-negative (Lin^-) cells ($CD3\square^-$, $CD19^-$, $NK.1.1^-$) expressing CD11c and MHC II (Figure 3.7A). As observed following influenza infection (66), IN infection with VSV resulted in the accumulation of significant numbers of DCs in the respiratory tract draining LNs (MdLN and cervical (cLNs)). Higher numbers of DCs were also observed in the peripheral LNs, the brachial (bLNs), and iLNs, although to a lesser extent. Furthermore, and specifically in the respiratory draining lymph nodes following IN VSV infection, a significant number of these cells expressed CD103, indicating that they may be migratory respiratory-tract derived DCs. A significant proportion of $CD103^+$ populations of cells were identified in the MLN, as previously described (220), but their frequency did not vary between infection routes. As $CD103^+$ DCs have been implicated in the differentiation of effector cells, (144), it is possible that the accumulation of this subset of DCs following respiratory infection, imparts effector $CD8^+$ T cells with an inferior ability to transition into long lasting memory cells. Intriguingly, however, a large proportion of these $CD103^+$ DCs also expressed CD11b, and had lower levels of MHC II expression (Figure 3.7C). While it has been reported that $CD11b^+$ DCs can express CD103 upon inflammation (221), and $CD103^+CD11b^+$ DCs have been described in the lamina propria of the gut (222), the lower level of MHC II expression indicates that these cells may not originate in the respiratory tract, but are instead derived from circulating monocyte

precursors (222). Further phenotypic analysis of these cells will aim to distinguish between these possibilities, and determine if a novel DC subset may be responsible for the substandard development of memory cells following respiratory infection.

Overall the data presented in this chapter shows that there is a common developmental pathway for memory $CD8^+$ T cells generated following respiratory infection. This pathway differs from the archetypes established in systemic models of infection, and offers an explanation as to why memory $CD8^+$ T cell development following vaccines and natural infections is neither as robust nor long lasting as those observed in systemic models of infection.

3.5 Discussion:

Systemic infection models have dominated the field of $CD8^+$ T cell memory development since its infancy (223). This is likely due to the ability to consistently produce large, traceable pools of T_{mem} using acute, viral, systemic infections (224). However, neither vaccines nor most naturally transmitted infections are acquired through an IV route. Thus the "ideal" $CD8^+$ T_{mem} derived from these models may be quite different than those generated via physiologically relevant routes. Indeed, Mueller et al. showed that infection via a mucosal route (IN influenza infection) results in qualitatively deficient memory $CD8^+$ T cells with reduced protective capacity compared to those acquired via a systemic route (IV LCMV) (212). How the respiratory environment regulates this response was unclear in these studies as distinct inflammatory and cytokine profiles elicited by the divergent priming viruses could not be eliminated as confounding variables. By using VSV infection as a model system, we directly tested the impact of the

respiratory environment on the developmental pathways responsible for anti-viral $CD8^+$ T cell development.

In our study, respiratory VSV infection recapitulated many features of anti-viral CD8 T cells derived from a native influenza infection, including the generation of a robust effector cell pool yet reduced frequencies of T_{mem} (Figure 3.1). The rate of attrition was most pronounced immediately after the peak number of $CD8^+$ T_{eff} were detected in all tissues (Figure 3.2) which led us to speculate that respiratory infection may differentially program anti-viral $CD8^+$ T cells in a way that favors short over long-term protection. Indeed, EECs which maintain the plasticity to differentiate into either MPECs or SLECs (129) were enriched and selectively maintained within the antigen-specific $CD8^+$ T cell pool after respiratory VSV infection, where they constituted ~ 1/3 of the antigen-specific response out to 15 dpi (Figures 3.4 and 3.5). The sustained EEC phenotype is also observed following influenza infection (Figure 3.4D), providing evidence that infection via the respiratory route may uniformly contribute to a developmental stall, preventing full transition to memory. Indeed, the sustained population of EECs generated following IN infection came at the expense of generating MPECs (Figure 3.4C and 3.4D) and correlated with the timing of the greatest loss of the IN specific $CD8^+$ T cells (Figure 3.2). Prior to our study it was unclear whether EEC maintained beyond contraction could convert to MPECs; our data suggests that they do not, however this remains a possibility. Nonetheless, the delayed appearance of $CD127^+$ (MPECs) after respiratory infection likely accounted for the greater loss of anti-viral $CD8^+$ T cells during contraction.

Limitations on T_{mem} in the respiratory tract makes teleological sense given the plethora of respiratory assaults an individual encounters over its lifetime and the limited space to harbor accumulating T_{mem} without compromising tissue function. However, reductions in T_{mem} were also observed in the spleen (Figure 3.1A &B), suggesting T_{mem} development was not selectively suppressed in the respiratory tract when exposure was by the respiratory route. Taking this into account, it is quite possible that while respiratory infection does not support T_{mem} development to the extent observed in systemic infection, certain subsets of T_{mem} may either selectively develop or remain unaffected by any respiratory derived developmental restrictions. Supporting this possibility, respiratory VSV infection resulted in lower expression of Eomes, as well as the Eomes-regulated IL-15 receptor, CD122 (Figure 3.4). Eomes expression is key factor relevant to maintaining systemically derived CD8 T_{mem} cells, partially due to its ability to up-regulate CD122 expression (130). However, we have previously shown that IL-15 is not required for the generation and maintenance of respiratory-derived $CD8^+$ T cells (141). Therefore, respiratory viral infections may favor memory cells which are IL-15 independent. Intriguingly, tissue resident memory cells (T_{RM}), defending in mucosal sites such as the lung express less CD122 than other T_{mem} subsets (225). Additionally a subset of T_{RM} isolated from the LNs of mice was found to develop independent of IL-15 signaling as well (226). Therefore, $CD8^+$ T cell programming after respiratory infection may favor the development of specific subsets of T_{mem}, many of which will provide protection at the site of infection (perhaps with reduced longevity), over large pools of "classical" memory cells. Since the role of IL-15 in $CD8^+$ T_{mem} generation and maintenance has not been well studied after oral or intra-vaginal infection it is impossible

to say if the respiratory environment exclusively provokes identical changes in T_{mem} derived from other mucosal immunizations.

The difference in T_{mem} programming after respiratory infection is likely associated with events occurring very early after infection as VSV is a promiscuous virus (227), eventually resulting in a brief systemic infection, even after IN delivery (228). Respiratory-resident $CD103^+$ DCs are well positioned to contact antigen early, are enriched in mucosal surfaces, and particularly important in the priming of naïve $CD8^+$ T cells after influenza infection (50, 66). Moreover, antigen-laden $CD103^+$ DCs begin to migrate to lung draining LNs as early as 6 hours post infection (68), resulting in peak priming ~3 dpi (50). Given that these DCs can influence effector cell differentiation and migration (144), we hypothesized that these migratory $CD103^+$ DCs were also responsible for the altered developmental phenotypes observed following intranasal infection. On first glance our data seemed to confirm this hypothesis, as $CD103^+$ T cells were specifically enriched in the respiratory tract draining lymph nodes following IN infection (Figure 3.7). However, these $CD103^+$ DCs expressed markers not typically associated with classic respiratory $CD103^+$ DCs including $CD11b^{hi}$ and low expression of MHC II, a phenotype more indicative of a monocyte-derived $CD103^+$ population of DCs (229). This DC phenotype is not observed after IV infection. Since many classical tissue-resident $CD103^+$ will die in the lung-draining LN after priming $CD8^+$ T cells (222), perhaps this newly recruited $CD103^+CD11b^{hi}$ pool not only develops to replace the tissue-resident $CD103^+$ DCs, but also participates in shaping the CD8 T_{mem} program. Whether the bona-fide tissue resident $CD103^+$ DCs or the monocyte derived $CD103^+$ DCs either support the altered the modified T_{mem} development or suppress the

development of classical $CD8^+$ T_{mem} through mechanisms including selective, sustained antigen presentation or modified co-stimulation are areas under active investigation. By comparing VSV infection delivered by the IN or IV route, we showed that the respiratory environment results in T_{mem} that is skewed from the archetypical memory developmental programs defined in systemic models of infection, resulting in numerically deficient memory (Figure 3.8). The implications of this work suggest that the induction of protective memory $CD8^+$ T cells should be studied in the context of appropriate infection route, as the developmental pathways and requirements for memory vary between routes of priming. As there continues to be a growing interest in developing CD8+ T cell based vaccines (particularly those which will induce respiratory specific responses)(13), it is imperative that we continue to improve our understanding regarding the mechanism of how the respiratory environment modifies T_{mem}. Fine tuning of the local respiratory environment via targeting specific DC pools or perhaps induction of responses via other routes mucosal infection may be necessary to secure the desired T_{mem} outcome.

3.6 Acknowledgements:

We would like to thank Dr. R. Tripp for access to the LSR II and David Rose for assistance with experiments.

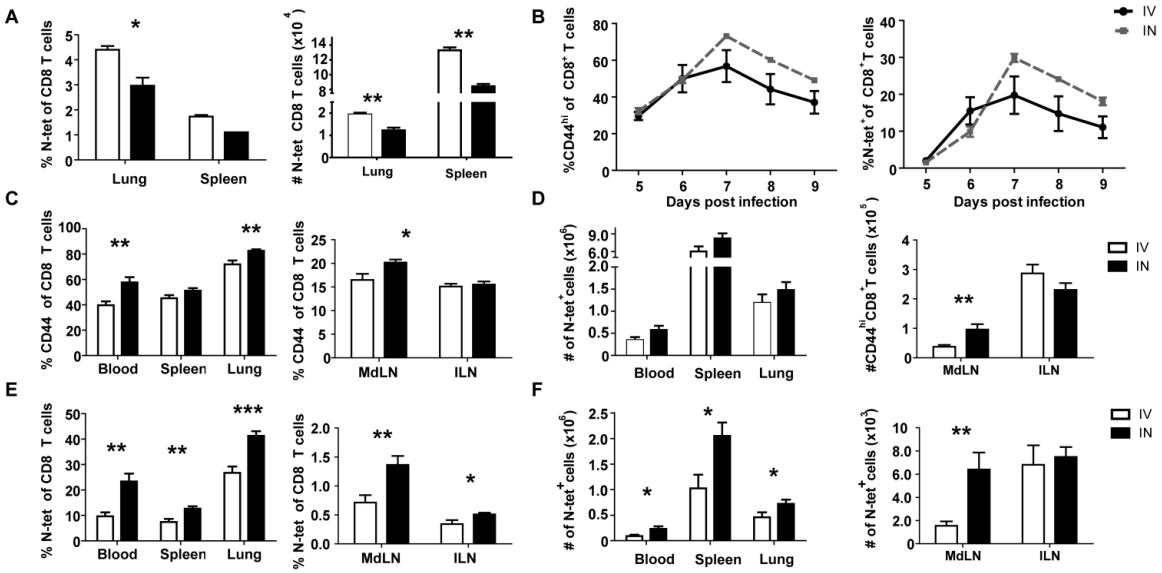

A
% N-tet of CD8 T cells
Lung
Spleen
N-tet CD8 T cells (x10 4)
B
%CD44hi of CD8+ T cells
Days post infection
%N-tet+ of CD8+ T cells
IV
IN
C
% CD44 of CD8 T cells
Blood
Spleen
Lung
MdLN
ILN
D
# of N-tet+cells (x10 6)
#CD44hiCD8+T cells (x10 5)
E
% N-tet of CD8 T cells
F
# of N-tet+cells (x10 6)
# of N-tet+cells (x10 5)

Figure 3.1. Respiratory infection results in quantitatively deficient memory pools, despite higher effector responses.

A) Mice were infected IN or IV with 10^4 pfu VSV and N-tet$^+$ CD8$^+$ T cell responses were assessed at 35 days post infection (dpi) and displayed as frequency of the CD8$^+$ T cell pool (left) and as total number (right); n=3 mice/group, experiment is representative of 3 independent experiments. B) Mice were infected IN or IV with 10^4 pfu VSV and CD8+ T cell responses were monitored in the blood between 5-9 dpi. Data are displayed as frequency of CD44^{hi} of CD8+ T cells (left) and frequency of N-tet$^+$ T cells (right); n=3 mice/group, data is representative of 2 independent experiments. C-F) Mice were infected with 10^4 pfu VSV by the IN or IV route and lymphocytes from the blood, spleen, lung, MdLN and iLNs were assessed for CD44 expression and N-tet reactivity in indicated tissues and displayed as total frequency of CD8$^+$ T cells (C+E, respectively) and quantified (D+ F); n=10 mice/group and data are representative of three independent experiments. Significance between groups was assessed using a two-tailed student t-test (*p=<0.05, **=p<0.01, ***=p<0.001).

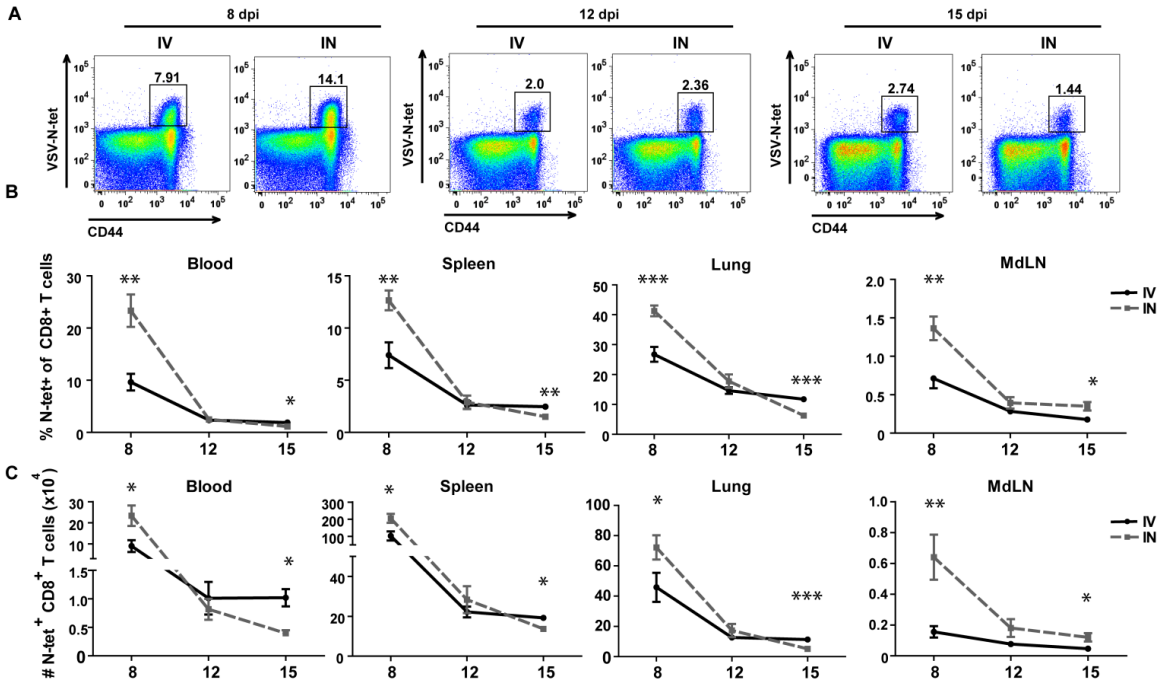

A
8 dpi
12 dpi
15 dpi
IV
IN
7.91
14.1
2.0
2.36
2.74
1.44
VSV-N-tet
CD44
B
Blood
Spleen
Lung
MdLN
% N-tet+ of CD8+ T cells
IV
IN
C
N-tet+ CD8+ T cells (x10^4)

Figure 3.2. Respiratory-derived $CD8^+$ T cell responses contract more rapidly, and to a greater extent, than those derived from systemic infection.

A) Representative flow plots of splenic lymphocytes isolated from mice infected with 10^4 VSV IN or IV, and sacrificed at 8, 12, and 15 dpi. Plots show populations previously gated on $CD8^+$ lymphocytes. B) Frequencies of VSV-N-tet^+ cells of $CD8^+$ T cells isolated from the Blood, Spleen, Lung, and MdLN at 8, 12 and 15 dpi. C) Total numbers of VSV-N-tet^+ $CD8^+$ T cells isolated from the indicated issues; n=5-10 mice/group. Data are representative of three independent experiments. Significance between groups was assessed using a two-tailed student t-test (*p=<0.05, **=p<0.01, ***=p<0.001).

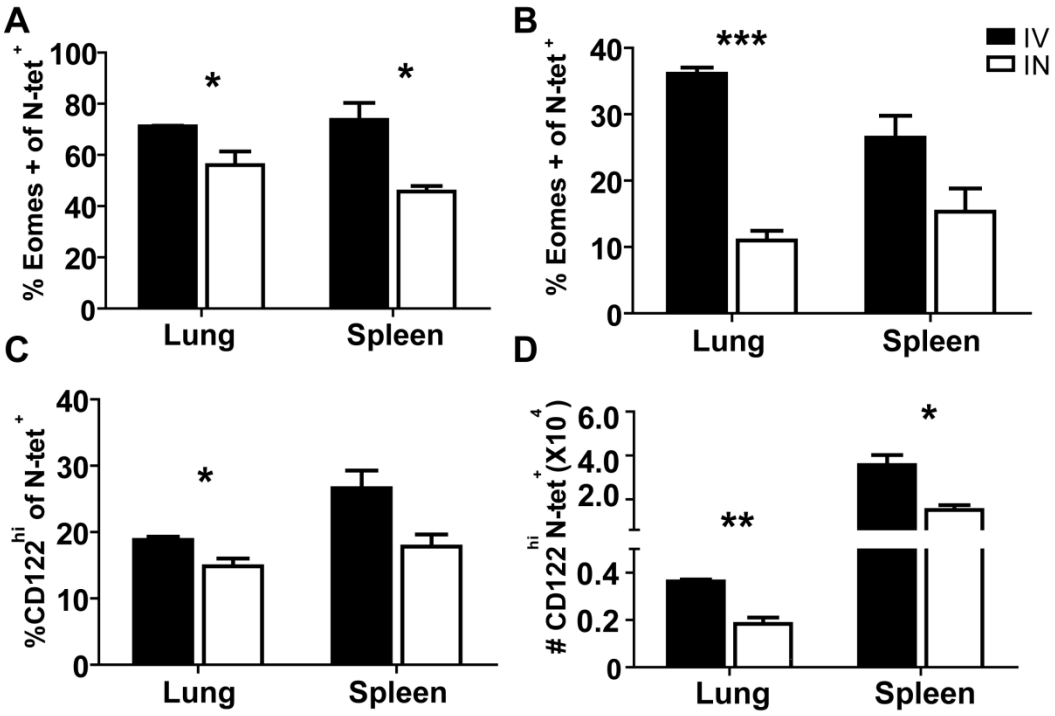

A
% Eomes + of N-tet+
100
80
60
40
20
0
*
*
Lung
Spleen
B
% Eomes + of N-tet+
40
30
20
10
0
***
IV
IN
Lung
Spleen
C
%CD122hi of N-tet+
40
30
20
10
0
*
Lung
Spleen
D
CD122hi N-tet+ (X10 4)
6.0
4.0
2.0
0.4
0.2
0
**
*
Lung
Spleen

Figure 3.3. Respiratory infection results in N-tet+ CD8+ T cells that have reduced Eomes and CD122 expression.

For all panels mice were infected by the IV route (black) or the IN route (white). A & B) Frequency of Eomes+ N-tet+ CD8+ T cells was assessed by intracellular staining at 6 (A) and at 35 dpi (B) (n=3mice/group, data are representative of 3 independent experiments). C&D) Frequency (C) and number (D) of $CD122^{hi}$ of N-tet+CD8+ T cells was assessed at 35 days post infection (n=3mice/group, data are representative of 3 independent experiments). Significance between groups was assessed using a two-tailed student t-test (*p=<0.05, **=p<0.01, ***=p<0.001).

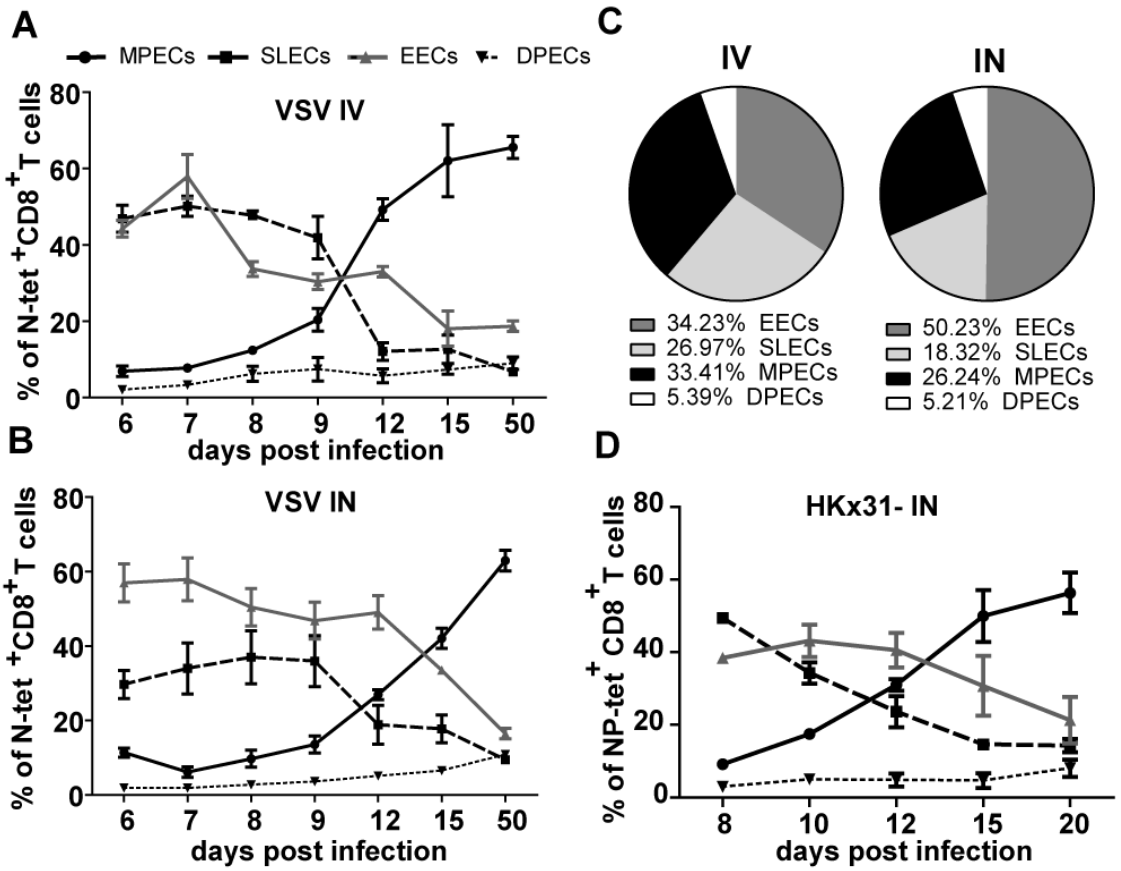

A
MPECs
SLECs
EECs
DPECs
VSV IV
% of N-tet +CD8+ T cells
days post infection
B
VSV IN
% of N-tet +CD8+ T cells
days post infection
C
IV
IN
34.23% EECs
26.97% SLECs
33.41% MPECs
5.39% DPECs
50.23% EECs
18.32% SLECs
26.24% MPECs
5.21% DPECs
D
HKx31- IN
% of NP-tet + CD8 + T cells
days post infection

Figure 3.4. Respiratory infection results in skewed populations of effector cells based on CD127 and KLRG1 expression.

VSV-N-specific CD8+ T cell responses were measured in the blood over time, populations were assessed for their expression of CD127 and KLRG1 and identified as previously defined effector/ memory cell subsets using these markers. Phenotypes after (A) IV-VSV infection (B) IN-VSV infection and C) pie charts of comparing subset representation at 12 dpi between IV (left) and IN (right) infected animals. D) Frequencies of memory phenotypes of NP-specific CD8 T cells following 10^3 pfu of Influenza A virus HKx31 in the blood at the indicated times post infection; n=at least 3mice/time point.

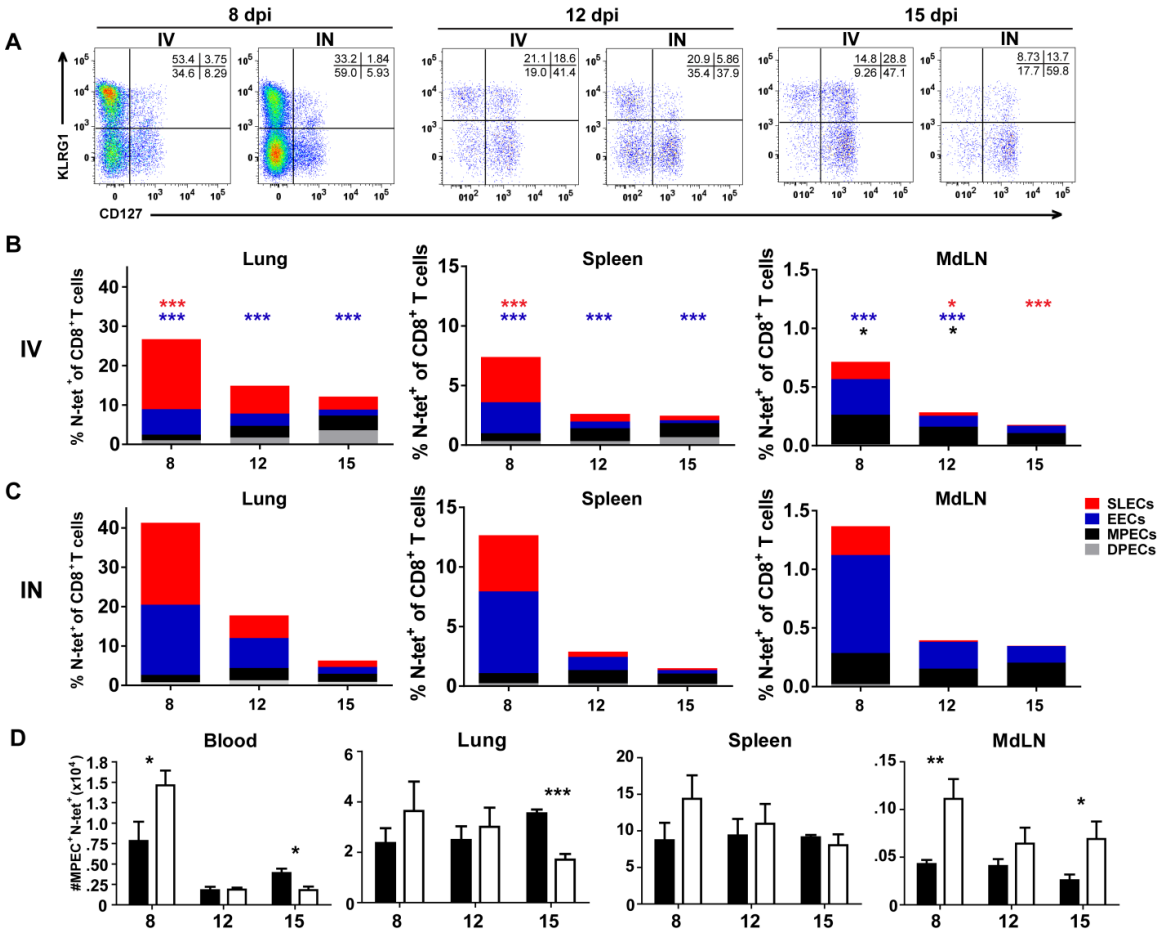

A
8 dpi
12 dpi
15 dpi
IV
IN
KLRG1
CD127
53.4 3.75 34.6 8.29
33.2 1.84 59.0 5.93
21.1 18.6 19.0 41.4
20.9 5.86 35.4 37.9
14.8 28.8 9.26 47.1
8.73 13.7 17.7 59.8
B
IV
C
IN
Lung
Spleen
MdLN
% N-tet+ of CD8+ T cells
SLECs
EECs
MPECs
DPECs
D
Blood
#MPEC+ N-tet+ (x10^4)

Figure 3.5. Respiratory infection results in increased and sustained proportions of EECs in tissues, resulting in inferior memory formation

A) Representative flow plots of splenic lymphocytes following IV or IN infection with 10^4 pfu VSV delievered by the IV or IN route, at 8, 12, and 15 days post infection as indicated. Data was previously gated on $CD8^+$ lymphocytes, followed by VSV-N-specific cells. B+C) Height of bar indicates the total frequency of VSV-N-specific $CD8^+$ T cells, while the colored portions within the bar indicate the proportion of these cells which is made up of the memory phenotypes (SLECs-red, EECs-blue, MPECs-black, and DPECs-gray). Colored asterixes indicate statistical significance between the same tissue and timepoint between the IV and IN infection (bars directly above/below one another). D) Total numbers of N-tet^+ cells with an MPEC phenotype from IV (black) or IN (white) infected mice in the indicated tissues at 8, 12 and 15 days post infection; n=5-10 mice/group. Data are representative of three independent experiments. Significance between groups was assessed using a two-tailed student t-test (*p=<0.05, **=p<0.01, ***=p<0.001)

Table 3.1. Frequencies of N-tet^{+} effector cell phenotypes characterized by CD127/KLRG1 expression.

		Blood			Spleen			Lung			MdLN		
		IV	IN	p-value	IV	IN	p-value	IV	IN	pvalue	IV	IN	p-value
8	EEC	33.01	46.77	**0.0011**	35.16	35.16	**<0.0001**	24.21	43.29	**<0.0001**	42.57	61.26	**<0.0001**
	SLEC	55.09	44.08	**0.0179**	51.35	37.23	**0.0003**	66.54	50.34	**0.0001**	20.72	18.07	0.3832
	MPEC	9.33	7.51	0.2376	8.954	6.674	0.0502	5.44	4.48	0.3577	35.3	19.53	**0.0109**
	DPEC	3.95	1.62	**0.0934**	4.531	1.973	**0.0004**	3.81	1.90	**<0.0001**	1.665	1.148	0.1493
12	EEC	30.44	45.52	**0.0123**	21.5	38.56	**0.0006**	21.50	42.94	**0.0005**	32.52	57.92	**0.0051**
	SLEC	40.64	24.54	0.1086	24.15	14.94	0.143	46.38	32.32	0.0771	10.51	3.19	**0.0119**
	MPEC	21.92	25.10	0.6805	41.76	38.38	0.6684	20.16	17.48	0.6038	54.4	38.38	**0.0368**
	DPEC	7.01	7.01	0.2914	12.57	8.12	0.2075	11.97	11.97	0.093	2.586	0.478	**0.015**
15	EEC	13.72	20.18	**0.0346**	9.142	19.33	**<0.0001**	9.52	27.18	**<0.0001**	34.8	40.45	0.2512
	SLEC	24.82	18.10	**0.0391**	15.86	10.58	0.0918	28.26	25.80	0.5723	4.568	0.763	**0.0002**
	MPEC	38.46	43.83	0.4106	47.82	57.78	0.0894	31.64	33.25	0.7042	55.12	57.2	0.6939
	DPEC	8.38	13.25	0.0525	27.18	12.31	**0.0024**	30.58	13.81	**0.0006**	5.26	1.327	**0.0205**

Table 3.2. Quantification of N-tet^{+} effector cell phenotypes characterized by CD127/KLRG1 expression.

		Blood/ml			Spleen			Lung			MdLN		
		IV	IN	p-value	IV	IN	p-value	IV	IN	p-value	IV	IN	p-value
8	EEC	3.06E+04	1.13E+05	**0.0137**	3.72E+05	1.11E+06	**0.0011**	1.12E+05	3.12E+05	**0.0007**	7.22E+02	3.98E+03	**0.0026**
	SLEC	4.91E+04	1.02E+05	**0.0494**	5.26E+05	7.51E+05	0.1646	3.06E+05	3.59E+05	0.4628	3.82E+02	1.23E+03	**0.0305**
	MPEC	7.81E+03	1.46E+04	**0.0348**	8.72E+04	1.44E+05	0.1733	2.36E+04	3.63E+04	0.3446	4.28E+02	1.11E+03	**0.0046**
	DPEC	2.88E+03	3.43E+03	0.5865	3.95E+04	4.14E+04	0.8444	1.77E+04	1.50E+04	0.6333	2.86E+01	2.86E+01	0.0772
12	EEC	2.87E+03	3.86E+03	0.468	4.83E+04	4.83E+04	0.086	2.68E+04	7.38E+04	**0.0428**	2.58E+02	2.58E+02	0.0701
	SLEC	4.64E+03	2.12E+03	0.1804	5.27E+04	5.27E+04	0.5607	5.99E+04	5.82E+04	0.9359	7.73E+01	7.29E+01	0.9134
	MPEC	1.79E+03	1.85E+03	0.9053	9.38E+04	1.10E+05	0.6617	2.48E+04	3.01E+04	0.5922	4.07E+02	6.42E+02	0.2338
	DPEC	8.00E+02	8.00E+02	0.1933	2.70E+04	1.87E+04	0.1898	1.50E+04	1.06E+04	0.144	1.83E+01	1.19E+01	0.406
15	EEC	1.40E+03	8.02E+02	0.0817	1.72E+04	2.63E+04	**0.0074**	1.07E+04	1.39E+04	0.1243	1.58E+02	4.85E+02	**0.018**
	SLEC	2.52E+03	7.15E+02	**0.0058**	3.07E+04	1.40E+04	**0.0144**	3.25E+04	3.25E+04	**0.0065**	2.15E+01	8.30E+00	0.0684
	MPEC	3.88E+03	1.80E+03	**0.0246**	9.10E+04	8.05E+04	0.4622	3.54E+04	1.71E+04	**0.0002**	2.59E+02	2.59E+02	**0.0428**
	DPEC	2.40E+03	4.17E+02	**0.0052**	5.29E+04	1.60E+04	**0.0028**	3.46E+04	7.07E+03	**0.0001**	2.15E+01	1.33E+01	0.0832

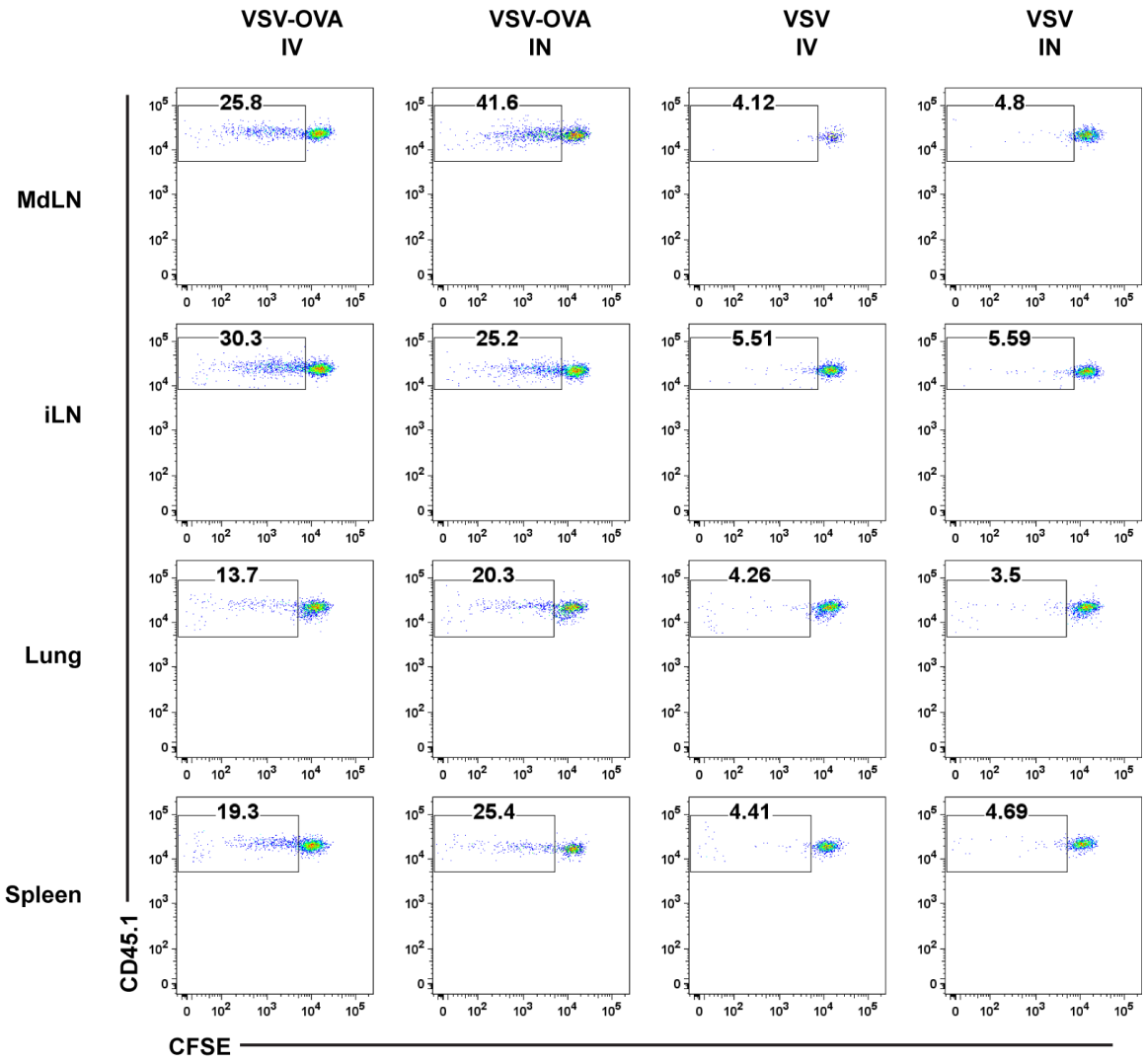

VSV-OVA IV
VSV-OVA IN
VSV IV
VSV IN
MdLN
iLN
Lung
Spleen
25.8
41.6
4.12
4.8
30.3
25.2
5.51
5.59
13.7
20.3
4.26
3.5
19.3
25.4
4.41
4.69
CD45.1
CFSE

Figure 3.6. Antigen persists through contraction following both IV and IN infection, resulting in proliferation of antigen-specific cells.

CD45.2 mice were infected with 10^4 pfu of VSV-OVA, or VSV via the IV or the IN route. 12 days post infection $2x10^6$ congenically labeled (CD45.1) OVA-specific OT-I cells were CFSE-stained and transferred into the infected mice via the tail vein. 5 days following transfer of the CFSE labeled OT-I cells, lymphocytes were isolated from the indicated organs and assessed for CFSE diltuion within the $CD45.1^+$ $CD8^+$ T cells. n=4 mice/group and data is representative of 2 independent experiments.

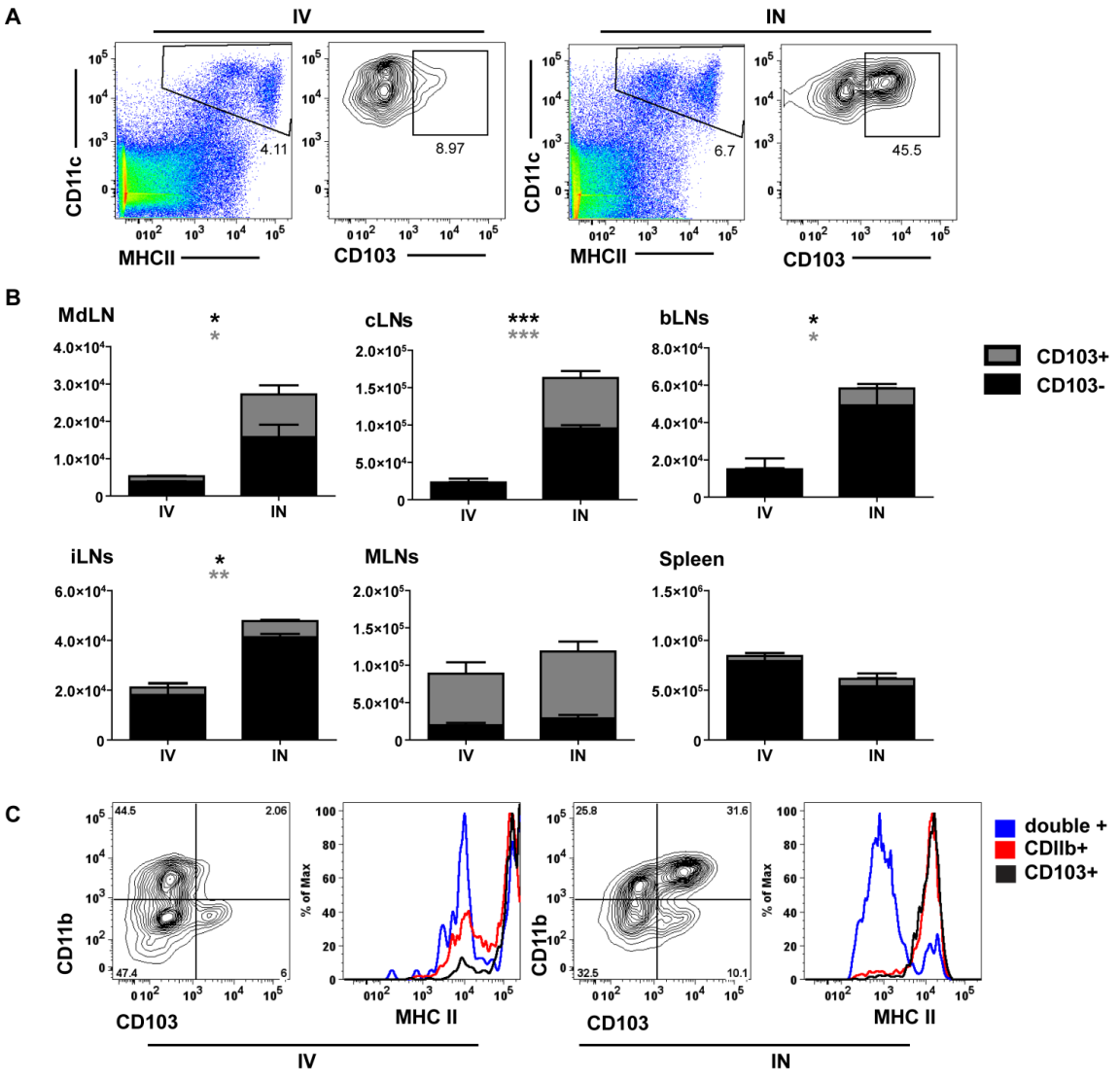

A
IV
IN
CD11c
MHCII
CD103
4.11
8.97
6.7
45.5
B
MdLN
cLNs
bLNs
iLNs
MLNs
Spleen
IV
IN
CD103+
CD103-
C
CD11b
CD103
MHC II
% of Max
44.5
2.06
47.4
6
25.8
31.6
32.5
10.1
double +
CDIIb+
CD103+

Figure 3.7. Repiratory infection results in distinct dendritic cell populations at sites of $CD8^+$ T cell priming.

A) Representative flow samples showing gating for DCs based on CD11c and MHC II (left), and for CD103 (right) expression on the gated DCs. Left panels were previously gated on single cells, followed by exclusion gating using a lineage cocktail ($CD3^-$,$CD19^-$,$NK1.1^-$ cells). B) Total numbers of DCs (total height of bar) in indicated tissues and numbers of $CD103^+$ DCs (gray portion of bar) isolated from indicated tissues. Black asterixes indicate signigicant differences between total numbers of DCs, while gray bars indicate difference between numbers of $CD103^+$ DCs. C). Phenotypes of DC polulations based on CD11b/CD103 expression (contour plots) and MHC II expression (histograms) for $CD103^+$/$CD11b^-$ DCs (black line) $CD11b^+$/$CD103^-$ (red line) and $CD103^+$/$CD11b^+$ DCs (blue line) N=3 mice/group, representative of two independent experiments.

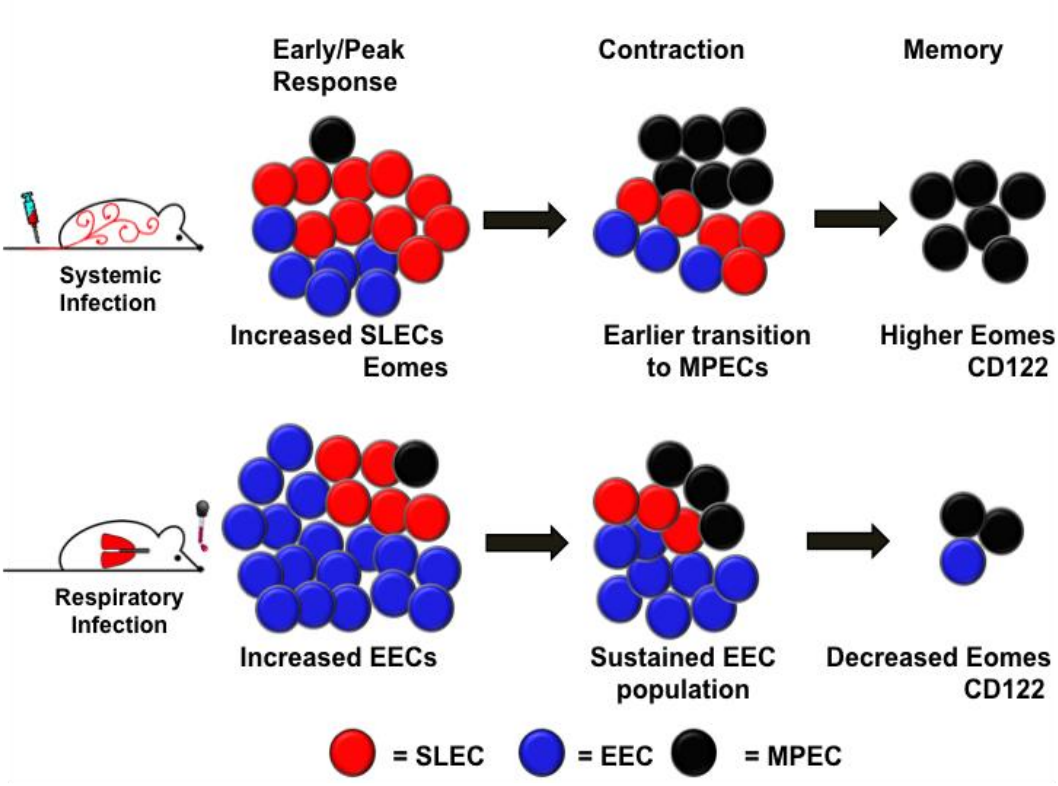
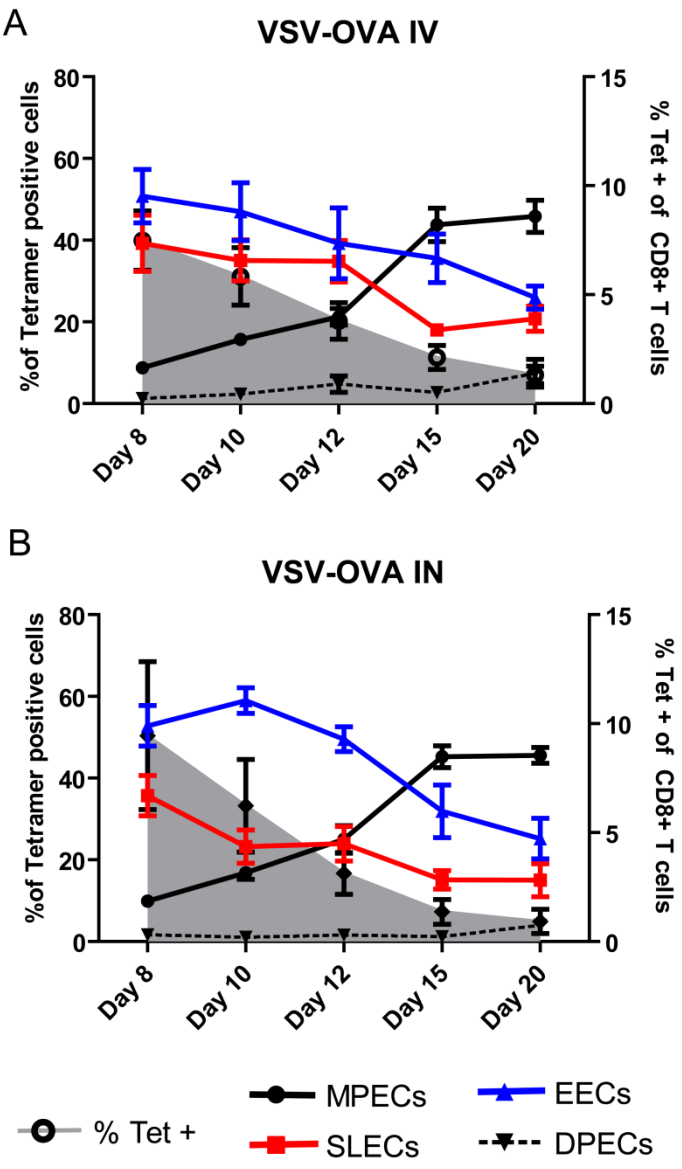

Early/Peak Response
Contraction
Memory
Systemic Infection
Increased SLECs
Eomes
Earlier transition to MPECs
Higher Eomes
CD122
Respiratory Infection
Increased EECs
Sustained EEC population
Decreased Eomes
CD122
= SLEC
= EEC
= MPEC

Figure 3.8. Respiratory infections generate a unique pool of effector CD8 T cells with altered differentiation potential. Early after infection, respiratory derived T_{eff} are generated at a greater number and favor the development of EEC over SLECs, which co-dominate after IV infection. While these stem-like EEC have the potential to develop into either MPECs, we believe that the EECs derived from respiratory infection do not fully execute the memory program resulting in cell loss. This is in part due to their decreased levels of Eomes and CD122 expression whereas systemically-derived T_{eff} are able to convert to MPECs. This rapid conversion to MPECs imparts a survival advantage resulting in enhanced Tmem after systemic infection.


A
VSV-OVA IV
%of Tetramer positive cells
% Tet + of CD8+ T cells
80
60
40
20
0
15
10
5
0
Day 8
Day 10
Day 12
Day 15
Day 20
B
VSV-OVA IN
%of Tetramer positive cells
% Tet + of CD8+ T cells
80
60
40
20
0
15
10
5
0
Day 8
Day 10
Day 12
Day 15
Day 20
% Tet +
MPECs
SLECs
EECs
DPECs

Supplemental Figure 3.1. Infection with VSV-OVA is phenotypically similar to infection with WT VSV in both IV and IN infection.

Mice were infected with 10^4 pfu VSV-OVA VSV either through the IV or IN route. OVA-specific CD8+ T cell responses were measured in the blood over time as indicated. Shaded area indicates the OVA-specific T cell frequencies and populations were assessed for their expression of CD127 and KLRG1, and designated as memory precursor phenotypes following IV-VSV-OVA infection (top) IN-VSV-OVA infection (bottom). N=3 mice/group, data is representative of two independent experiments.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

The formation of a protective pool of memory $CD8^+$ T cells following infection is simple in concept, but incredibly complex in reality, with multiple pleomorphic signals either promoting or prohibiting memory development. These signals are spatially and temporally regulated, and often act in a graded fashion, where the same signal can result in multiple outcomes depending on the level, duration, the timing of its availability, and the availability of appropriate receptors. It is the total culmination of these factors that not only decides if a particular cell will survive into memory or die, but if it is to become a memory cell, what phenotype it will exhibit and what function it will have upon recall responses (117). As our understanding of T cell memory continues to evolve, we are learning that the requirements for memory development and maintenance are quite distinct, and can vary depending on the type of infection as well as the route through which the infection is acquired; the importance of regional immune responses has become particularly evident in recent years. Here, we have investigated the impact that the respiratory environment has on the $CD8^+$ T cell response to acute viral infection, with an emphasis placed on the development and maintenance of $CD8^+$ T cell memory. We have demonstrated in this dissertation that the respiratory environment can influence memory development of $CD8^+$ T cells in ways that are distinct from systemic models of infection, where $CD8^+$ T cell development and maintenance has been primarily studied.

Immune responses in the respiratory tract must balance a very fine line between the development of appropriate effector responses and the prevention of immunopathology (230). The result of the inability to maintain this balance is apparent in certain pandemics, where normally healthy young adults have made up the greatest percentage of mortality; one such example of this is the 1918 influenza pandemic (231). It is currently believed that the majority of severe symptoms, and ultimate cause of death, in many of these individuals was due to 'inappropriate' immune responses, or the massive infiltration of cells into the lungs (230). It is not surprising then, that there are mechanisms in place which regulate immune responses at this site (91) and that these mechanisms of regulation may impact the development of memory populations.

$CD8^+$ T cells elicit the greatest levels of immunopathology when viral titers are high, or when the $CD8^+$ T cells are unable to control infection (232). This would argue that the appropriate response to respiratory infection would favor a strong, yet short-lived effector $CD8^+$ T cells at the site of infection, eliminating virus-infected cells before titers reach an uncontrollable level. Data presented here, in both Chapter 2 and Chapter 3 of this work, support the generation of a robust, but short-lived $CD8^+$ T cell responses to respiratory viral infection. In Chapter 2 we showed that the cytokine TSLP acts directly on $CD8^+$ T cells, resulting in a proliferative burst of these cells in, and proximal to, the lung and lung airways. However, due to the temporal regulation of the cytokine (Figure 2.1) as well as the cytokine receptor (174), TSLP-induced proliferation is short lived. Interestingly, signaling through the TSLP-R (or perhaps the TSLP-induced proliferation) results in memory cells that have reduced CD62L expression, or more of a T_{EM} phenotype, a population of cells that has reduced longevity compared to T_{CM} cells (205).

In Chapter 3, respiratory derived $CD8^+$ T cells displayed a greater level of activation, as well as numerically greater effector responses than their systemically derived counterparts (Figure 3.1). However, these cells contracted rapidly following day 8-post infection, becoming numerically deficient as compared to systemic infection by 15 dpi (Figure 3.2). Thus, respiratory infection seems to favor a strong, but short-lived response to infection, therefore providing a mechanism by which viral infection can be controlled early, without resulting in detrimental immunopathology.

While this regulated response may be important for limiting immunopathology in the lung following primary respiratory infection, it also seems to negatively impact the creation of long-lived protective populations of memory $CD8^+$ T cells. In murine models of influenza infection, protective $CD8^+$ T cell responses (in the lung and lung airways) are lost several months following infection (96), to a level where they can no longer provide heterosubtypic immunity (24, 26). Furthermore, we have shown that respiratory infection results in reduced pools of memory (compared to systemic), even in sites distal to the site of infection (Figure 3.1) (141), indicating that this is not just a loss of cells in the respiratory tract, but an overall quantitative defect in the development of memory cells. To attempt to understand *how* respiratory infection results in numerically deficient $CD8^+$ T cell memory pool we examined the impact that the route of infection has on memory development following respiratory infection, in direct comparison to systemic infection using a VSV model (Chapter 3). This work revealed that priming via the respiratory route led to the development of memory cells that would not be considered "ideal" as defined by models of systemic infection. The populations of cells resulting

from respiratory infection had reduced Eomes expression and CD122 expression (Figure 3.3), which classically define long lived memory cells (130, 217).

Another significant aspect of this work is the finding that phenotypes that are used to predict the generation of memory cells following systemic infections, based on the expression of CD127 and KLRG1, are not analogous following respiratory infection (Figure 3.4 & 3.5). Respiratory infection with both VSV and influenza results in elevated and sustained levels of EECs. Following systemic infection EECs are found early in the response, but rapidly convert to either SLECs or MPECs (129, 233). Therefore it seems that following respiratory infection these cells are either developmentally stalled (perhaps lacking a signal that would result in their conversion to one phenotype or the other). While it has not been directly tested, our data would suggest that these EECs are unable to survive through the contraction, perhaps due to a lack of survival signals normally conferred through CD127 expression (116)). It would be interesting to compare the development of these effector cell populations following infection at other mucosal sites (GI tract or reproductive tract) to determine if this skewing is indeed respiratory specific, or if is a more broad phenomenon observed outside of the systemic context of infection.

The mechanism of this distinct memory pathway has not been fully elucidated, although the current evidence leads us to believe that these developmental responses are initiated quite early in the immune response (123), perhaps imparted by different antigen presenting cells. As one might expect, VSV infection by the IN *vs.* IV route results in distinct differences in both overall numbers of DCs and the phenotypes of these DCs in site-specific draining lymph nodes (Figure 3.7). Particularly interesting is the identification of a subset of cells in the respiratory tract draining lymph nodes that is

phenotypically distinct ($CD103^+CD11b^+$ and MHC II^{lo}) from currently described DC subsets involved in priming of respiratory T cell responses (46). $CD103^+CD11b^+$ DCs have been described in the lamina propria of the small intestine (234, 235), where they have been described to promote Th17 differentiation (236) and maintain T_{regs} in the gut (235). Yet, these cells had not yet been described in the context of the respiratory tract and the response to respiratory infection. Their high level of presence in the respiratory LNs during peak times of T cell priming implicate them as potential cells which may influence memory formation. While it is tempting to speculate that these DCs may result in the regulated memory formation that is observed following respiratory infection, this has not been directly tested at this time. Future work will aim to extensively phenotype this DC subset, to determine if these cells are resident in the lung or if they form from monocyte precursors (229). Furthermore, functional analysis of these cells will be performed, using *ex vivo* studies to determine if these cells present antigen (and if so in what manner) and can drive the proliferation and/or skew the phenotypes of effector $CD8^+$ T cells. We have some evidence that these cells also exist following influenza infection, and these cells will be characterized in the context of both infection models.

Another interesting aspect of memory development in the respiratory tract, although not directly tested in this work, is the development of resident memory cells (T_{RM}) following infection. The development T_{RM} has not been fully elucidated, yet transcriptional studies have shown that these cells are distinct from T_{EM} and T_{CM}, and that these cells have some unique profiles, depending on their tissue of residence (237). Interestingly, T_{RM} cell in multiple sites express reduced levels of CD122 (Table 1.1), indicating that like respiratory derived $CD8^+$ T cells (141), they develop and are

maintained in the absence of IL-15 signaling (226). Respiratory infection, then, may preferentially give rise to these populations of T_{RM} cells at the expense of creating T_{CM} cells. However, as previously noted, in the respiratory tract these cells are not maintained long term (26), while T_{RM} cells at other locations do not display the same attrition as T_{RM} cells in the lung and lung airways (238). Therefore, if respiratory infection favors the development of these cells, yet the respiratory environment does not support their persistence, it could lead to the loss of heterosubtypic immunity that we see in murine models of infection. Once again, the attrition of these T_{RM} cells in the lung and lung airways may be a consequence of the regulatory environment of the lung. It has been known for some time now that instead of having direct cytolytic function, T_{RM} cells in the lung/lung airways function by producing cytokines to induce the migration of circulating memory (non-specific) $CD8^+$ T cells into the lung early after infection(37). Recently, it has also been shown in the female reproductive tract T_{RM} cells themselves can be activated in a non-specific manner (239). If potentially any antigen encountered by the T_{RM} cells could stimulate the mass migration of $CD8^+$ T cells in the lung it is easy to imagine that this could lead to vast immunopathology. Therefore, the long term persistence of T_{RM} in the lung airways *could* end up being detrimental to the host. Studies to address the potential consequences of long-lived T_{RM} cells in the lung this will be difficult in animal models, where most are kept in (somewhat) sterile environments and should not be subjected to the same stimuli as a human. However, these are important considerations to be made in rational vaccine design.

If respiratory infection leads to the preferential development of short-lived $CD8^+$ memory T cells, as our data suggests, a major question then still remains; can $CD8^+$ T

cells be targeted for the production of protective immunity against respiratory viruses? A common argument against the potential effectiveness of $CD8^+$ T cell based vaccination is that natural infection with influenza does not prevent an individual from developing subsequent infections. Indeed, by the very nature of how $CD8^+$ T cells work (recognizing peptide/MHC I complexes on the surface of infected cells) $CD8^+$ T cells will not be able to produce sterilizing immunity. However, evidence in human studies suggests that disease severity is reduced when individuals harbor populations of memory $CD8^+$ T cells (28, 107), and reduction of disease severity would be a major goal for a "universal vaccine". Another interesting observation from human studies, is that when $CD8^+$ T cell responses have been evaluated in the blood of patients, there is a correlation between $CD8^+$ T cell levels and protection from severe disease (28, 107). This suggests one of two things: that the measurement of $CD8^+$ T cells in the blood is a strong indicator that there are cells in the lung and the lung airways that are able to provide heterosubtypic immunity, or that in humans cells other than those in the lung and lung airways are able to provide heterosubtypic immunity. One caveat of studies where heterosubtypic immunity has been tested in murine models is that the dose of the challenge virus is often quite high. While this high dose gives credence to the ability of $CD8^+$ T cells in the lung/lung airways to provide protection against severe disease, the amounts of virus encountered and the severity of disease is not physiologically relevant. It is possible that the memory $CD8^+$ T cells located in lymphoid organs are able to provide a degree of protection that is not observable in this system. This possibility is an area where more research is needed, as it could modify what populations of $CD8^+$ T cells we should be targeting for effective vaccination. If it would be appropriate to target peripheral

responses, based on the data presented in chapter 3, it may be best to target immune responses systemically, or perhaps in a way that will reduced the level of skewing away from "classical" memory cells.

The growing popularity and usage of the live-attenuated influenza vaccine (Flumist) will provide researchers/epidemiologists with an interesting opportunity to see if a respiratory vaccine will be able to produce strong cellular immunity against influenza infection. Several studies have already shown that the Flumist vaccine can induce $CD4^+$ and $CD8^+$ T cell responses following vaccination (210, 240), but the longevity of these responses are not known, and only account for the populations of cells that are found circulating in the blood. In mice, a single dose of the Flumist vaccine results in the production of effector $CD8^+$ cell responses, but negligible populations of memory (211). Importantly, memory populations can be increased by a second round of vaccination, but the longevity of these cells is unknown (211). By tracking and evaluating individuals who receive the Flumist vaccine yearly, and comparing to those individuals who may have received the vaccination only once we may be able to gain some valuable insight as to how long lasting respiratory-induced responses are in humans.

It is clear that effective targeting of memory $CD8^+$ T cells in vaccine design is an area where more research is needed. Comparatively, the design of humoral vaccines is quite easy, and considerations do not need to be made concerning the location of B cells, nor their ability to traffic to/persist in the site of infection to provide immunity. However, there are certainly limitations in the protective qualities of antibodies (largely the inability to produce heterosubtypic immunity), that can be overcome by protective $CD8^+$ T cell responses, making this an important cell population to target in vaccine design. This

research focused on how the respiratory environment influences anti-viral $CD8^+$ T cells and the development of $CD8^+$ T cell memory. Overall, in this dissertation we have demonstrated that respiratory specific factors can have a direct and distinct impact on anti-viral $CD8^+$ T cell responses. These respiratory factors seem to developmentally favor strong effector cell responses, while limiting overall memory formation and perhaps its longevity. Importantly, we have shown that the developmental pathway for memory cells derived from a respiratory infection is distinct from those derived from a systemic infection. We believe that the work presented in this dissertation will have important implications for the understanding of ant-viral (or vaccine derived) $CD8^+$ T cell memory development following respiratory priming and will be important for consideration in both the production and the evaluation of $CD8^+$ T cell based vaccines against respiratory pathogens.

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