THE ROLE OF IL-15 IN LYMPHOCYTE MIGRATION AND MEMORY FOLLOWING INFLUENZA INFECTION

by

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(Under the Direction of Kimberly Klonowski)

ABSTRACT

Mechanisms by which the immune system eliminates influenza virus involve the coordinated actions of the innate and adaptive immune systems. Influenza-specific CD8 T cells have been characterized as the primary mediators of viral clearance, and a crucial role for natural killer (NK) cells has also been described, namely that NK cells provide short-term control of viral replication prior to T cell activation. In order to fulfill their functions, both NK and CD8 T cells must migrate from the periphery to the site of infection, a process dependent on the production of chemokines and cytokines in the lung airways. This study shows that following influenza infection, localized increases in the homeostatic cytokine IL-15 are responsible for the migration and accumulation of both NK cells and influenza-specific CD8 T cells in the lung airways. Entry of NK cells and CD8 T cells into the site of infection is delayed in the absence of IL-15, and this reduction in lymphocyte numbers results in disregulated control of early viral replication. By the same principle, viral control can be therapeutically enhanced via intranasal administration of exogenous IL-15 complexes. Additionally, continued administration of IL-15 complexes throughout the contraction phase of the anti-influenza CD8 T cell response magnifies the resultant CD8 T cell memory pool generated *in situ* and provides protection to heterosubtypic viral challenges, even though these cells themselves do not

require IL-15 for long-term maintenance. Our data suggest that mucosally-generated memory CD8 T cells may be independent of IL-15 for their homeostasis. Together this work suggests that local deposits of IL-15 regulate the coordinated innate and adaptive immune responses to influenza and emphasizes different cytokine requirements by CD8 T cell responses to mucosal or systemic viral infections.

INDEX WORDS: Influenza, NK Cells, CD8 T cells, IL-15

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# DEDICATION

I would like to dedicate this work to my husband, Calen Verbist, for his never-ending love and support and, importantly, his encouragement for me to maintain a sense of wonder at the world.

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## 1.1 Introduction

Influenza, commonly referred to as the flu, is an infectious disease caused by RNA viruses of the family Orthomyxoviridae. Although some of the symptoms associated with influenza infection—fever, coughing, fatigue, headache and muscle aches—can be confused for those associated with milder respiratory illnesses such as the common cold, influenza is a more severe disease and may be lethal, especially in the very young, the very old, and the immuno-compromised. Influenza spreads around the world in seasonal epidemics, resulting in the deaths of 250,000-500,000 people every year, and these numbers increase when pandemic viruses emerge [1]. The most devastating pandemic occurred in 1918, when it is estimated that 2.5% to 5% of the world's population was killed by the Spanish Flu [2]. Because the introduction of antibiotics has helped manage secondary infections, subsequent influenza pandemics have been less severe. Even so, the three pandemics since 1918—the 1957 Asian Flu (type A, H2N2 strain), the 1968 Hong Kong Flu (type A, H3N2 strain), and the 2009 Swine Flu (type A, H1N1 strain)—have claimed the lives of 2-3 million individuals [3,4]. Combined with the economic burden imposed by missed work during circulations of seasonal influenza strains, influenza infection constitutes a substantial public health problem, one that necessitates research into mechanisms of invasion and host defense as well as the development of vaccination strategies and novel immune therapies.

Individuals typically contract the influenza virus via inhaled aerosols, which are transmitted either by coughs and sneezes or by contact with aerosol-contaminated fomite surfaces. It is currently unclear which mode of transmission contributes more

substantially to the spread of seasonal epidemics [5]. The spread of seasonal epidemics has been slowed by the introduction and availability of influenza vaccines and anti-viral therapies, at least in developed countries. The most common of these human vaccines is the trivalent influenza vaccine (TIV), which contains purified and inactivated antigens against three viral strains: two influenza A virus subtypes and one influenza B virus subtype [6]. Immunological protection by the TIV depends on the induction of a robust humoral (antibody) response to viral coat proteins, primarily the hemagglutinin. Unfortunately, viral coat proteins are subject to a high degree of change due to the processes of antigenic drift and shift [7]. Therefore, each year the virus strains that comprise the TIV must be selected based on predictions about predominant circulating strains, which may or may not be accurate. Furthermore, responding to novel strains is a time consuming and expensive process because the appropriate vaccine must be grown in eggs before it is ready to be distributed [6]. A vaccine produced in this manner could be too late entirely. The production of TIV, which is cumbersome and costly, along with its potential ineffectiveness renders our current vaccine strategy rather inefficient and demands investigation into alternative strategies. Antiviral drugs such as the neuraminidase inhibitor oseltamivir are also available and have been used to treat influenza. Their effectiveness, however, is difficult to determine because much of the data that concerns these treatments remains unpublished [8].

If contracted, infection with influenza virus is localized to the upper respiratory tract, with viral replication largely limited to epithelial cells [9]. Mechanisms by which the immune system eliminates influenza have been well studied and are known to involve the coordinated actions of the innate and adaptive immune systems. It is important to note

that a murine model for studying both arms of the immune response to influenza virus has been established and well-characterized. Studies presented in this dissertation, therefore, employ a mouse model of influenza infection to explore the mechanisms by which both innate and adaptive effectors of the immune system respond to influenza virus. The findings presented here contribute to the scientific community's overall understanding of host defense against this virus.

## 1.2 Influenza Immunobiology

### *Innate Immune Response to Influenza Infection*

Innate immunity refers to mechanisms of host defense that are directly encoded by the genome, in the absence, generally speaking, of pathogen-specific responses or immunological memory. Innate immunity is typically thought of as a rapid, first-line defense against foreign antigens and is vital for the control of most (if not all) pathogens, either directly by eliminating the pathogen or indirectly by shaping the development of subsequent adaptive responses. After infection with influenza virus, it takes 5-7 days before adaptive responses, which include specific antibodies and T cells, are present at the site of infection and engaged in viral clearance [10]. During this substantial span of time, therefore, innate responses are critical for controlling influenza replication and initiating the appropriate adaptive responses. The innate response to influenza infection is complex, but for the sake of discussion it can be roughly divided into the following phases: 1) inhibition of viral invasion by soluble proteins in mucosal secretions, 2) detection of infection by soluble and/or cellular sensors, 3) limitation of viral replication and dissemination, and 4) recruitment and modulation of additional innate and/or adaptive immune effectors for viral clearance [11].

The respiratory tract is lined by alveolar cells and ciliated epithelial cells, the apical surface of which is covered by a layer of mucus. The physical properties of this mucociliary escalator may trap influenza viruses and then clear them by moving them into the gastrointestinal tract. It is the chemical properties of mucins, however, that are thought to be more important in inactivating virus and preventing infection [12,13]. Influenza invades respiratory epithelial cells through the binding of viral hemagglutinin to α(2,3)- or α(2,6)-linked sialic acids on the epithelial cells, and mucins greatly retard this process by providing sialic acid mimics that serve as hemagglutinin ligand decoys [13]. Collectins such as SP-A, SP-D, and mannose-binding lectin (MBL) present in the mucus and bronhcoalveolar lavage (BAL) also aid in the innate responses to influenza. As their name implies, collectins consist of a lectin moiety bound to a collagen domain and contain a carbohydrate recognition domain that is capable of binding the carbohydrates found in viral envelope and neutralizing the virus [11]. Furthermore, the interaction between collectins and viral particles activates the complement cascade, components of which are present in the fluid of the alveolar lining [14]. Finally, immune cells release several soluble mediators such as defensins (broad spectrum antimicrobial peptides released by neutrophils) and natural IgM [11,15,16].

Despite a wide array of soluble proteins that are highly effective at neutralizing influenza, at a sufficient infectious dose some viral particles will successfully invade the epithelial layer. Infection is then detected by a range of cellular sensors. Perhaps the best characterized mechanism of sensing and "reporting" infection is that of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). In regards to sensing influenza infection, it is known that two endosomal RNA-recognizing TLRs (TLR3 and

7) and one cytoplasmic sensor, Retinoic Acid-Inducible Gene-I-like receptor (RIG-I) are important activators of immune responses [17]. Expression of these receptors is upregulated in the lungs in response to influenza infection, and signals emanating from these receptors lead to activation of NF-κB and robust induction of type 1 interferons (IFNs), especially by resident plasmacytoid dendritic cells (pDCs) [18]. The PRR RIG-I, although classically associated with the recognition of dsRNA, recognizes the 5' capped ssRNA of influenza [19]. Similar to activation of TLR3 and 7, signals downstream of RIG-I activation result in activation of NF-κB and type I IFNs [17]. Interestingly, the NS1 protein of many influenza viruses blocks recognition by RIG-I, dampening the type I IFN response [20].

It is clear from this discussion that NF-κB activation and type I IFN induction are two key responses to influenza infection. Type I IFNs (IFNα and IFNβ) induce an antiviral state that induces the apoptosis of infected cells, limits viral replication, and makes surrounding cells more resilient to viral invasion [21]. Type I IFNs also potentiate responses of innate and adaptive immune effector cells either directly or indirectly by activating interferon response factors (IRFs) or interferon-stimulated genes (ISGs) in the promoters of many genes downstream in the inflammatory response [21]. Similarly, the activation of NF-κB is a ubiquitous and well-conserved pathway that regulates the expression of a variety of proinflammatory cytokines, including IL-1, IL-6, IL-8, and TNFα. Aside from the intrinsic anti-viral activity of many of these cytokines, this response is most important for modulating the responses of cellular effectors. Cytokines downstream of the type I IFN and NF-κB pathways regulate the trafficking and activation status of many immune cell types addressed below.

The epithelial cells that line the upper respiratory tract are the first target of influenza invasion and the first to mount an innate immune response [11]. The primary means by which epithelial cells respond to infection include release of type I IFNs and release of IL-8. Production of IL-8, in turn, promotes the recruitment of neutrophils to the site of infection [22]. Neutrophils represent the predominate cell type recruited into the lung airways during the first days post infection (p.i.), but their role in viral clearance is controversial. Blockades of neutrophil influx have had different effects on the outcome of infection, exacerbating infection in some models and improving outcome in others [23-26]. If primary responses by epithelial cells and neutrophils fail to adequately control virus replication, released virions and the contents of apoptotic cells can be phagocytosed by antigen presenting cells (APCs) resident in the lung and lung airways. Alveolar macrophages are abundant in the respiratory tract and are critical in controlling inflammatory responses there [27]. These cells may also contribute to the priming of adaptive responses, but this role is not yet well-defined [28]. Resident DCs largely consist of two populations (at least in rodents, though their likely counterparts have been identified in humans) distinguished by differential expression of CD103 and their distribution in the respiratory tract, with $CD103^{+}$ DCs more closely associated with epithelial cells and $CD103^{-}$, $CD11b^{hi}$ DCs more abundant in the lung interstitium [29,30]. These DC subsets are the most important mediators of subsequent adaptive responses. Under inflammatory conditions, monocyte-derived DCs (MHC $II^{lo}$, $CD11c^{lo}$) traffic into the lung airways, take up particulates, and migrate to draining lymph nodes [29]. Furthermore, pDCs are widely recognized as the major producers of type I IFNs

following infection. These monocyte-derived DCs and pDCs, however, are weak activators of naive T cells [28].

Another innate immune effector recruited into the lung and lung airways during influenza infections is the natural killer (NK) cell. A large body of literature emphasizes the importance of NK cells in the host response to influenza virus infection, and recent studies elucidate mechanisms through which NK cells recognize and become activated in response to influenza. As early as 1983, it was recognized that NK could be found in lungs of influenza-infected mice, contributing to antiviral immunity [31]. Follow up studies revealed that depletion of NK cells via anti-asialo GM1 in hamsters and mice resulted in severely increased morbidity and mortality [32]. Enhanced NK cell activity has also been correlated with improved outcomes to influenza infection [33]. NK cells, however, exert a variety of anti-viral functions such as direct lysis of infected cells, production of anti-viral cytokines, and recruitment and activation of other cell types [34], so how NK cells contributed to anti-influenza responses was not apparent. The activity of NK cells is known to be controlled via a balance of activating and inhibitory receptors whose ligands are expressed on infected and uninfected cells [34-36]. Among these receptors, NKp46 (NCR1 or Ly94) is specifically expressed on NK cells [37]. Gazit *et al.* have shown that mice lacking NKp46 have increased mortality after influenza viral infection [38]. Importantly, it has been demonstrated that influenza virus can be directly recognized by NK cells, in part, due to binding of the activating receptors NKp44 and NKp46 directly to viral HA [39,40]. This recognition not only enables direct lysis of infected epithelial cells, but also provides specificity to the interaction of DCs with NK cells, leading to further activation of the NK cells [39]. Moreover, this recognition of

influenza-infected cells by NK cells appears to be a phenomenon that occurs across a range of infections with different hemagglutinins [41].

NK cells at the site are also important modulators of T cell responses, both through cytokine secretion and cross-talk with resident DCs [42]. This role for NK cells in aiding the priming of anti-viral CD8 T cells has been shown to be especially important in influenza infection, since neither human CD8 T cells (in vitro) or murine CD8 T cells (in vivo) developed into effector cytotoxic T lymphocytes (CTLs) capable of killing influenza-infected targets in the absence of NK cells [43]. The significant contributions NK cells make to the control of influenza infection continue to be recognized, as it was reported that severe to lethal infections with the CA 09 H1N1 pandemic virus correlated with reduced NK responses [44]. Thus, NK cells have emerged as an innate immune effector that has tremendous effects on the outcomes of influenza infection, through both direct control of viral replication and modulation of adaptive immune responses.

*Adaptive Immune Response to Influenza Infection*

Adaptive responses to influenza infection are initiated by cells of the innate immune system, antigen presenting cells. As noted above, $CD103^+$ and $CD103^-$, $CD11b^+$ resident DCs are positioned in the intraepithelial layer and the lung interstitium, respectively, where they continuously sample antigens in the environment [29]. Upon infection, these subsets migrate to draining lymph nodes (DLNs) (cervical lymph nodes for the upper respiratory tract and mediastinal lymph nodes [MdLN] for the lower respiratory tract) along a chemokine gradient dependent on CCR7 expression by the DCs [45]. Once in the lymph nodes, antigen-bearing DCs interact with naïve T cells circulating through secondary lymphoid organs. The initial wave of antigen presentation

(days 1-4 p.i.) in the DLNs following influenza infection is predominantly carried out by $CD103^+$ DCs [29]. Thereafter, influenza antigens are continuously presented by migratory $CD103^-$, $CD11b^{hi}$ DCs and monocyte-derived DCs [28,46]. The temporal regulation of antigen presentation by different APC subsets likely affects the activation profile and transcriptional program of responding T cells and might influence the immunodominance hierarchy [47].

Naïve T cells bearing influenza-specific T cell receptors engage APCs presenting their cognate antigen in DLNs and become activated there. Once activated, T cells initiate a program of proliferation and differentiation, resulting in the expansion of effector T cells (Teff) that have the capacity to migrate to the site of infection and clear it [48]. This process of migration into the lung and lung airways begins around day 6 p.i. [49-51]. The signals and mechanisms by which these effector cells accomplish this migration are largely unidentified. Unlike effector cells trafficking to other mucosal sites such as the skin or gut which are known to be dependent on expression of CCR4 and CCR9, respectively, no such "imprinting" has yet been identified for trafficking to the lung or the airways. Teff found in the lung following influenza infection do express CXCR3, and this molecule is likely important for migration as well as modulating the effector-memory differentiation [52,53]. CXCR3 expression is important for extravasation of T cells in general, though, so the signals that direct specific trafficking of Teff to the lung remain to be characterized. The lung has two circulatory systems: the bronchial circulation supports the cells making up the lung tissue, and the pulmonary system (which branches to form the alveolar capillary beds) is optimized for gas exchange. Entrance of T cells into the lung from the bronchial circulation is dependent on the interactions of selectins that cause

the T cell to slow and roll along the vessel wall which is laced with glycosaminoglycan-bound chemokine gradients. The interactions between chemokines on the vessel wall and chemokine receptors and integrins on the T cells enable extravasation [54]. The capillary beds of the pulmonary circulatory system, however, are optimized for gas exchange and, as such, are very low pressure. This low pressure enables T cell extravasation independent of the specific interactions described above [55]. It is possible, then, that immune cell trafficking into and out of the lung airway space is under less regulation at the level of extravasation than it is at other mucosal sites. Chemotactic signals, however, must be present in order to direct Teff to the site upon infection, but further investigation into identifying these signals is clearly needed.

Once in the lung airways, effector T cells at the site have an immediate impact on viral load via direct cytolysis and production of antiviral cytokines [49-51]. Effector T cell numbers peak days 9-10 p.i., concurrent with or slightly after viral clearance (days 8-9 p.i.) [49-51]. CD8 Teff are known to be especially important mediators of viral clearance [56,57]. CD4 T cells may have some direct effect on viral titers through the production of antiviral cytokines and are unarguably important in helping antibody responses [58,59], but depletion of CD4 cells has little impact on the course of infection [60]. Antibody responses, while absolutely essential to the optimal control of secondary infections, also appear to have little impact on the outcome of a primary infection with most influenza serotypes. Seroconversion is typically not detectable until about day 14 p.i., well after viral clearance, and complete clearance in mice without antibody responses has been demonstrated [59,61,62]. CD8 T cells, however, are thought to be the primary mediators of viral control and are known to clear influenza through granule-mediated

mechanisms such as perforin and granymes as well as Fas-Fas ligand interactions [63,64]. Interferon gamma (IFNγ) production, a well-described effector mechanism of CD8 T cells (and NK cells), does not appear to be critical for the control of influenza virus directly, but it may be important in recruiting additional CD8 Teff and enhancing cytolytic activity of these cells [65,66]. Therefore, understanding how to augment specific CD8 T cell responses, their trafficking to the lung airways, and their cytolytic activity is critical to our understanding of how to control primary infections with influenza.

Once virus is cleared, influenza-specific CD8 T cells undergo a contraction phase and establish a pool of memory cells that can be found throughout the animal, including in the lung airways [10]. The memory CD8 T cells (Tmem) resident in the lung airways are phenotypically distinct from those in the periphery, or even the lung parenchyma. Lung airway-resident CD8 Tmem are predominantly CD62Llo, CCR7lo "effector memory" cells whereas the CD8 Tmem in peripheral sites are a mixture of effector memory (Tem) and "central memory" (Tcm) cells, which express high levels of CD62L and CCR7 (molecules that contribute to preferential homing to secondary lymphoid organs) [10,67,68]. Airway-resident CD8 Tmem also express high levels of CD44 and CXCR3, variable levels of CD127 (IL-7Rα), and low levels of CD122 (IL-2/IL-15Rβ) [69,70]. Also in contrast to peripheral CD8 Tmem which express high levels of the integrin CD11a (LFA-1), the majority of Tmem in the BAL are $CD11a^{lo}$, because this molecule is downregulated upon entry into the lung airways [71,72]. Another unique characteristic of the Tmem in the lung airways is that they do not proliferate in this site; rather, this population must be continually seeded by cells proliferating in the periphery,

which hemeostatically proliferate for the lifetime of the animal [73-75]. Tmem in the BAL are also capable of producing cytokines such as IFNγ in response to proinflammatory cytokines without any specific interaction with cognate antigen [76].

The unique phenotype and location of CD8 Tmem in the BAL enables them to be poised and ready to respond to secondary infection, and these cells are important mediators of protection against viral challenge [10]. Although large numbers of influenza-specific CD8 Tem can be recovered from the BAL about a month post infection, in the months that follow, this number declines, eventually reaching steady state numbers approximately 3 months p.i. in mice and 6 months p.i. in humans [69,77]. CD4 Tmem, which represent a very small proportion of the Tmem in the BAL one month p.i. decline even more rapidly and are no longer detected by 2 months p.i. [78]. Concomitant with the decline to CD8 Tmem in the BAL is a decline in heterosubtypic immunity, that is, protection against influenza serotypes different from the primary infection [77]. While neutralizing antibodies are very efficient at providing protection against a subsequent infection with the same strain of influenza, neutralizing antibodies providing cross-protection against different strains are less effective [79]. Thus, the correlation between the decline of CD8 Tmem in the BAL and the loss of cross-protection implies that CD8 Tmem contribute more to heterosubtypic immunity than cross-protective antibodies that circulate for the lifetime of the animal [79]. Furthermore, studies in which protection against Sendai virus could be transferred to naïve mice by the intratracheal transfer of Tmem have directly implicated airway-resident Tmem in providing protection to challenge infections [80].

Given the great expense incurred in developing current influenza vaccines, which only provide immunity to strains that are contained within the vaccine, there is considerable interest in elucidating the mechanisms of heterosubtypic immunity. It is widely accepted that one such mechanism is the population of CD8 Tmem in the lung airways, but unfortunately the hetersubtypic immunity that this population provides diminishes in strength as the population declines in number. There are, then, two important questions concerning influenza-specific CD8 T cells that these observations about heterosubtypic immunity raise: 1) what signals induce CD8 T cells to migrate into the lung airways? and 2) what signals are required for their long-term survival there? In the research that predicated this dissertation, we hypothesized that the cytokine IL-15 was an important component in the answer to each of these questions.

**1.3 Interleukin-15**

IL-15 is a common gamma chain (γc) cytokine in the same family as IL-2, IL-4, IL-7, IL-9, and IL-21, which all utilize the γc as a component of their receptors, leading to redundancy in signaling and function. IL-15 was independently identified by two groups in 1994 based on its ability to stimulate the proliferation of the murine T cell line CTLL-2 [81,82]. Now, human, murine, bovine, porcine, feline, and rabbit IL-15 have all been cloned with 70-80% structural homology [83]. Like IL-2, IL-15 is a 14-15 kDa glycoprotein member of the four α-helix bundle-containing cytokines [82,84,85]. Both cytokines signal through trimeric receptors that utilize γc, and CD122 (IL-2 Rβ) [82,86,87]. Specificity for each cytokine is conferred to by a private receptor α chain—CD25 or IL-15Rα for IL-2 and IL-15, respectively [88]. IL-15 has been shown to be presented in trans to responsive cells expressing CD122 and CD132 by cells expressing

the cytokine itself bound to a membrane form of the receptor alpha chain [89], and a similar mode of signaling may also exist for IL-2 [90]. With so many shared structural and signaling components, it is not surprising that IL-2 and IL-15 also share many functional redundancies including induced proliferation of NK and CD8 T cells and enhanced CTL activity in these cell types [87,91,92]. Both cytokines also induce the proliferation and differentiation of stimulated human B cells [93]. Despite the many overlapping functions between IL-2 and IL-15, however, it has become abundantly clear that IL-15 exclusively mediates many immune functions. Whereas IL-2 has a critical role in activation-induced cell death (AICD), IL-15 appears to always oppose AICD by acting to prolong the survival of T lymphocytes [94,95]. IL-15 is also exceptional in its ability to support the homeostasis of natural killer (NK cells) and memory phenotype and antigen-specific memory CD8 T cells, and it is probably best characterized for its role in maintaining these pools of cells [96]. Therefore, despite the high degree of redundancy in cytokine signaling within the immune system, IL-15 clearly mediates many important unique aspects of immunity.

Emerging literature is revealing many divergent functions for IL-15 within and outside of the immune system, but many of these functions—and mechanisms by which these functions are differentiated in various cell types—are not well understood. IL-15 transcripts are constitutively expressed in a variety of tissues has been implicated in a variety of physiological processes. IL-15 expression in all tissues is heavily regulated at the posttranscriptional level (Waldman and Tagaya [97] and Budagian [83] review these modifications at length). Overall, IL-15 translation is inefficient due to multiple layers of negative regulation. Such abundant regulatory mechanisms may reflect on IL-15's

potency as a pro-inflammatory cytokine. Unchecked IL-15 expression could easily lead to a various inflammatory and autoimmune disorders, and indeed, IL-15 is implicated in the pathology of many of these diseases including inflammatory bowel disorder, celiac disease, and rheumatoid arthritis [98]. However, IL-15 expression induced by an infectious agent is a very important part of transforming NK cells, CD8 T cells, and other cells of the immune system into functional effectors capable of efficiently eliminating pathogens. Both innate and adaptive immune responses can be ramped up or dampened down by increasing or decreasing IL-15 availability, respectively, and a large body of literature emphasizes the many roles IL-15 plays in modulating immune responses to viral pathogens.

The cell types best capable of engaging infected cells directly and mediating antiviral responses through cytokine release such as IFN γ are NK cells, lymphocytes of the innate immune system, and cytotoxic CD8 T cells, lymphocytes of the adaptive immune system. As recently reviewed by Sun and Lanier [99], these cell populations bear many parallels, including their professional killing capacities via release of perforin and granzymes, their development from common lymphoid progenitors, and notably, their dependence on γc cytokine signals for their development and homeostasis. Both NK cells and CD8 T cells must become activated in the presence of foreign antigens, migrate to sites of infection, and retain be able to survive and expand in order to maximally exert effector functions. Although a variety of cytokine signals are important in this process, IL-15 is a potent activator, chemotactic agent, and homeostatic signal for NK cells and CD8 T cells.

**1.4 IL-15 and Natural Killer Cells**

NK cells constitute the third population of cell types that originate from a common lymphoid progenitor in addition to B and T cells. Despite their lymphocyte origin, NK cells have been classified as innate immune cells because they do not use recombination-activating gene (RAG) enzymes to generate specific antigen receptors, and, as such, are able to respond rapidly to infected cells without any prior sensitization [99]. Although IL-15 is an important modulator of many different functions of innate immune cells such as neutrophils, basophils, and eosinophils, NK cells are perhaps the innate immune effector most dependent on IL-15 signaling for development, homeostasis, and function.

All stages of developing NK cells express high levels of CD122, suggesting that IL-15 could act on these cells throughout the entire course of their development [100]. Accordingly, analyses of IL-15$^{-/-}$ and IL-15Rα$^{-/-}$ animals revealed severe reductions in numbers of NK cells, implicating an important role for IL-15 in the development of NK cells [101-103]. The earliest NK cell progenitor does not have any known lineage-specific markers, but is currently identified as a non-stromal bone marrow cell that expresses CD122 and IL-15Rα [104,105]. The necessary expression of CD122 and IL-15Rα by NK cell progenitors highlights how important IL-15 signaling is in the early development of NK Cells, and in vitro studies have confirmed that IL-15 is sufficient in inducing differentiation of NK cells from early haematopoietic cells [105]. How IL-15 signaling in early haematopoietic cells results in NK cell differentiation is somewhat less clear, but many studies have contributed significantly to understanding the induction of NK cell development by IL-15. IL-15 expression in bone marrow stromal cells is

upregulated by IRF-1 acting on an IRF response element in the IL-15 promoter (IRF-E) [106]. The induced IL-15 then acts on NK and NK T cell progenitors, stimulating their subsequent maturation [106,107]. Therefore, upstream expression of the IRF-1 transcription factor is important for IL-15 expression and subsequent NK cell development, transcription factors downstream of IL-15 signaling within the NK cell progenitor have also been identified. The transcription factor E4BP4 (also known as NFIL3) has been proposed as a NK cell lineage-specifying factor, since E4BP4-deficient mice show a severe reduction in NK cell numbers [108,109]. EBP4 was shown to be downstream of IL-15R signaling when addition of exogenous IL-15 was unable to rescue NK cell development in E4BP4-deficient progenitor cells [108]. Thus, transcription factors influence the expression of IL-15 (and probably its receptor), and lie downstream of the IL-15R signaling pathway in NK cell progenitors to control NK cell development.

Another way in which IL-15 signaling may affect NK cell development is through the induction of Ly49 receptors. Ly49 receptors mediate activating and inhibitory signaling in mature NK cells through interactions with MHC class I elements and are increasingly more appreciated as important mediators of "education" or "licensing" in developing NK cells [35,99]. Some reports indicate that in NK cells lacking IL-15Rα, Ly49 expression is reduced [110]. Others have indicated that immature NK cells in $IL\text{-}15^{-/-}$ mice do express Ly49 receptors, but these cells do not phenotypically develop beyond the "minor but discrete $CD11b^{-}CD27^{+}$ $Dx5^{hi}$ $CD51^{dull}$ $CD127^{dull}$ $CD122^{hi}$ stage" [111]. Still others have used a model in which bone marrow (BM)-derived dendritic cells were prepared from mice transgenically modified to express varying amounts of IL-15α to demonstrate that NK cell homeostasis, NK cell differentiation, and acquisition of Ly49

receptor and effector functions by NK cells require different levels of IL-15 trans-presentation input to achieve full status [112]. IL-15 promotes not only early NK cell development but is also suggested to be important the differentiation of $CD11b^{+}$ $CD27^{+}$ NK cells into $CD11b^{+}CD27^{-}$ fully matured NK cells, as monocytes need to express Tbet and IL-15Rα for this maturation to occur [113]. Although many differences exist between mouse and human NK cell development, recombinant human interleukin-15 (rhIL-15) or an Ad-vector expressing human IL-15 is able to significantly enhance NK cell development and maturation in the bone marrow and liver of Balb/c $Rag2^{-/-}$ $gamma(c)^{-/-}$ mice reconstituted with human hematopoietic stem cells [114]. Thus, varying levels of IL-15 stimulation in different microenvironments may induce different stages of NK cell development, and only further study will determine when, where, and how much IL-15 is available in vivo to the various stages in the NK cell lineage.

In addition to the requirement for IL-15 in optimal NK cell development and maturation, NK cells require IL-15 for their homeostasis in the periphery—that is to maintain normal numbers. Mature NK cells adoptively transferred into $IL\text{-}15^{-/-}$ or $IL\text{-}15R\alpha^{-/-}$ mice fail to proliferate, and their half-life of 7-8 days is reduced to only 2 days [115,116]. The mechanisms by which IL-15 influences the homeostasis of NK cells are known to dependent on IL-15's ability to induce Bcl-2 expression [115] and suppress both the forkhead box O3A (FOXO3A) and the pro-apoptotic factor BCL-2-interacting mediator of cell death (BIM) transcription factors [117]. Interestingly, these vital signals are dependent on the transpresentation of IL-15 by IL-15Rα-expressing dendritic cells [96]. Whereas mixed bone chimeras generated from $IL\text{-}15R\alpha^{-/-}$ mice and $IL\text{-}15R\beta^{-/-}$ mice could support the survival of adoptively transferred NK cells, mixed bone chimeras

generated from IL-15Rα$^{-/-}$ and IL-15$^{-/-}$ mice could not, indicating that IL-15 and its Rα chain must be expressed by the same cells types to support the survival of NK cells [116,118]. In complimentary experiments, forced expression of IL-15Rα on only dendritic cells (DCs) or treatment of mice with soluble IL-15/IL-15Rα complexes promotes the expansion of NK cell populations [119,120]. In human NK cells, IL-15 sustains the expression of its high-affinity receptor, leading to long-lasting STAT5 phosphorylation and Bcl-2 expression important for the long-term survival of these cells [121].

Although survival of mature NK cells is in the periphery is strictly dependent on transpresented IL-15, this requirement can be by-passed by transgenically forcing the constitutive expression of Bcl-2 [122]. However, even though numbers of circulating NK cells could be restored in these transgenic animals, the NK cells in these mice were impaired in their cytolytic activity [122]. More recent research suggests that IL-15 works additively with IL-10 to increase cytotoxicity of NK cells [123]. Glucocorticoids may also work synergistically with IL-15 to mediate activation of NK cells. Glucocorticoids when combined with IL-15 in cultures of peripheral blood (PB)-derived CD56$^{+}$ cells induce increased high cytolytic activity, IFN-gamma production, and expression of NKp30, NKp44, NKp46, 2B4, NKG2D and DNAM-1 [124].These data imply that IL-15 is also an important signal in the activation of NK cells. Dendritic cells (DCs) are known to activate NK cells [125], and IL-15Rα expression by the DC is an essential component in its ability to activate NK cells, enhancing both cytolytic activity and IFNγ production [126]. Upon transient or prolonged exposure to IL-15 complexed to a soluble form of its receptor alpha chain in vivo, NK cells also undergo distinct phenotypic changes [127].

Importantly, transient stimulation augmented the NK cell pool and leads to a more activated phenotype including increased CD69, NKG2D, and NKp46 expression; whereas chronic stimulation with IL-15 complexes appeared to impair NK cell activation and function [127]. Therefore, although future study is clearly warranted in this area, IL-15 may provide important signals for the activation of mature NK cells.

Mature NK cells are largely localized in the red pulp of the spleen and the sinusoidal regions of the liver; however, during viral infection they infiltrate the splenic white pulp and migrate rapidly to sites of infection [128]. The precise signals required for NK cell traffic in different situations are not well defined, but a small but convincing body of literature implicates IL-15 in the migration of NK cells from the circulation to sites of immunological insult. Only three years after the discovery of the cytokine, it was shown that NK cells would migrate to IL-15 in *in vitro* checkerboard chemotaxis assays and that these cells adhered more readily to a vascular endothelial cell line after stimulation with IL-15 [129]. Although IL-15 does appear to induce the migration of NK cells, it is unclear whether this is direct chemotaxis to the cytokine or an indirect effect due to modulation of other cell surface receptors. Stimulation with IL-15 is known to modulate CD11a expression on NK cells, thus increasing their adhesion to vascular endothelium [129]. Additionally, stimulation with IL-15 is known to alter the expression of several different chemokine receptors on the surface of NK cells. Expression of CX3CR1 is well described as a chemokine receptor whose expression is negatively regulated by IL-15 signaling through NFAT-dependent mechanisms [130,131]. This effect appears to be specific to CX3CR1, however, as expression of CCR5 by NK cells is increased upon stimulation with IL-15, and expression of CXCR4 remains unaltered upon

IL-15 stimulation [132]. In combination with glucocorticoids, treatment of PBMC-derived NK cells with IL-15 significantly increased surface expression of CXCR3 and CXCR4 [124].

Overall, IL-15 is a key regulator of NK cells, modulating all aspects of NK cell biology, including their development and maturation, survival and proliferation, activation and cytoxicity, and migration to sites of inflammation. Thus, IL-15 is a vital part of NK cell responses, effecting a population of innate cells in sufficient number and capacity to respond to infection and limit viral replication.

**1.5 IL-15 and Antigen-Presenting Cells**

Although the immune system is frequently and conveniently broken down into innate and adaptive immunity, in reality there is no clear demarcation in vivo indicating the end of innate responses and the initiation of adaptive responses. Rather, a cascade of innate effectors is followed fluidly by a cascade of adaptive effectors whose functions are shaped by the signals of their predecessors. Although almost all cells of the innate immune system can affect those of the adaptive immune system, the key regulators of adaptive responses and long-considered "bridges" of innate and adaptive immunity are antigen presenting cells. Antigen presenting cells including dendritic cells and monocytes/macrophages are not only profoundly affected by IL-15 signals, but are also key producers of IL-15. Thus, IL-15 represents a cytokine signal that influences and informs both arms of the immune system and brings them together into a single, functional unit.

In response to infection, antigen-presenting cells must functionally mature to optimally prime cells of the adaptive immune system, and IL-15 plays an important role

in this process. IL-15 stimulation enhances the phagocytic uptake of microbial pathogens by monocytes and macrophages and induces the production of pro-inflammatory factors such as IL-8 and monocyte chemotactic protein 1 (MCP-1) by these cells [133]. IL-15 signaling in DCs may also lower the peptide concentration required for priming, as this has been shown to be the case in IL-15-stimulated DCs used to prime CTL responses to melanoma antigens [134], possibly because IL-15 stimulation increases levels of CD11c and MHC molecules, as well as CD40, CD80, and CD86 [135]. Similarly, IL-15 stimulation of DCs results in upregulated costimulatory factors, enhances IL-12 production and responsiveness, and increased IFNγ production [83]. Furthermore, DCs from IL-15 deficient mice are impaired in their ability to prime delayed type hypersensitivity reactions or produce IL-2 and thus stimulate T cell proliferation [136,137]. Monocytes differentiated in vitro into DCs with GM-CSF and IL-15 initiate Th1 and Th17-type responses [138], and enhance NK cell production of IFNγ, leading to enhanced CD8 T cell induction [139]. It is important, then, that antigen-presenting cells are responsive to IL-15 signals for shaping subsequent lymphocyte responses.

Not only must antigen-presenting cells be able to receive signals from IL-15 to be fully functional, but in order to instruct the eventual functions of the cells they prime, antigen-presenting cells provide soluble IL-15 or transpresent it to the cells that they interact with. APCs matured with pathogenic stimuli such as poly IC and LPS produce IL-15 in response [140-142]. The stimulation of DCs with covalently linked extracllular CD40L domains also induces the production of IL-15 and enhances T cell proliferation to *C. albicans* antigen [143]. In anti-viral immune responses, type 1 IFNs serve as a potent inducer of IL-15 expression, since the IL-15 promoter contains an interferon regulated

element [141,144]. Once antigen-presenting cells are induced to express IL-15, NK and T cells are able to respond to these signals, continuing the evolution of the immune response to the invading pathogen. It has also been shown, for example, that monocytes activate NK and NKT cell proliferation and LMP1 expression through transpresented IL-15 [145]. Additionally, DC-derived IL-15 is required for tumoricidal NK activity [146] as well as CD69 expression, IFNγ secretion, and proliferation [147]. IL-15 presented by dendritic cells to anti-viral CD8 T cells is also important for the generation and maintenance of these responses in both systemic viral infections [148] and at specific sites following tissue-specific infections [149].

**1.6 IL-15 and CD8 T Cells**

CD8 T cells express all three components of the IL-15 heterotrimeric receptor. Although T cells constitutively express very low levels of IL-15Rα [85], expression of this receptor chain is induced by stimulation with IL-2, anti-CD3 antibody, or phorbol-myristate acetate [150]. CD122 is also constitutively expressed by T cells; although, CD8 T cells express much higher levels of this receptor chain than CD4 cells, and memory subsets express higher levels than naïve subsets [151]. In general, IL-15 signals are thought to be more important for the homeostasis of memory CD8 T cells than other T cell subsets. Signaling through the IL-15 receptor is known to be coupled to Jak1 and Jak3 activation and phosphorylation and nuclear translocation of STAT3 and STAT5 [152,153]. Additionally, IL-15 signaling in T cells results in the phosphorylation p56lck and p72syk, induction of the MAP kinase pathway, and induction of Bcl-2 [83,154]. These signals are important for the survival of T cell populations, especially memory

populations whose survival is dependent on cytokine stimulation and independent of antigen.

Paralleling NK cells, IL-15 signaling affects all phases of CD8 T cell biology, including their development, activation, proliferation, survival, function, and migration. NK cells, however, appear to have much stricter requirements for IL-15 for their development than CD8 T cells. Original analyses of IL-$15^{-/-}$ and IL-15R$\alpha^{-/-}$ mice revealed reductions in numbers of CD8 T cells that could be found in the periphery, but these deficiencies were largely restricted to the memory ($CD44^{hi}$) population [101,102]. These data would suggest that the early development of naïve TCRαβ CD8 T cells is minimally dependent on IL-15. Indeed, these findings are consistent with earlier studies that suggested that IL-15 is expressed only by thymic stromal epithelial cells and only at very low levels, which is important for promoting the development of TCRαβ T cells from thymic bipotential T/NK progenitor cells, as high concentrations of IL-15 promoted the development of TCRγδ CD8 T cells and NK cells instead [155]. Nonetheless, thymic IL-15 may subtly affect naïve CD8 T cell development. Although IL-7 is well-known to be critical in normal thymocyte development, $\gamma c^{-/-}$ mice have a more severe thymocyte defect than IL-7R$\alpha^{-/-}$ mice [156,157]. Furthermore, IL-7R$\alpha^{-/-}$ IL-2R$\beta^{-/-}$ double mutant mice display a similar thymic phenotype to $\gamma c^{-/-}$ mice, while IL-7R$\alpha^{-/-}$ IL-$2^{-/-}$ double mutant mice phenotypically resemble IL-7R$\alpha^{-/-}$ mice [158]. These observations suggest that IL-7Rα independent signals that utilize IL-2Rβ and γ chains independently of IL-2 (likely IL-15) are important in fine-tuning thymocyte development [159].

Once CD8 T cells have made their exodus from the thymus, IL-15 provides CD8 T cells in the periphery proliferative signals in steady-state conditions. This has been

evidenced by the fact that lymphocytes from un-immunized IL-15Rα$^{-/-}$ mice proliferate at a lower rate in vivo compared with lymphocytes from normal mice [101]. In reverse experiments, injection of IL-15 causes the selective proliferation of memory phenotype CD8$^{+}$ T cells [144]. Consistent with these findings, IL-15 transgenic mice that overexpress IL-15 have increased numbers of memory phenotype CD8 T cells [160,161], and delivery of IL-15 complexed to its Rα chain induces the robust proliferation of memory phenotype CD8 T cells and (to a lesser extent) naïve CD8 T cells [120]. There is evidence, however, that even this naïve CD8 T cell response to IL-15 complex depends on interactions with MHC I and the avidity of the TCR [162]. Thus, in the absence of an antigenic stimulus, IL-15 delivers a proliferative signal to CD8 T cells; however, it remains unclear how important this signal is to naïve CD8 T cells in the periphery, as all of these studies together implicate a more important role for IL-15 in the proliferation of memory (or at least memory phenotype) CD8 T cells.

While IL-15 may support the basal proliferation of naïve CD8 T cells to some degree, it is probably more important for their proliferation in situations of lymphopenia. When T cells are transferred into lymphopenic conditions, IL-15 is a potent inducer of homeostatic proliferation. In experimental models of homeostatic proliferation, small numbers of T cells (e.g. $(1–2) \times 10^{6}$ cells per mouse) are transferred into either constitutively (e.g. RAG-1$^{-/-}$ or RAG-2$^{-/-}$ mice), or transiently (e.g. sublethally irradiated or anti-TCR mAb treated) lymphopenic mice. Transfer of naive T cells into sublethally irradiated IL-15$^{-/-}$ hosts leads to reduced proliferation compared with normal hosts (although this deficit is not as profound as in IL-7$^{-/-}$ hosts) [163-165]. The increased expression of IL-2Rβ on naive T cells undergoing homeostatic proliferation suggests that

signals delivered through IL-2Rβ containing receptors may be involved in this process, and IL-15 appears to be an important signal for maintaining homeostatic proliferation [166]. Interestingly, naïve CD8 T cells homeostatically proliferating or proliferating in response to IL-15 complexes acquire a memory-like phenotype, expressing CD44, Ly6c, and CD69 [120,167]. Thus, IL-15 contributes to the sustained homeostatic proliferation and possibly the phenotypic conversion of naive cells during this process.

In addition to providing the stimulus for CD8 T cells to proliferate, IL-15 has been shown to be an important survival factor for these cells. IL-15 can protect lymphocytes from undergoing programmed cell death; although, this function may not be independent of its ability to promote proliferation, as IL-15 appears partially influence cell survival by causing them to enter the cell cycle [159]. In vitro, the induction of anti-Fas-mediated apoptosis can be prevented in cultures of concanavalin A-treated human T lymphocytes by the addition of IL-15 to these cultures [168]. Co-culture with IL-15 also rescued ex-vivo T cells from AD10 transgenic mice that were induced to undergo apoptosis by previous injection of with the Vβ3 family inducing superantigen SEA [169]. A role for IL-15 in preventing cell death has also been demonstrated in vivo by protection of mice injected with an anti-Fas antibody with administration of a chimeric human IL-15-murine IgG2b fusion protein [168]. Thus, IL-15 is able to protect T cells against apoptotic cell death. All the mechanisms by which IL-15 protects cells from undergoing programmed cell death in different circumstances have not been fully delineated, but IL-15 is known to upregulate expression of anti-apoptotic proteins (most notably bcl-2) and cause entry into the cell cycle.

Initial activation of CD8 T cells is a consequence of engagement of specific TCR and peptide-loaded MHC I molecules on antigen-presenting cells (APCs) and non-specific engagement of costimulatory molecules. These events proceed independently of any cytokine signaling; however, these events bring T cells into close and prolonged interactions with APCs during which APCs can provide multiple cytokine stimuli, an important one of which is transpresented IL-15. Expression of both IL-15Rα and CD122 increases after TCR activation, and heterologous IL-15 can support the survival and/or proliferation of activated T cells [169]. Similarly, addition of IL-15 to mice augmented specific T cell proliferation and cytoxicity to SIINFEKL-pulsed DCs [170]. Contact with IL-15 and especially signaling through CD122 seem to be essential factors in differentiating activated CD8 T cells. Whereas strong, prolonged signaling through CD122—effected by IL-2 binding—promotes terminal effector differentiation, weaker and intermediate-strength signaling through CD122—effected by IL-15 binding—promotes central memory development and survival [171]. This is consistent with evidence that IL-2, but not IL-15, is essential for the generation of primary effector responses [172] and the dispensability of IL-15 in primary CD8 T cell responses to various pathogens (discussed below). Although these cells are generated in the absence of IL-15 signaling, the prolonged survival of effector CTLs through the contraction phase is highly dependent on IL-15 [173,174], and this is especially true of $CD127^{lo}$, $KLRG\text{-}1^{hi}$ short-lived effector cells [174,175].

As early as 1995—only a year after its discovery—a role for IL-15 in the chemoattraction of T cells was described [176]. IL-15-induced migration of T cells is likely very important for the arrival of effector CTLs at sites of inflammation and/or

infection. In models of rheumatoid arthritis, for example, IL-15 is abundant in synovial fluid and can induce the migration of human PBMCs in vitro [177]. Similar to the IL-15-induced migration of NK cells, it is unclear whether chemoattraction of T cells by IL-15 is a result of direct chemotaxis, indirect modulation of molecules important for lymphocyte migration, or some combination of the two. IL-15 has been shown to induce the expression of both chemokines and their receptors in T cells, and IL-15 promotes T cell extravasation through endothelial cells via a CD44-dependent mechanism [178]. The expression of the adhesion molecules CD44, LFA-1, and CD62-L are aberrantly expressed on CD8 T cells from IL-15Rα$^{-/-}$ mice, and splenocytes from IL-15Rα$^{-/-}$ mice were less efficient than normal splenocytes at entering peripheral lymphoid organs after intravenous injection [101].

Therefore, in parallel to the many ways in which IL-15 regulates NK cell biology, IL-15 represents an integral part of shaping anti-viral CD8 T cell responses. IL-15 promotes the survival and proliferation of CD8 T cells at all stages of the immune responses (especially the memory phase), enhances the cytoxicity of effector CD8 T cells, and signals T cells either directly or indirectly to enter infected locales.

## 1.7 IL-15 and Anti-Viral Immunity

*Systemic Viral Infections*

The effects of IL-15 on the outcome of other virally-mediated diseases vary greatly from virus to virus. Because IL-15 is a potent activator of NK and CD8 T cell populations, viral diseases whose control largely depends on NK and CD8 T cell may be aggravated by an IL-15 deficiency whereas viruses that cause lympho-proliferative disorders may be ameliorated be the loss of IL-15. One such example of the latter is

human T cell lymphotropic virus type 1 (HTLV-1). HTLV-1 causes a neurological disease termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) with clinical manifestations similar to those of multiple sclerosis. The increased proliferation of lymphocytes in HAM/TSP-infected individuals can be partially attributed to increased expression of IL-15Rα and increased production of the cytokine itself, and blocking antibodies against IL-15 or its receptor blocks some proliferation [179,180]. In addition to increasing their proliferation, IL-15 signaling may also increase the activation status of lymphocytes in these patients by enhancing their effector capabilities, causing spontaneous degranulation and IFNγ secretion [181]. Thus, in diseases such as HAM/TSP, wherein viral infection results in expansion of the lymphocyte pool, blocking IL-15 signaling may be an important therapeutic option. In fact, the humanized antibody Mikβ1, a blocking antibody against CD122 is in clinical trials for HAM/TSP patients (http://clinicaltrails.gov).

In viral infections, far more common than the desire to reduce lymphocyte proliferation and activation is the desire to augment lymphocyte numbers and cytotoxicity to such a degree that they are potent effectors able to control and eliminate the viral pathogen. The potential role for IL-15 in this process has been investigated in a variety of models of viral pathogens. An absence of IL-15 poses no problems in the acute phase of infection with either vesicular stomatitis virus or lymphocytic choriomeningitis virus, as IL-15$^{-/-}$ mice clear these viruses [148,182,183]. In contrast to the unaltered control of viral load in IL-15$^{-/-}$ mice infected with VSV or LCMV, infection of IL-15$^{-/-}$ mice with vaccinia virus results in a loss of control of viral replication, and these animals succumb to infection [102]. These differences likely reflect differential roles for NK cells

in immune responses to these viruses. NK cells are thought to help control the initial replication of vaccinia virus, but robust NK cell responses to LCMV play little role in viral clearance and may actually be immunoregulatory, suppressing T cell responses that are critical in controlling this infection [184]. Regardless of whether or not IL-15 is necessary for NK cell responses and/or viral clearance, IL-15 is not unimportant in immune responses to VSV and LCMV infections. Models of intravenous infection of mice with these viruses have revealed that IL-15 is important for the generation of antigen-specific memory CD8 T cells responding to VSV infection [148]. While a similar requirement for IL-15 in the generation of memory CD8 T cells was not observed following LCMV infection, in both viral infection models, IL-15 is requisite for the long-term maintenance of the antigen-specific memory CD8 T cells, as this population wanes dramatically over time following infection [148,182,183].

*Tissue-Specific Viral Infections*

In contrast to the gradual attrition of antigen-specific memory CD8 T cells in IL-$15^{-/-}$ mice infected with VSV or LCMV, IL-$15^{-/-}$ mice infected with latent gamma herpes virus (MHV-68) show no defects in the antigen-specific memory CD8 T cells [185]. This difference may be partially accounted for by the continual presence of antigen from reactivation of the gammaherpes virus [185], but viral persistence is likely not the main factor maintaining memory CD8 T cells in the absence of IL-15. IL-15-independent memory CD8 T cells may be a result of the mucosal environment in which these CD8 T cells are primed [186]. Immune responses primed in specific tissues may impart transcriptional programs on responding CD8 T cells that renders these cells more or less dependent on IL-15 for their homeostasis, but this remains to be formally demonstrated.

IL-15 is not required for positive disease outcome or maintenance of a memory CD8 T cell population in the aforementioned tissue-specific viral infection, but IL-15 is not unimportant in tissue-specific viral infections. It has been repeatedly observed that viruses induce expression of IL-15, and this expression is important for anti-viral responses by different lymphocyte populations. Infection with hepatitis B, for example, induces IL-15 expression, and low levels of circulating IL-15 are associated with high viremia and poor disease outcome [187]. In individuals infected with hepatitis C, there is no correlation between the disease and IL-15 expression, but in vitro studies have shown that IL-15 treatment promotes the proliferation and survival of NK cells, a cell population that is known to be important in the control of hepatitis C replication in the liver [188]. Thus, in both diseases, suboptimal production of IL-15 is correlated with the likelihood of the virus entering a chronic phase, presumably because protective NK cell responses cannot be maintained in absence of IL-15. IL-15 production is also known to be upregulated in response to respiratory viruses such as RSV [189]. In the latter, local IL-15 production appears to be important for the prolonged survival of CD8 T cells in the lung airways [70,149].

Following infection herpes simplex virus-2 (HSV-2), IL-15$^{-/-}$ mice are unable to control the pathogen and the infection is lethal—effects thought to be mainly mediated by the lack of NK and NKT cells in these animals [190]. In vitro evidence corroborates these conclusions, as IL-15 has been shown to have antiviral effects on isolated human PBMCs infected with HSV-1, Epstein barr virus, or herpes virus-6, mainly through its action on NK cells [191], but additional studies with HSV-2 report a critical role for IL-15 in the innate immune response for protection independent of any action on natural killer cells

[192]. These studies were further corroborated by more recent studies in which IL-15$^{-/-}$ mice were not protected against HSV-2, but wild type animals depleted of natural killer cells were [193]. Although it is somewhat unclear as to the mechanism by which IL-15 mediates protection in this herpes model, it is also known that effective CD4 responses are essential for viral clearance, and IL-15 supports the basal proliferation of CD4 cells and enhances the TCR-dependent proliferation of Th1/Th17 (IFNγ, IL-17 double positive) cells [194]. On the whole, how IL-15 signaling affects the cell population most important for control of a pathogen determines the importance of IL-15 as an immune modulator in that system. Thus, it is of critical importance that the role of IL-15 be examined in individual infections: systemic or mucosal.

*Chronic Viral Infection/HIV*

The chronic virus-mediated disease in which immune intervention via IL-15 targeting has received the most attention is Human Immunodeficiency Virus. IL-15 treatment has been explored as a method of enhancing NK cell responses to control viral replication, immune restoration in patients in whom antiretroviral therapy is sufficient to suppress viral load but T cell counts remain low, and as a potential adjuvant for vaccines designed to elicit robust T cell memory [195]. In vitro, IFNγ production by CD8 T cells from SIV and SHIV-infected macaques was increased by IL-15 stimulation [196]. When administered as a treatment in vivo to chronically SIV infected macaques, IL-15 augmented populations of effector memory CD8 T cells and NK cells [197]. IL-15 treatment has also been shown to augment populations of effector memory CD4 T cells in anti-retroviral treated animals [198]. Although IL-15 treatment had no effect on viral load in either of these studies, the ability to expand effector memory CD8 and CD4 T cell

compartments reveal potential for this mode of treatment in immune reconstitution. Additionally, IL-15 treatment alone or in combination with IL-2 was effective at inducing new CD4 and CD8 memory to influenza and tetanus toxins, indicating that IL-15 treatment may also be effective in ameliorating the severe consequences of coinfection in HIV-infected individuals [199]. In studies of human immunodeficiency virus (type 1), in vitro experiments have revealed that IL-15 can increase IFNγ production and chemokine secretion by NK cells isolated from HIV-1-infected subjects [200]. Increased IFNγ production, enhanced cytotoxicity, and protection from apoptosis upon IL-15 treatment in vitro has also been shown for CD8 T cells isolated from HIV-1-infected subjects [201]. Because IL-15 treatment expands T cell populations, however, IL-15 treatment could increase viral targets and lead to increased viral load [195]. In models of acute SIV infection, IL-15 treatment increased viral setpoint by 3 logs and accelerated disease progression in 2 out of 6 animals [202].

Observations that IL-15 could increase viral replication lead to the hypothesis that IL-15 might be an effective vaccine adjuvant, increasing the immunogenicity of the vaccination. Indeed, HIV DNA vaccines co-infected with the IL-15 gene enhanced CD8 T cell responses [203], and similar platforms in which DNA vaccines encoding SIV antigen were coinjected with an IL-15 containing plasmid were associated with enhanced protection from an SHIV (SHIV 89.6P) challenge when compared to animals receiving the vaccination alone [204]. Comparable studies, however, report no clinical differences between vaccinated and vaccinated plus IL-15-adjuvanted groups in terms of viral load, although adjuvanted animals did exhibit increases in SIVgag-specific T cell responses [205]. Finally, when IL-15 was employed to adjuvant a vaccine to a more virulent

challenge virus, SIV mac251, the IL-15-induced proliferation of viral targets appeared to abrogate any benefits from the vaccine itself [206]. Therefore, while IL-15 seems to have little potential as an HIV vaccine adjuvant, the consistently reported expansion of effector memory T cell populations and no disease exacerbation in the chronic phase does suggest that IL-15 may be an effective therapeutic for immune reconstitution in HIV-infected individuals.

*IL-15 and Immune Intervention to Viral Pathogens*

Because IL-15 production in response to viral infection appears to an important mechanism of immune control of that virus, there has been considerable interest in trying to use IL-15 expression as a method of adjuvanting vaccines and/or improving immune responses to viral pathogens. These interests have been further encouraged by the development of IL-15-expression vectors and therapeutics for immune-intervention and reconstitution for HIV, and their application has been gradually expanded to include various other viral pathogens. Indeed, such strategies have met some successes. A vaccinia-virus-based vaccine platform was engineered to express the hemagglutinin and neuraminidase from H5N1 influenza viruses as well as IL-15 induced cross-neutralizing antibody responses and robust cellular immune responses that conferred cross-clade protection to mice challenged with different H5N1 viruses [207]. Additionally, a smallpox vaccine with integrated IL-15 has been shown to protect cynomolgus monkeys from a lethal dose of monkeypox virus administered three years post vaccination [208]. Enhanced immune responses to plasmid DNA vaccination by IL-15 was also reported against hepatitis B surface antigen [209] and the HSV-1 glycoprotein B [210]. However, not all attempts have yielded such promising results. Immunizing rhesus macaques with

different doses of IL-15 expressing plasmid in an influenza immunogenecity model, Yin et al. found that whereas low doses of IL-15 were effective at improving T cell responses, high doses of IL-15 (4mg) decreased production of IFNγ and T cell proliferation [211]. These data highlight the importance of optimizing adjuvants but overall reveal a significant potential for IL-15 as an effective strategy for improving vaccines to viruses.

**1.8 Structure of the Dissertation**

IL-15 represents a prime candidate for immune intervention in a variety of pathological settings, including many viral diseases. IL-15 emerged as a candidate for immunotherapy because of its potency in promoting the activation, migration, survival, and proliferation of NK and CD8 T cells, lymphocyte populations that are primary effectors of innate and adaptive immune responses. Although both of these cell populations are known to participate in the immune response to influenza infection, there remains a gap in our knowledge of how these lymphocytes migrate into the lung airways to control primary infections and how these cells might be maintained in the lung airways long term to control secondary infections. We hypothesized that influenza-induced expression of IL-15 would induce the migration of CD8 T cells to the lung airways and that limited availability of this cytokine after viral clearance would cause the decline of these cells in this site. In chapter two of this dissertation we demonstrate that influenza infection induces the local production of IL-15 and describe a role for this cytokine in inducing the migration of CD8 Teff to the lung airways. In chapter 3, we further explore the role of IL-15 in mediating migration of lymphocytes to the lung airways following influenza infection and propose that CD8 T cell migration to the lung airways may be a consequence of the direct recruitment of NK cells by IL-15. Finally, in chapter 4, we

investigate IL-15's role in maintaining influenza-specific memory CD8 T cells. The final chapter of this dissertation goes through the key findings of our work, explores the implications of these findings in the context of scientific community's current understanding of antiviral immune responses, and suggests avenues for further research that might deepen that understanding.

# CHAPTER 2

## A ROLE FOR IL-15 IN THE MIGRATION OF EFFECTOR CD8 T CELLS TO THE LUNG AIRWAYS FOLLOWING INFLUENZA INFECTION[1]

[1] Verbist, K., C. Cole, M. Field, and K. Klonowski. 2011. *The Journal of Immunology*. 186:174-182. Reprinted here with permission of publisher.

## 2.1 **Abstract**

The cytokines generated locally in response to infection play an important role in CD8 T cell trafficking, survival, and effector function, rendering these signals prime candidates for immune intervention. In this paper, we show that localized increases in the homeostatic cytokine IL-15 induced by influenza infection is responsible for the migration of CD8 effector T cells to the site of infection. Moreover, intranasal delivery of IL-15–IL-15Ra soluble complexes (IL-15c) specifically restores the frequency of effector T cells lost in the lung airways of IL-15–deficient animals after influenza infection. Exogenous IL-15c quantitatively augments the respiratory CD8 T cell response, and continued administration of IL-15c throughout the contraction phase of the anti-influenza CD8 T cell response magnifies the resultant CD8 T cell memory generated in situ. This treatment extends the ability of these cells to protect against heterologous infection, immunity that typically depreciates over time. Overall, our studies describe what to our knowledge is a new function for IL-15 in attracting effector CD8 T cells to the lung airways and suggest that adjuvanting IL-15 could be used to prolong anti-influenza CD8 T cell responses at mucosal surfaces to facilitate pathogen elimination.

## **2.2 Introduction**

Current influenza vaccines rely on the induction of a neutralizing Ab response to viral coat proteins that are subject to variation from antigenic drift and shift [7]. An alternate vaccination strategy is to immunize against conserved, internal viral proteins recognized by CD8 T cells, a strategy that confers protection against multiple serotypes, or heterosubtypic immunity [77,212,213]. Such a “universal” vaccine could provide cross-protection against the emerging and imminent threats of avian H5N1 and swine-origin H1N1 pandemics. However, despite such promise in generating a cross-protective

vaccine, the number of memory T cells (Tmems) in the lung airways that differentiate from local effector cell precursors declines over time, eventually plateauing ~3 mo postinfection (p.i.) [69]. Curiously, protective heterosubtypic immunity declines concomitantly, despite the large reservoir of Tmems in the spleen that continues to migrate to the lung airways and maintains a local steady-state pool of Tmems [74,77]. Overall, these data suggest that the effector T cells (Teffs) that gain access to the airways early p.i. and differentiate to Tmems in situ are responsible for the limited heterosubtypic immunity. Thus, understanding how to augment the initial seeding pool of Teffs in the lung airways may represent a unique methodology to increase the resultant pool of Tmems that develops in the respiratory tract and to coordinately prolong protection after a challenge infection.

IL-15 is a common g-chain cytokine sharing overlapping signaling and biological properties with IL-2 as a result of their mutual usage of the IL-2/-15β and common γ-chain (γc) receptor subunits [81,82]. Cells coexpressing IL-15 with IL-15Rα on their surfaces present the cytokine in trans to responsive IL-2/-15b/γc–expressing lymphocytes, a process referred to as transpresentation [89]. Although production of IL-2 is confined to periods of lymphocyte activation, the constitutive expression of IL-15 maintains the homeostatic proliferation of lymphocytes, most notably memory CD8 T cells, in the steady-state through the sustained expression of bcl-2 [83,102,144,182]. Pathogenic triggers such as type I IFNs, however, can induce the expression of IL-15, and Teffs upregulate expression of IL-2/-15β after activation [141,144,214]. In addition, systemic administration of IL-15 or soluble IL-15–IL-15Rα complexes (IL-15c) during the contraction phase of the immune response against Listeria

monocytogenes and Mycobacterium bovis prolongs Teff survival and results in the preferential accumulation of short-lived KLRG-1hi Ag-specific Teffs [215]. Taken together, these observations suggest that infection-induced alterations in IL-15 expression could modulate regional CD8 Teff responses.

Recent studies indeed demonstrated that a subset of dendritic cells (DCs) in the lung transpresent IL-15 to Teffs that have migrated to the respiratory tract after influenza infection [149]. Removal of IL-15–producing DCs from the respiratory tract or IL-15 blockade decreases the frequency of Ag-specific CD8 Teffs recovered from the lung. Although the attrition of Teffs in the lung is accelerated in the absence of IL-15, it remains unclear whether this is solely due to the loss of a pertinent survival signal or also the impaired ability of Teffs to traffic to the lung airways, because IL-15 is chemotactic in certain inflammatory milieus [176,177]. In this article, we demonstrate that influenza infection potentiates IL-15 levels in the respiratory tract that drives the migration of Teffs into the lung airways. Moreover, the therapeutic provision of IL-15 during this effector phase of the immune response translates into a greater proportion of locally derived Teffs that differentiate into Tmems in situ. Overall, our studies describe a function for IL-15 in T cell migration in which IL-15c can be used to augment respiratory CD8 T cell effector responses to extend the duration of heterosubtypic immunity and provide long-term immunological protection to subsequent influenza virus infections.

## 2.3 Materials and Methods

*Mice, viruses, and infection*

C57BL/6 mice were purchased from NCI or Taconic, and age and sex-matched IL-15$^{-/-}$ mice were generously provided by Dr. Leo Lefrançois (University of Connecticut, Farmington, CT) or obtained from Taconic. Influenza viruses A/HK-x31(x31, H3N2) and A/PR/8 (PR8, H1N1) were generously provided by Dr. S. Mark Tompkins (University of Georgia, Athens, GA). The recombinant x31-ova expressing the CD8 H2-Kb restricted SIINFEKL epitope was generously provided by Dr. Peter Dougherty (St. Jude Children's Research Hospital, Memphis, TN) [216]. Animals were infected with either $10^3$ pfu of x31 or $5x10^3$ pfu of PR8 intranasally in 50 µl PBS.

*Quantitative RT-PCR*

Total RNA was purified from tissues of naïve or $10^3$ pfu x31-infected C57BL/6 mice using the RNeasy Plus Mini Kit (Qiagen). Reverse transcriptions were primed with random primers and performed using the High Capacity cDNA Reverse Transcription Kit from ABI (Foster City, CA). Quantitative PCR assays were done using the ABI TaqMan Gene Expression Master Mix from ABI 7500 Real Time PCR System. Only ratios with a SE<0.2 log (95% confidence limits) were considered for determination of induction levels. Quantitative real-time RT-PCR was performed using IL15-FAM (#mm00434210_m1) and 18s-VIC (#4319413E) assays (ABI). Thermal cycling conditions were 30 min at 48°C, 10 min at 95°C, and 40 cycles of denaturation (95°C for 15sec) and annealing (60°C for 60 sec). Samples were analyzed in triplicate, normalized against 18s and expressed relative to mock infected animals. The results are expressed as fold induction of gene expression (RQ) using the ΔCt method [217].

*IL-15c ELISA*

The Ready-Set-Go Mouse IL-15/IL-15R Complex ELISA kit was purchased from eBioscience. Whole blood was collected from IL-15$^{-/-}$, naïve, and influenza-infected animals at various time points post influenza infection, allowed to clot for 1 hour at RT, and clarified via centrifugation at 3,000 g for 10 min. Lungs from the same animals were homogenized in 0.5 ml of PBS and the lysate was used for analysis. Supernatants (100 μl) of samples were used in duplicate. ELISA plates were incubated with samples at RT for 2.5 hours. Plates were read with a Molecular Diagnostics Vmax microplate reader (Molecular Devices Corporation, Sunnydale, CA) at 450 nm and analyzed with Softmax software (Molecular Designs). The standard curve had a minimal regression coefficient of 0.99.

*Tissue preparation and flow cytometry*

Single-cell suspensions were obtained from spleens and lymph nodes by passing homogenized organs through cell strainers. Spleens were depleted of erythrocytes using Tris-buffered Ammonium Chloride. Peripheral blood was collected from the retro-orbital sinus and also depleted of erythrocytes. Lung airway-resident cells were harvested following intratrachial introduction and recovery of 1ml PBS 3-5x. Following BAL recovery, lymphocytes were isolated from the perfused lung parenchyma. Following perfusion with ~25 mL PBS/heparin, lungs were excised, minced, and incubated at 37°C with 1.25 mM EDTA for 30 min followed by 6 mg/mL collagenase for 60 min at 37°C. After passage through cell strainers, cells were resuspended in 44% Percoll underlayed with 67%, centrifuged, and lymphocytes at the interface were collected.

The influenza NP MHC class I (H-2D(b)/ASNENMETM) tetramer was generated by the NIAID Tetramer Facility (Emory University). Tetramer staining was conducted at RT for 1 hr in conjunction with other mAbs from eBioscience (PerCP/Cy5.5-conjugated αCD4 or αCD8, and Pe/Cy7-conjugated αCD44 or αCD45.1), BD Pharmingen (Pe-Cy7-conjugated αCD11a and APC/Cy7-conjugated αCD8), or Caltag (FITC-conjugated αCD122). Stained cells were analyzed using a BD LSR II digital flow cytometer and BD FACs Diva software.

*Enrichment for NP-specific naïve CD8 T cell precursors*

Single cell suspensions from spleens and all visible lymph nodes (cervical, axillary, mesenteric, and inguinal) were depleted of erythrocytes, washed, and stained with both PE- and APC-labeled influenza-NP specific MHC class I (H-2D(b)) tetramers, anti-CD8-PerCP, and Fc block for 1 hr RT in 1 mL FACS buffer. Cells were subsequently washed, resuspended in 500 μl MACS buffer (PBS with 0.1% NaN3, 0.5% BSA, and 2mM EDTA, degassed), and labeled with 50 μl anti-PE microbeads (Miltenyi Biotec) for 20 minutes at 4°C. Cells were again washed and passed over a magnetized LS column (Miltenyi Biotech). Bound cells were eluted from the columns and stained with a PeCy7-labeled dump gate (including anti-CD4, 19, and CD11c). The frequency and number of NP-specific cells in each cohort of animals was determined by gating on $CD8^+$, $dump^-$ lymphocytes.

*Adoptive transfers and migration and proliferation assays*

CD8 T cells were enriched to >88% purity from spleens or lung tissue by negative enrichment per the manufacturer's instructions (Dynal). For *in vitro* migration assays, cells from the indicated tissues were placed in the top insert of a 0.4 μm chemotaxis

Transwell (Fisher Scientific). The bottom chamber contained either warm media alone or supplemented with 100 ng/ml recombinant murine IL-15/IL-15Rα Fc-chimeric complexes (R&D Systems). Percent migration was calculated as # NP-tet+ CD8 T cells in the bottom chamber / # NP-tet+ CD8 T cells in the input sample. For *in vivo* migration assays, IL-15c consisting of 1.5 µg recombinant murine IL-15 and 7 µg IL-15Rα Fc-chimera (R&D Systems) were generated by incubation at 37°C for 20 min followed by incubation at 4°C for 10 min and delivered i.n. in 72.5 µl PBS. Similarly, IL-2c and IL-7c consisting of 1.5 µg recombinant murine IL-2 (ebioscience) or IL-7 (Peprotech) and 7 µg mAb (R&D Systems) were generated by incubation at 37°C for 20 min followed by incubation at 4°C for 10 min and delivered i.n. in 72.5 µl PBS. IL-15c were also used to treat animals throughout the contraction phase of the anti-influenza response and were delivered i.n. in 36.25 µl PBS every other day for 10 days. To measure cell survival and proliferation, animals were pulsed i.p. with 200 µl of 10 mg/ml of BrdU (Sigma). Cells isolated from these animals 12 hr later were stained with 20 µl 7-AAD (BD Pharmingen) at 4°C for 20 min, surface stained as previously described, and intracellularly stained with a FITC-labeled αBrdU mAb (BD Pharmingen).

*Plaque assays*

Plaque assays were performed as previously described [218]. Briefly, lungs from x31-immune WT and IL-15$^{-/-}$ mice challenged with PR8 were lysed at the indicated times in 1ml 1x MEM+1µg/ml TPCK-treated trypsin. Confluent monolayers of MDCK cells were incubated with 10-fold serially diluted 10% homogenate for 1 hr at 37°C. The inoculums were removed and cells were washed with PBS. Monolayers were then overlaid with MEM containing 1.2% Avicel microcystalline cellulose (FMC BioPolymer,

Philadelphia, PA) [218], 0.04M HEPES, 0.02 mM L-glutamine, 0.15% $NaHCO_3$ (w/v), and 1µg/ml TPCK-trypsin. 72 hrs post infection, monolayers were fixed with cold methanol: acetone (60%:40%) and stained with crystal violet.

*Statistics*

Where appropriate, an unpaired two-tailed student's T test was applied using Prism Graphpad software. P values are indicated in the figure legend where statistical significance was found. For multiple comparisons, an analysis of variance was applied with a Tukey's *post hoc* analysis using Prism Graphpad software. P values less than 0.05 were considered significant.

## 2.4 Results

*Infection with influenza virus induces localized IL-15 expression in the respiratory tract*

IL-15 is constitutively expressed systemically by DCs and macrophages but can also be expressed in mucosal tissues by epithelial cells [83,96,219]. Moreover, cellular expression of IL-15 is regulated by various pathogenic triggers (15). Namely, the IL-15 promoter contains a type I IFN regulatory element [141,214], and IL-15 expression is increased in response to type I IFNs [144] which are abundant following influenza infection [220]. Indeed, influenza infection has been shown to induce the expression and transpresentation of IL-15 in the lungs at day 6 p.i. [149]; however, we wished to more carefully characterize the kinetics of IL-15 expression following respiratory infection to better define how pathogen-induced IL-15 impacts local CD8 T cell responses. Since IL-15 protein has been historically difficult to detect in biological solutions, we first monitored IL-15 mRNA expression in the lung airways (via broncheoalveolar lavage [BAL]), lung parenchyma, lung-draining mediastinal lymph nodes (MdLN), and spleens

of C57BL/6 mice following intranasal (i.n.) infection with influenza A/Hong Kong x31(x31). By 3 days post infection (p.i.), there was a four-fold induction in the relative expression of IL-15 in the lung airways of infected animals when compared to naïve, i.e. mock-infected, animals, and IL-15 mRNA levels remained elevated until day 7 p.i. (Fig. 1). While IL-15 transcription in the airways began to drop by day 10 p.i., it remained threefold higher than the levels in naïve mice. Expression of IL-15 also increased in the lung parenchyma, MdLN, and spleen following infection, albeit to a much slighter degree. Additionally, a newly developed ELISA which specifically detects soluble IL-15/IL-15Rα complexes (IL-15c) confirmed that expression of IL-15c in the lung lysate was greatly enhanced and quantitatively much greater (than the serum) of influenza-infected animals on day 3 p.i. when compared to naïve mice (Supplemental Fig. 1). Therefore, infection with influenza virus initiates an inflammatory cascade which results in a local increase in the expression of IL-15 suggesting that this cytokine may specifically regulate anti-influenza CD8 Teff responses in the lung airways.

*IL-15 is responsible for the early accumulation of anti-influenza Teff in the lung airways*

IL-15 prolongs the survival of activated CD8 Teff during the contraction phase of the immune response to systemic viral infection, and systemically administered IL-15 can specifically restore the numbers of Teff lost in IL-15$^{-/-}$ mice [174,175,221,222]. Recent data also suggests that IL-15 modulates local respiratory CD8 Teff responses via sustained survival signals provided by lung-resident DCs [149]. However, given that transient augmentation of IL-15 mRNA expression immediately precedes the initial influx of Teff to the lung airways (Fig 1; Teff first detectable ~5.5 days p.i. (data not shown)), the documented chemotactic properties of IL-15, and the fact that lung

parenchymal cells (including influenza-infected lung epithelial cells) can also express IL-15 [223], we hypothesized that in addition to supporting the survival anti-influenza CD8 Teff, IL-15 could also participate in the recruitment of the Teff to the site of infection. To that end, IL-15$^{-/-}$ and WT animals were infected i.n. with x31, and the kinetics of the CD8 T cell response against influenza nucleoprotein (NP) was assessed using MHC class I tetramers on lymphocytes isolated from the BAL, lung parenchyma, spleen, and MdLN. Since IL-15$^{-/-}$ animals have half the total number of CD8 T cells as WT animals [101,102], and correspondingly half the number of NP-specific precursors as wild type mice (but the same frequency) (Supplemental Fig. 2A-C), the anti-NP CD8 T cell response is expressed as a percentage of total CD8 T cells to accurately compare rates of expansion.

Interestingly, the lung airways of IL-15$^{-/-}$ mice harbored only half the frequency of influenza-specific Teff at day 7 p.i., (Fig. 2A) and only 25% at day 12 p.i. (Fig. 2B). This reduction in the frequency of NP Tet+ CD8 T cells was observed as early as day 6 (~40%) but resolved by day 15 p.i. (data not shown). Moreover, when the total number of NP-specific cells recovered from the airways of IL-15$^{-/-}$ at d6.5 p.i. was assessed, the loss of NP-specific Teff in IL-15$^{-/-}$ mice was greater than the 50% numerical reduction resulting from their reduced NP-specific precursor number (Supplemental Fig. 2D). Only the CD8 and NK cell pools were numerically deficient in the BAL from infected IL-15$^{-/-}$ mice, and the numbers of B220+ B cells and populations of CD11b and CD11c+ cells were unaffected (data not shown). Furthermore, these deficiencies in CD8 Teff accumulation were specific to the lymphocytes recovered from the BAL, as the percentage of influenza-specific Teff in the lymphoid tissues of IL-15$^{-/-}$ mice was not

compromised (Fig. 2A-B). The frequency of NP-specific CD8 T cells was also reduced in the lung parenchyma of IL-15$^{-/-}$ animals at day 12 p.i., though not to significant levels. Thus, an IL-15 deficiency resulted in a specific reduction in the frequency of influenza-specific CD8 Teff at the site of infection.

In order to confirm our finding that the absence of IL-15 selectively reduces influenza-specific CD8 Teff in the lung airways with equal Ag-specific precursors, we adoptively transferred 500 ovalbumin (ova)-specific CD8 T cells from congenic (CD45.1$^{+}$) OT-I mice into WT and IL-15$^{-/-}$ CD45.2$^{+}$ mice. Recipient animals were subsequently infected with x31-ova, and on day 12 p.i., lymphocytes were isolated from the BAL, lung, and lymphoid tissues, and the level of donor OT-I expansion was calculated. The quantity of OT-I cells recovered from the spleen, MdLN, and lung parenchyma was similar between WT and IL-15$^{-/-}$ recipients (Fig. 2C). We did observe, however, a 45% reduction in the number of donor OT-I cells isolated from the BAL of IL-15$^{-/-}$ mice. These data confirm our findings that the absence of IL-15 results in a tissue-specific reduction of Ag-specific CD8 Teff in the lung airways of influenza-infected animals.

*Delivery of exogenous IL-15c intranasally enhances the accumulation of influenza-specific CD8 $T_{eff}$ at the site of infection*

While intravenous administration of γc cytokines can affect CD8 T cell responses systemically, it is unclear whether such cytokine therapies can be modified to spatially limit their function. Since an IL-15 deficiency resulted in the selective reduction of CD8 Teff in the respiratory tract, we sought to determine whether administration of exogenous IL-15 to the lung airways could rescue this defect. To that end, we intranasally delivered

either PBS or IL-15/IL-15Rα complexes (IL-15c) to WT or IL-15$^{-/-}$ animals 12 days p.i. with x31. Delivery of cytokine/ receptor complexes increases the cytokine's potency *in vivo* presumably by increasing its half-life [120,224]. Twelve hours post treatment with IL-15c, cells from the BAL were collected and analyzed for tetramer reactivity. As shown previously, the frequency of NP Tet+ CD8 T cells isolated from the lung airways of IL15$^{-/-}$ mice was reduced by 50% compared to WT animals; however, this percentage was restored to WT levels when exogenous IL-15c was administered to the respiratory tract of these mice (Fig. 3A).

IL-15 is known to prolong the survival of CD8 Teff mainly through downstream signaling events that result in the enhanced expression of the anti-apoptotic molecule Bcl-2 (26). Accordingly, it has recently been demonstrated that, following influenza infection, IL-15 transpresented to CD8 Teff in the lung airways is able to protect this population from rapid apoptosis [149]. It remains unclear, however, how much of the accumulation of lung-airway-resident CD8 Teff is the result of improved survival and how much is due to IL-15-induced migration into the site. The idea that IL-15 can induce the chemotaxis of lymphocytes is not unprecedented. Natural killer cells, which are dependent on IL-15 for their development, will migrate to IL-15 in an *in vitro* chemotaxis assay, and overnight treatment of these cells with IL-15 increases their binding to cultured endothelial cells [129]. Moreover, T lymphocytes can invade IL-15-containing collagen gels and transmigrate through endothelium in response to IL-15 [176,225]. Despite these *in vitro* assays demonstrating the chemotactic potential of IL-15, there has been limited inquiry into whether or not IL-15 participates in the recruitment of CD8 T cells to sites of infection *in vivo*.

Because of the documented chemotactic properties of IL-15, the concentrated regional expression of IL-15 following influenza infection, and the failure of Teff to accumulate in the lung airways of IL-15$^{-/-}$ mice following influenza infection, we hypothesized that IL-15 participates in recruiting influenza-specific CD8 Teff to the site of infection. To test this hypothesis, we first tested whether locally instilled exogenous IL-15 could induce the chemotactic migration of NP-specific CD8 Teff to the lung airways. WT animals were infected with x31 and 6.5, 12, or 40 days later, we intranasally administered either PBS or IL-15c. We monitored alterations in the accumulation of NP-specific CD8 Teff in the lung airways 12 hrs post treatment to distinguish the effects of IL-15c on migration from its potential effects on proliferation or protection from apoptosis. Administration of exogenous IL-15c increased the number of NP-specific CD8 T cells in the lung airways 8 fold (or ~70 x $10^3$ more cells) than PBS treated mice at d12 p.i. (Fig. 3C) while ~50x$10^3$ NP-specific CD8 Teff were correspondingly lost from the splenic pool of Teff (data not shown) suggesting that this Teff reservoir may be recruited to the BAL in response to IL-15c treatment. Moreover, local delivery of IL-15c induced the migration of activated CD44$^{hi}$ CD8 and CD4 T cells to the lung airways (Fig. 3 C), while naïve (CD44$^{lo}$) CD8 and CD4 T cells and B220+ cells did not accumulate (data not shown). The specific augmentation in the number of influenza-specific CD8 Teff could only be observed as early as day 6.5 p.i. (Fig. 3B) since few NP-tet+ CD8 T cells could be recovered from the BAL at earlier time points, but this effect was abrogated by the memory phase of the response (day 40 p.i.) (Fig. 3 D). These data indicate that either Tmem are refractory to IL-15-mediated migratory signals or are incapable of accumulating in measurable numbers considering that the vasculature beds may be less

permissive for lymphocyte entry in the absence of inflammation, irrespective of a chemotactic signal. IL-15 was specifically responsible for eliciting the migration of influenza-specific CD8 Teff to the lung airways since the intranasal instillation of complexes of the common gamma chain cytokines IL-2 or IL-7 failed to induce the trafficking of CD8 Teff to the lung airways (Fig. 3 E).

Recently, it has been reported that pulmonary DCs promote the survival of influenza-specific CD8 Teff through transpresentation of IL-15 [149]. In our studies, it was possible that, in addition to the effect of IL-15 on the migration of CD8 Teff, IL-15-dependent proliferation and/or enhanced survival were also contributing to the observed accumulation of CD8 Teff in the lung airways following IL-15c treatment. To test whether administration of IL-15c affects CD8 Teff proliferation, we infected animals with x31, and, 12 days later, we pulsed animals with 5-bromo-2'-deoxyuridine (BrdU) and simultaneously administered PBS, IL-15, IL-7, or IL-2 complexes i.n.. Compared to PBS treated animals, treatment with any of the γc cytokine complexes did not augment the levels of BrdU incorporation by BAL resident anti-influenza Teff (Fig. 3 F-G). To test whether IL-15c enhanced the survival of Teff otherwise destined to die, CD8 Teff isolated from the lung airways 12 hrs post γc cytokine complex treatment were stained with 7-AAD, a fluorescent dye excluded from viable cells. Analysis of NP-specific CD8 T cells from IL-15c, as well as IL-7 and IL-2 complexes, revealed that all three cytokine treatments enhanced the survival of CD8 Teff since ~50% more of these CD8 T cells excluded 7-AAD (Fig. 3 F-G). In accordance with previous studies, these data confirm that IL-15 provides sustained survival signals to influenza-specific Teff in the absence of proliferation [149]. However, IL-2 and IL-7 complexes were also effective at enhancing

the survival of influenza-specific Teff without affecting Teff accumulation in the lung airways as observed with IL-15c treatment (Fig. 3 E). Thus, these data show that exogenous IL-15c delivered to the lung airways uniquely induced the migration of CD8 Teff to the site where interaction with IL-15 also augmented survival *in situ*.

*IL-15 induces the migration of influenza-specific CD8 Teff from the systemic Teff pool*

Our data demonstrate that localized *in vivo* administration of exogenous IL-15c recruited influenza-specific CD8 Teff to the lung airways; however, it is unclear whether this IL-15-induced migration was the result of direct chemotaxis to the IL-15c, modification of chemokine receptor expression by IL-15c treatment, or an alteration of a secondary chemotactic signal by an accessory cell affected by the IL-15c treatment. To begin to distinguish between these possibilities we monitored the direct effects of IL-15c on $T_{eff}$ migration in an *in vitro* chemotaxis assay. Single cell suspensions from the lung parenchyma, BAL, spleen, lung-draining MdLN, and non-draining inguinal lymph nodes of day 12 x31-infected animals were placed in the top chamber of a transwell chemotaxis chamber while media or media supplemented with IL-15c was placed in the bottom. After 90 minutes incubation, cells in the bottom chamber were collected, counted, and the percentage of NP-specific CD8 T cells which had migrated towards the IL-15c was determined. While NP-specific CD8 Teff isolated from both LNs migrated fairly efficiently towards the IL-15c, the Teff isolated from the lung parenchyma and spleen exhibited a 10 fold increase in migration to IL-15c (Fig. 4 A). However, the Teff isolated from the BAL migrated poorly towards IL-15c, presumably because they have down-regulated expression of IL-2/15Rβ (CD122) (Fig. 4 B) following IL-15-induced migration and arrival at their terminal destination.

Overall, how Teff cells enter the lung airways is not well understood because two routes of entry are available to them. Lymphocytes can enter the lung airways by either traversing the lung parenchyma or by bypassing the lung tissue and entering directly from the circulation [226]. Since CD8 Teff isolated from both the spleen and the lung tissue readily migrated to IL-15c *in vitro*, we asked whether influenza-specific CD8 Teff from one anatomical location preferentially migrated to IL-15c *in vivo*. To pursue this question, donor CD8 Teff were enriched from the lung or the spleen of day 12 x31-infected CD45.2+ WT donor mice and independently transferred into congenic CD45.1+ recipients that were 12 days p.i. with x31 (Fig. 5 A). At the time of adoptive transfer, recipient animals were given either IL-15c or PBS i.n., and, 12 hours later, the BAL from these animals was collected and analyzed for the presence of donor-derived CD8 Teff. Donor Teff were detected in the BAL of IL-15c treated animals only, confirming that IL-15 is capable of inducing the migration of CD8 Teff *in vivo* (Fig. 5 B-C). Interestingly, IL-15 elicited the migration of donor Teff isolated from the spleen but not the lungs (Fig. 5 C), suggesting that the majority of cells recruited into the lung airways post influenza infection are likely entering the lung airways from the circulation rather than by trafficking through the lung parenchyma. Together with the data presented in Fig. 2A,B wherein frequencies of NP-specific CD8 T cells are unaltered in the lung parenchyma but reduced in the BAL, these data indicate that circulating, Teff cells are partially dependent on IL-15 for their immigration into the lung airways and those Teff which have mobilized into the lung parenchyma either do not migrate to IL-15 or utilize unique IL-15-independent mechanisms to traverse into the airways.

*Mucosal delivery of IL-15c during the effector phase of the anti-influenza CD8 T cell response enhances the resultant Tmem pool*

Since IL-15 recruits influenza-specific CD8 Teff to the lung airways and prolongs their survival, adjuvanting IL-15 in the respiratory mucosa could numerically boost the CD8 Tmem generated from Teff maintained at this site. To test this hypothesis, WT mice infected with x31 were given IL-15c or PBS i.n. every other day between day 10-20 p.i.. Animals were rested until memory (day 31 p.i.) when cells from the lung airways and other indicated tissues were collected, counted, and analyzed for the presence of NP-specific CD8 T cells. Animals receiving IL-15c retained a higher frequency (2x) and number (2.5x) of influenza-specific CD Tmem in their lung airways compared to animals receiving PBS alone (Fig. 6A-B). To test the quality of this quantitatively augmented memory CD8 T cell pool, these animals were challenged on day 45 p.i. with a lethal dose of the heterologous H1N1 influenza A/Puerto Rico 8 (PR8). At 3, 6, and 8 days after receiving the challenge infection, lungs from both PBS-treated and IL-15c treated memory mice were collected and viral titers determined. At the earliest time (d3) post infection, the lungs of both WT and IL-15$^{-/-}$ mice contained less virus than those of naive controls; however, the lungs of IL-15c-treated animals had lower viral titers than those of PBS-treated animals (data not shown). More importantly, no detectable virus was isolated from IL-15c treated mice as early as day 6 post challenge while WT mice did not clear virus until 2 days later (Fig. 6C and data not shown). Thus, by altering the dynamics of the CD8 Teff initially seeding the respiratory tract, IL-15c quantitatively and qualitatively augmented the resultant Tmem developing in the lung airways, suggesting IL-15c therapy could be used as a means of prolonging heterosubtypic immunity to influenza infection.

## 2.5 Discussion

The prevention of influenza epidemics is reliant on the generation of a potent and long-lived pool of CD8 Tmem capable of cross-protecting hosts from multiple strains of virus which are continually evolving between and within humans and other mammalian and avian hosts. Unfortunately, the duration of the protection afforded by respiratory CD8 Tmem is limited, as these cells are lost over time [69,77]. Given that the factors responsible for this attrition are ill defined, a reasonable methodology to prolong long-term immunity to influenza infection is to reset the initial frequency of antigen-specific CD8 Teff seeding the lung airways which would coordinately increase the numerical output of Tmem. This methodology requires a deeper understanding of the factors and mechanisms used by antigen-specific CD8 Teff to migrate into and survive within the lung airways following influenza infection for exploitation as a vaccine adjuvant.

In the fifteen years since its discovery, IL-15 has been implicated in the development, activation, or maintenance of many cell types both inside and outside the immune system [83]. The ability of IL-15 to mediate so many effects on divergent cell populations requires regulation of both IL-15 and its receptors. Our studies define a new function for IL-15, namely modulating the trafficking of Teff to the lung airways. This function of IL-15 may require regulation distinct from our traditional view of how IL-15 delivers signals for the survival and proliferation of CD8 Teff and Tmem. In these scenarios, IL-15 is transpresented by DCS and macrophages to CD8 Tmem that express CD122 [227-229]. While DCs can transpresent IL-15 in the lung airways after influenza infection, and this method supports Teff survival, substantial levels of IL-15 are produced by non-DC populations. Airway epithelial cells (AECs) constitutively express IL-15 and

IL-15Rα on their apical surfaces and are directly juxtaposed to the vascular endothelium [230]. During inflammation, AECs can be induced to express IL-15 on the basolateral surface, potentially making the cytokine available to cells in the vasculature [230]. Viral infection also induces the expression of metalloproteases such as ADAM17, which can cleave IL-15Rα from the surface of cells in order to create soluble IL-15/IL-15Rα complexes, similar to our intranasally delivered IL-15c [231]. Indeed, soluble IL-15c can be detected as early as d3 p.i. in the lung as well as in the serum (Supplemental Figure 1). With IL-15 message levels continuing to rise locally in the lung (in the absence of sustained or increasing levels of IL-15c), it is likely that these complexes are shed locally only during a distinct time frame, and subsequent responses to IL-15 in the lung are due to interaction with membrane bound complexes (Fig. 1, Supplemental Figure 1, (16)). These two forms of IL-15 are likely important in creating a chemotactic gradient for migrating CD8 Teff which instructs them leave the circulation and enter the lung airways. The spatial and temporal expression of the soluble vs membrane forms of IL-15 could regulate the migration and pro-survival functions of IL-15, respectively. Ongoing studies will explore these possibilities.

How CD8 T cells “see” these complexes is unclear; IL-15 has dual binding sites for IL-15Rα, and therefore CD8 T cells responding to these IL-15 complexes could potentially receive signals through either IL-15Rα or CD122, which are both expressed on the surface of Ag-experienced CD8 T cells [83,148]. Studies thus far have only focused on IL-15 signaling through CD122, however, IL-15Rα can transmit signals either alone or in conjuction with γc and the newly identified transmembrane tyrosine kinase Axl [232]. The differential coupling and signaling through these IL-15 receptors may

help integrate IL-15 signals into unique cascades that result in different biological functions, including survival, proliferation, and migration. Future studies in our lab will define how IL-15 signaling regulates Teff migration to the lung airways.

While signaling through CD122 can enhance the survival of KLRG-1$^{hi}$ Teff [175,233], and pulmonary dendritic cells can provide survival signals to resident influenza-specific CD8 Teff through IL-15 transpresentation [149], our data shows for the first time that IL-15 also evokes the chemotactic migration of pulmonary CD8 Teff to the site of infection. Anti-NP CD8 Teff isolated from secondary lymphoid tissues are only minimally affected by an IL-15 deficiency, despite the fact that influenza infection increased IL-15 mRNA levels in these sites, whereas respiratory bound anti-influenza effectors are numerically compromised. In addition, the timing of the influenza-induced IL-15 expression in the lung airways precedes and coincides with Teff influx and the anti-influenza CD8 Teff in the lung airways are enriched by 8-fold only 12 hours after IL-15c treatment, a timeframe more consistent with enhanced migration than survival (Fig. 3). While our *in vitro* chemotaxis assays suggest that the enhancement of Teff migration is a direct effect of IL-15 signaling, IL-15 can induce the transcription of chemokine receptors of the CC family in human PBLs [178]. Lymphocytes exposed to IL-15 also morphologically resemble motile T cells with polarized expression of chemokine receptors at the leading edge and adhesion molecules on the uropod [234]. Moreover, IL-15 specifically increases the expression of LFA-1, an integrin required for lung-specific trafficking of T cells [74,129]; however, none of these IL-15-induced effects on T cell migration have been studied in an infection model.

Perhaps the most significant finding of our work, however, is that IL-15c instilled directly into the respiratory tract can augment regional CD8 Teff responses and coordinately prolongs the persistence of immunological memory to influenza infection. Memory mice treated with IL-15c during the effector phase exhibited more rapid clearance of a heterologous influenza infection (Fig. 6), even when challenged a full 25 days after treatment had discontinued, implying that augmenting the number of Teff gaining access to the lung airways concordantly augments the resultant Tmem that differentiate *in situ* and mediate protection. Importantly, the use of IL-15 as a therapeutic or vaccine adjuvant may not be limited to influenza virus as infection with dual recombinant vaccinia viral vectors expressing both gp160 and IL-15 resulted in a heightened CD8 Teff and Tmem response against this HIV antigen [235]. While γc cytokines, most notably IL-2, have been used as adjuvants with vaccines against specific cancers and infectious diseases [236], toxicity and half-life issues have precluded their success in clinical trials. However, by complexing IL-15 to its receptor and delivering intranasally, direct communication between the cytokine and responding Teff can be established locally and temporally regulated without affecting lymphocytes in other sites.

In summary, our experiments demonstrate for the first time that IL-15c can be used therapeutically to augment the number of Teff that traffic to and survive in the lung airways, suggesting that IL-15c can be used as a vaccine adjuvant to boost site-specific immunity. The implications of continual enhanced recruitment of Teff to the lung airways could be extended to multiple disease models, including cancer and other chronic infections, but is especially relevant to influenza infection where the efficacy of CD8 T cell-mediated protection and viral clearance is linked to the number of virus-specific

CTLs present directly in the mucosa prior to challenge [69,77]. Periodic recruitment and augmentation of T cells reactive to conserved influenza epitopes by an adjuvanted IL-15c boost could sustain Teff above numerical thresholds and restore their protective capacity to both seasonal and pandemic strains of influenza.

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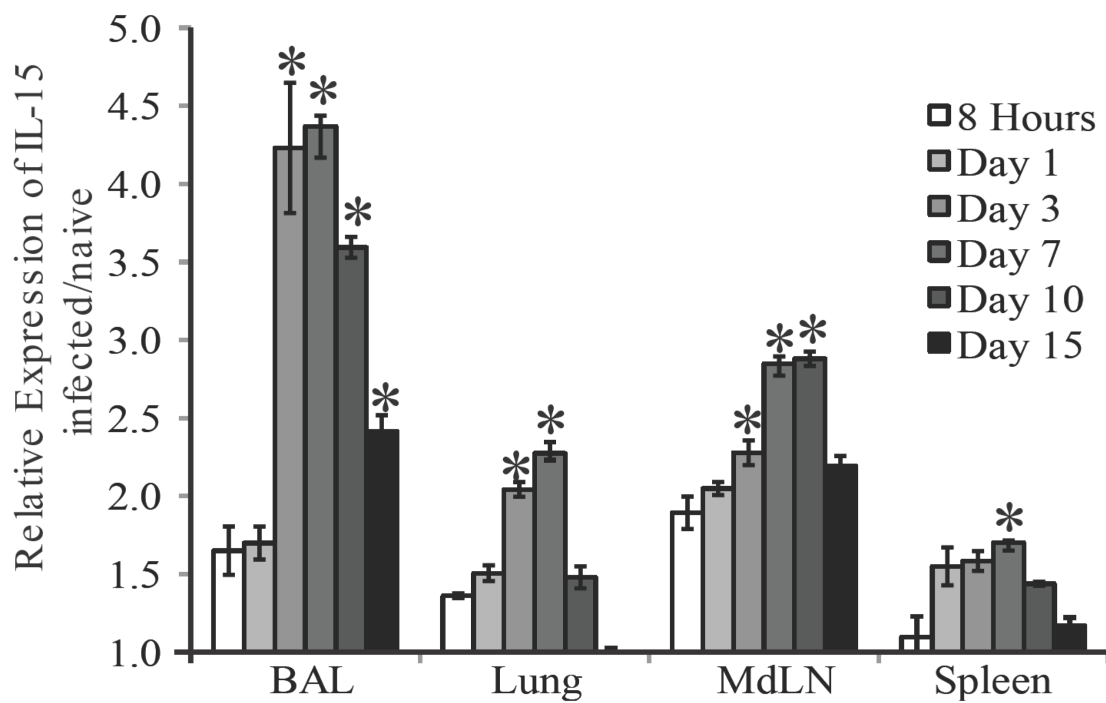


Relative Expression of IL-15 infected/naive
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
BAL
Lung
MdLN
Spleen
8 Hours
Day 1
Day 3
Day 7
Day 10
Day 15

**Figure 2.1. Influenza infection induces the local expression of IL-15 in the lung airways.** Gene expression levels were quantified from BAL, lung tissue, spleen and MdLN by RT-qPCR at the indicated time points post infection with x31, and are expressed as relative expression above a baseline in naïve animals normalized to 1 using the ΔCt method. Values are represented as mean ± SD (n=3; *p < 0.05 by ANOVA with Tukey's *post hoc* analysis) compared with mock-infected mice.

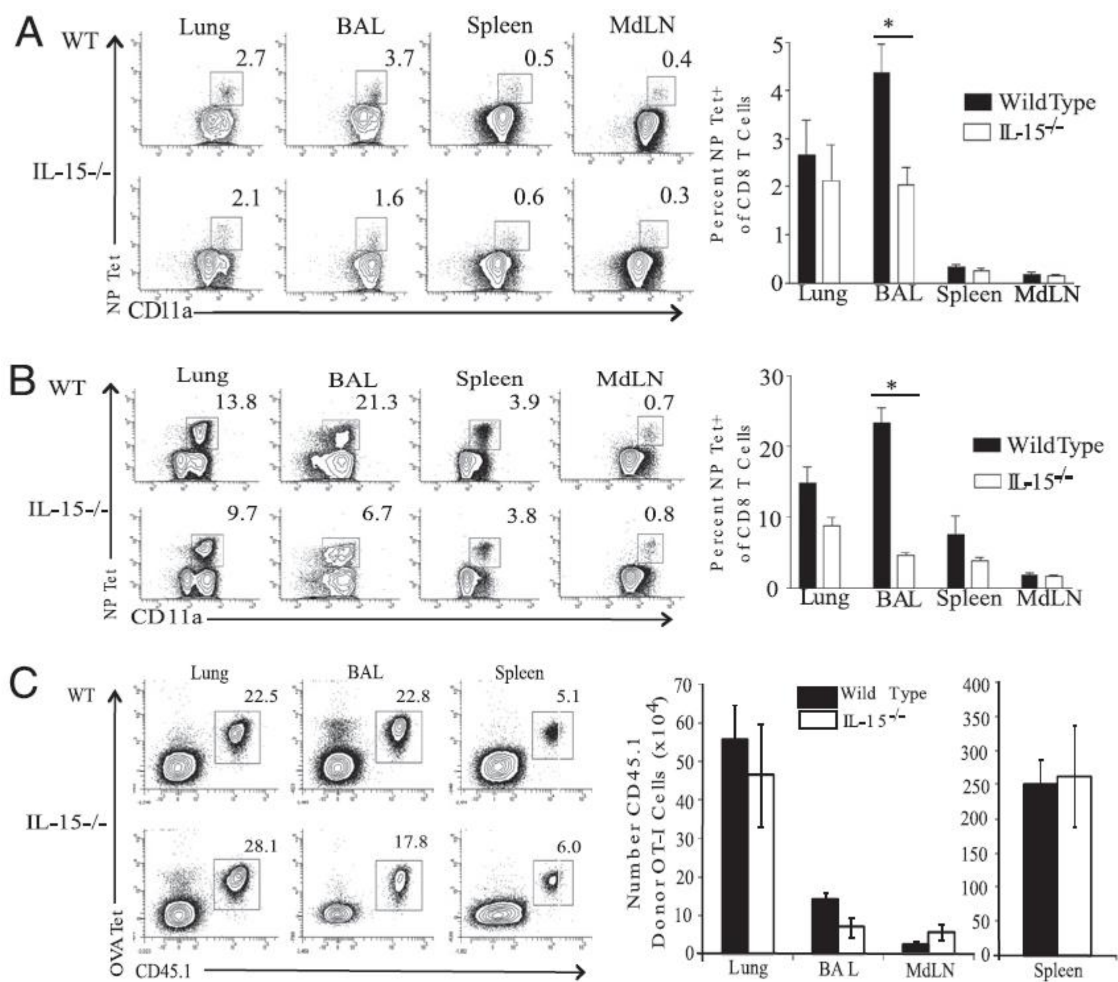
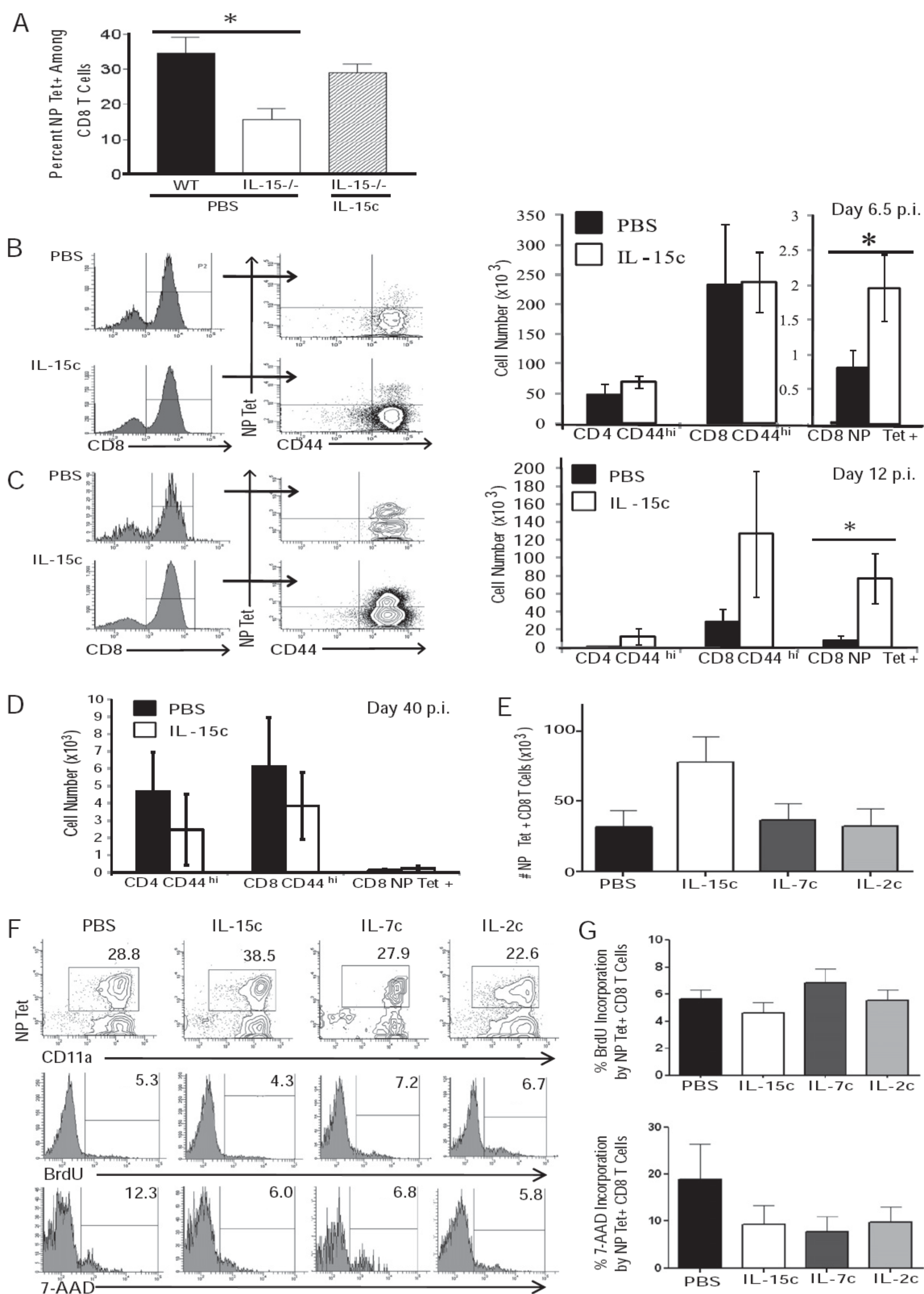

A
WT
IL-15-/-
Lung
BAL
Spleen
MdLN
2.7
3.7
0.5
0.4
2.1
1.6
0.6
0.3
NP Tet
CD11a
Percent NP Tet+ of CD8 T Cells
Wild Type
IL-15-/-
B
WT
IL-15-/-
Lung
BAL
Spleen
MdLN
13.8
21.3
3.9
0.7
9.7
6.7
3.8
0.8
NP Tet
CD11a
Percent NP Tet+ of CD8 T Cells
Wild Type
IL-15-/-
C
WT
IL-15-/-
Lung
BAL
Spleen
22.5
22.8
5.1
28.1
17.8
6.0
OVA Tet
CD45.1
Number CD45.1 Donor OT-I Cells (x10^4)
Wild Type
IL-15-/-
Lung
BAL
MdLN
Spleen

**Figure 2.2. An IL-15 deficiency results in a reduced frequency of influenza-specific effector CD8 T cells in the lung airways.** Lymphocytes from the indicated tissues were isolated and analyzed for tetramer reactivity. Representative flow plots for WT and IL-$15^{-/-}$ animals are shown for day 7 (A) and day 12 (B) p.i. with x31 i.n.. The mean percent tetramer positive among CD8 T cells for WT (shaded bars) and IL-$15^{-/-}$ (open bars) on day 7 p.i. (A) is represented ± SEM (n=3 mice/group; *p=0.0282) and on day 12 p.i. (B) ± SEM (n=3 mice/group; *p=0.0011). Data are representative of two independent experiments. (C) 500 naïve CD45.1+ OT-I CD8 T cells were adoptively transferred into WT or IL-$15^{-/-}$ CD45.2+ recipients subsequently infected with x31-ova. Representative flow plots from the BAL, lung, and spleen are shown (left). The mean number of donor OT-I CD8 Teff isolated from indicated tissues at day 12 p.i. is plotted ± SEM (n=4-5 mice/group) on right. Data are representative of three independent experiments.



A
Percent NP Tet+ Among CD8 T Cells
WT
IL-15-/-
IL-15-/-
PBS
IL-15c
B
PBS
IL-15c
CD8
NP Tet
CD44
Day 6.5 p.i.
PBS
IL-15c
Cell Number (x10$^3$)
CD4 CD44$^{hi}$
CD8 CD44$^{hi}$
CD8 NP Tet +
C
PBS
IL-15c
CD8
NP Tet
CD44
Day 12 p.i.
PBS
IL-15c
Cell Number (x10$^3$)
CD4 CD44$^{hi}$
CD8 CD44$^{hi}$
CD8 NP Tet +
D
PBS
IL-15c
Day 40 p.i.
Cell Number (x10$^3$)
CD4 CD44$^{hi}$
CD8 CD44$^{hi}$
CD8 NP Tet +
E
# NP Tet + CD8 T Cells (x10$^3$)
PBS
IL-15c
IL-7c
IL-2c
F
PBS
IL-15c
IL-7c
IL-2c
NP Tet
CD11a
28.8
38.5
27.9
22.6
5.3
4.3
7.2
6.7
BrdU
12.3
6.0
6.8
5.8
7-AAD
G
% BrdU Incorporation by NP Tet+ CD8 T Cells
PBS
IL-15c
IL-7c
IL-2c
% 7-AAD Incorporation by NP Tet+ CD8 T Cells
PBS
IL-15c
IL-7c
IL-2c

**Figure 2.3. Exogenous IL-15 results in the accumulation and enhanced survival of influenza-specific CD8 Teff at the site of infection**. (A) Twelve days p.i. with x31, WT or IL-15$^{-/-}$ received PBS or IL-15/IL-5Rα complexes (IL-15c) as indicated below horizontal line. BAL was collected 12 hrs post treatment, and isolated lymphocytes were analyzed for tetramer reactivity. The mean percent NP-tetramer positive among CD8 T cells is represented ± SEM (n=4 mice/group; *p=0.0291). Data are representative of two independent experiments. (B) 6.5 or (C) 12 days following x31 infection, animals received either PBS or IL-15c. 12 hours later, BAL were analyzed for CD4, CD8 and CD44 expression and tetramer reactivity. Mean cell number is plotted ± SEM (n=3 mice/group; *p=0.0024) in corresponding right panels. Data are representative of 3 independent experiments. (D) 40 days following x31 infection, animals received either PBS or L-15c. 12 hours later, BAL were analyzed for CD4, CD8 and CD44 expression and tetramer reactivity. Mean cell number is plotted ± SEM. (E) 12 days following x31 infection, animals received PBS (black bar), IL-15c (open bar), IL-7/anti-IL-7 mAb complexes (IL-7c; darkly shaded bar), or IL-2/anti-IL-2 mAb complexes (lightly shaded bar) i.n.. 12 hours later, BAL were analyzed tetramer reactivity. Mean cell number is plotted ± SEM (n-4 mice/group; *p=0.0400). Data are representative of two independent experiments. (F-G) At d 12 p.i., WT mice were pulsed with 2 mg of BrdU i.p. and simultaneously received either PBS, IL-15c, IL-7c, or IL-2c i.n.. Twelve hours later, BAL was collected and stained with 7-AAD and subsequently intracellularly stained for BrdU. Representative flow plots (F) and mean percent of BrdU (top) or 7-AAD (bottom) positive among NP-tet+ CD8 T cells (G) is plotted ± SEM (n=4 mice/group).

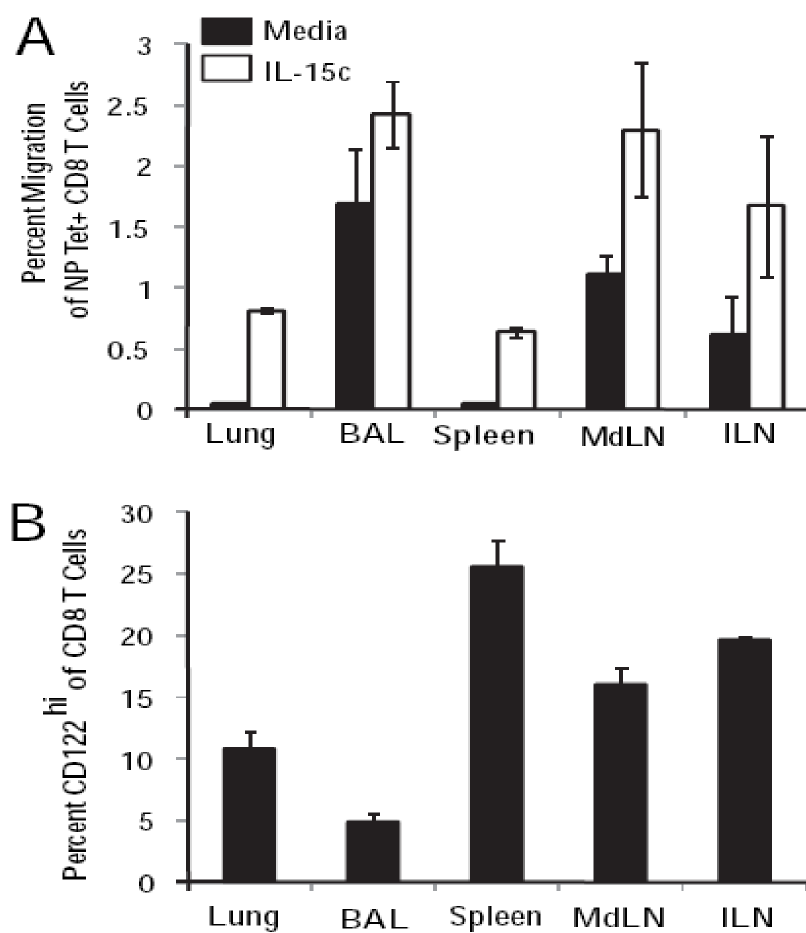

A
Media
IL-15c
Percent Migration of NP Tet+ CD8 T Cells
3
2.5
2
1.5
1
0.5
0
Lung
BAL
Spleen
MdLN
ILN
B
Percent CD122hi of CD8 T Cells
30
25
20
15
10
5
0
Lung
BAL
Spleen
MdLN
ILN

**Figure 2.4. IL-15 is chemotactic for influenza-specific CD8 Teff *in vitro*.** (A) $1x10^6$ bulk cells isolated from the indicated tissues of WT animals infected 12 days previously with x31 were placed in the upper chamber of a 0.5μm Transwell with media alone or supplemented with IL-15c in the bottom chamber. Percent migration was calculated as # NP-tet+ CD8 T cells in the bottom chamber / # NP-tet+ CD8 T cells in the input sample. Mean percent migration is plotted ± SEM among three replicates for each tissue sample. (B) The mean percent of CD122 positive among day 12 CD8 Teff was determined and plotted ±SEM (n=3 mice/ group).

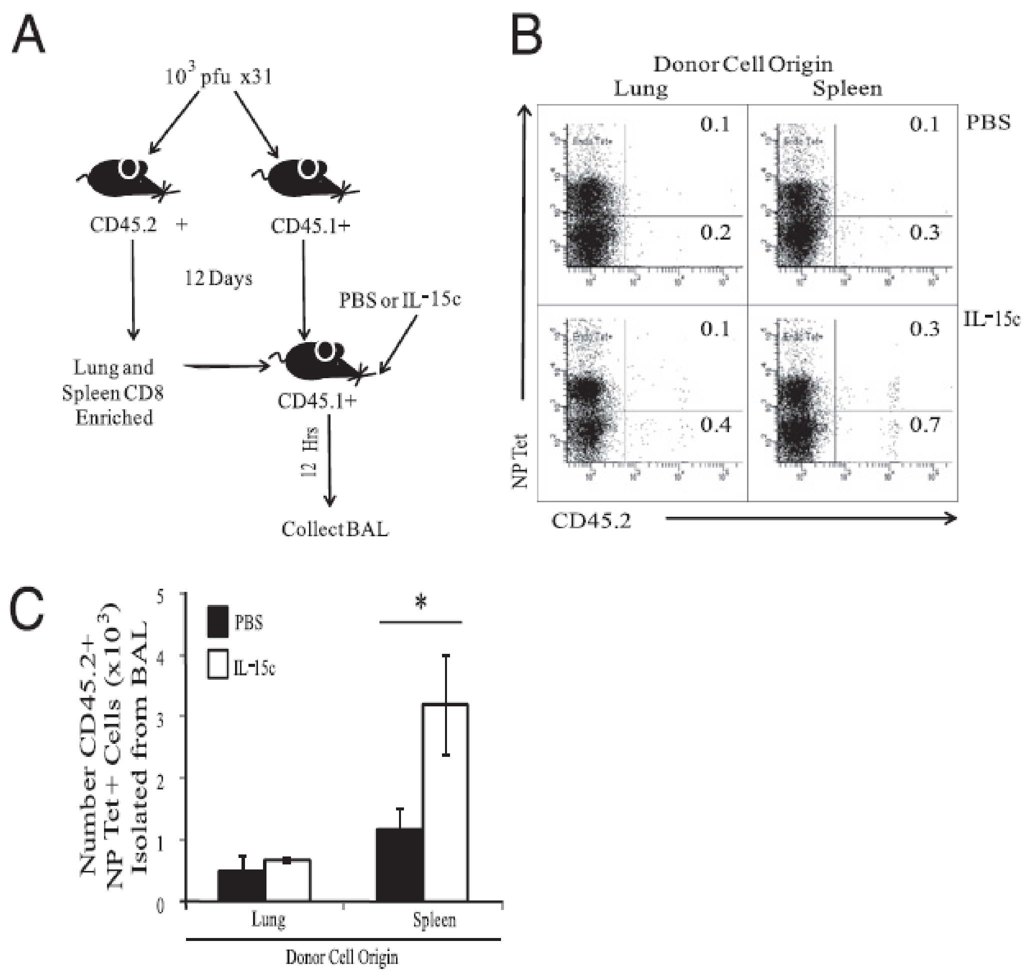

A
$10^3$ pfu x31
CD45.2 +
CD45.1+
12 Days
PBS or IL-15c
Lung and Spleen CD8 Enriched
CD45.1+
12 Hrs
Collect BAL
B
Donor Cell Origin
Lung
Spleen
PBS
IL-15c
0.1
0.2
0.1
0.3
0.1
0.4
0.3
0.7
NP Tet
CD45.2
C
Number CD45.2+ NP Tet + Cells ($x10^3$) Isolated from BAL
PBS
IL-15c
*
Lung
Spleen
Donor Cell Origin

**Figure 2.5. IL-15 induces the migration of influenza-specific CD8 Teff *in vivo*.** (A) Methods schematic for panels B and C. (B) $2.5x10^6$ CD8 T cells from the spleens and lungs of $CD45.1^+$ mice 12 days post x31 infection were adoptively transferred i.v. into identically infected $CD45.2^+$ recipients. One hour post transfer, recipients received either PBS or IL-15c i.n. and 12 hours later, BAL was collected and the number of donor (CD45.2+) NP-specific CD8 Teff migrating into the lung airways was determined by flow cytometry. (C) Mean number of donor cells recovered is plotted $\pm$ SEM (n=6 mice; p=0.0245).

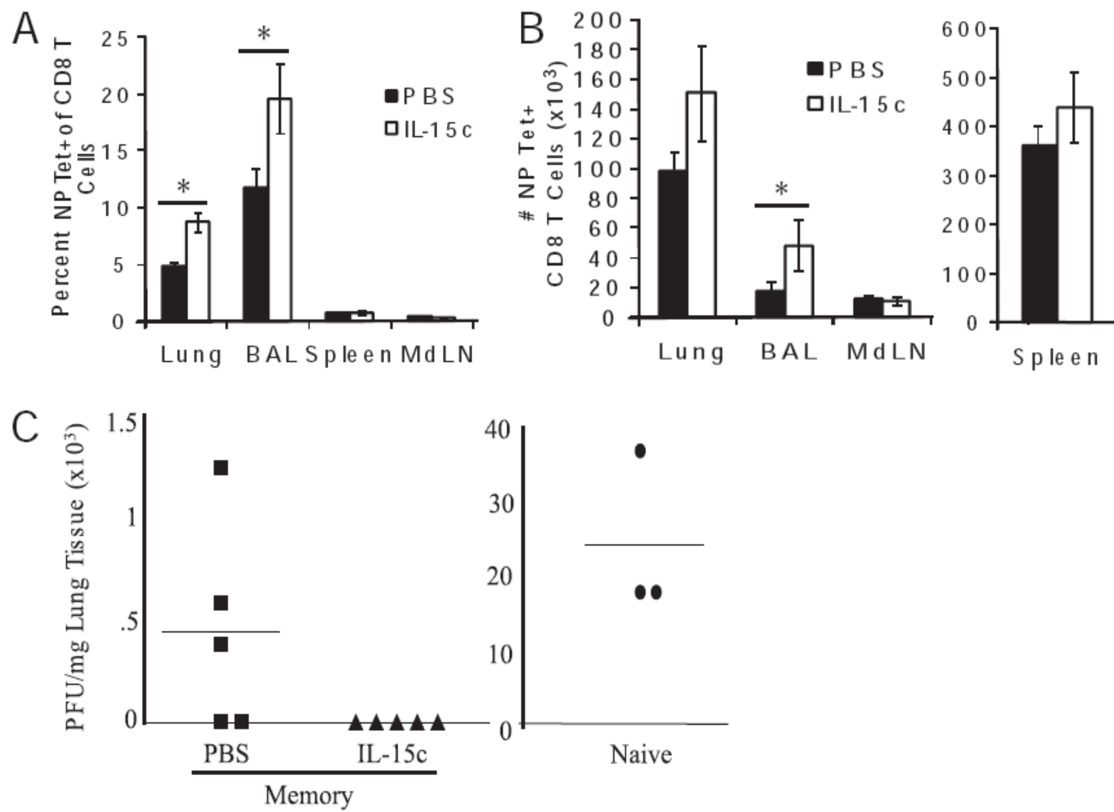
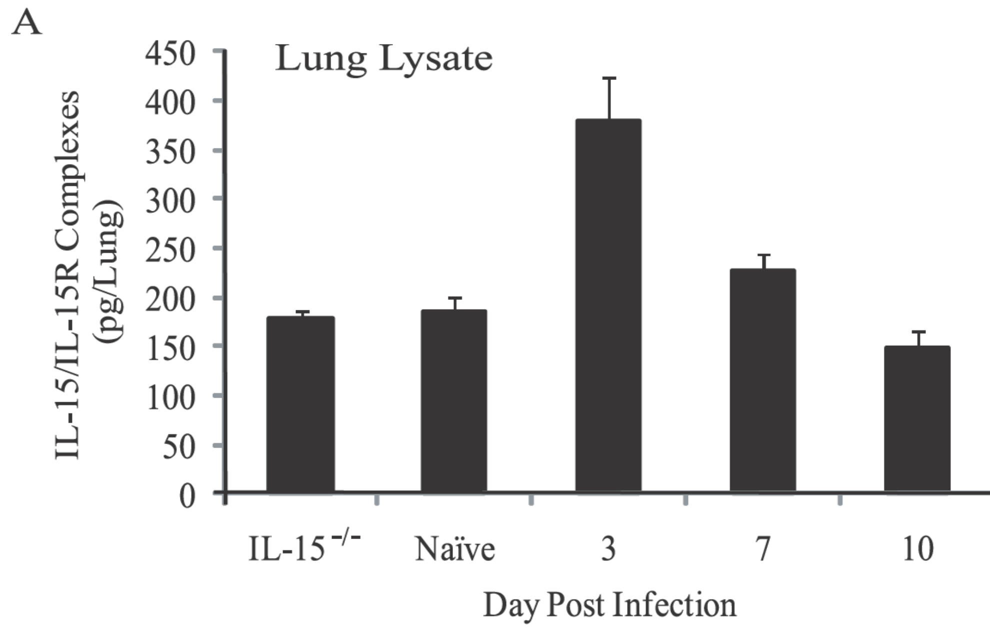
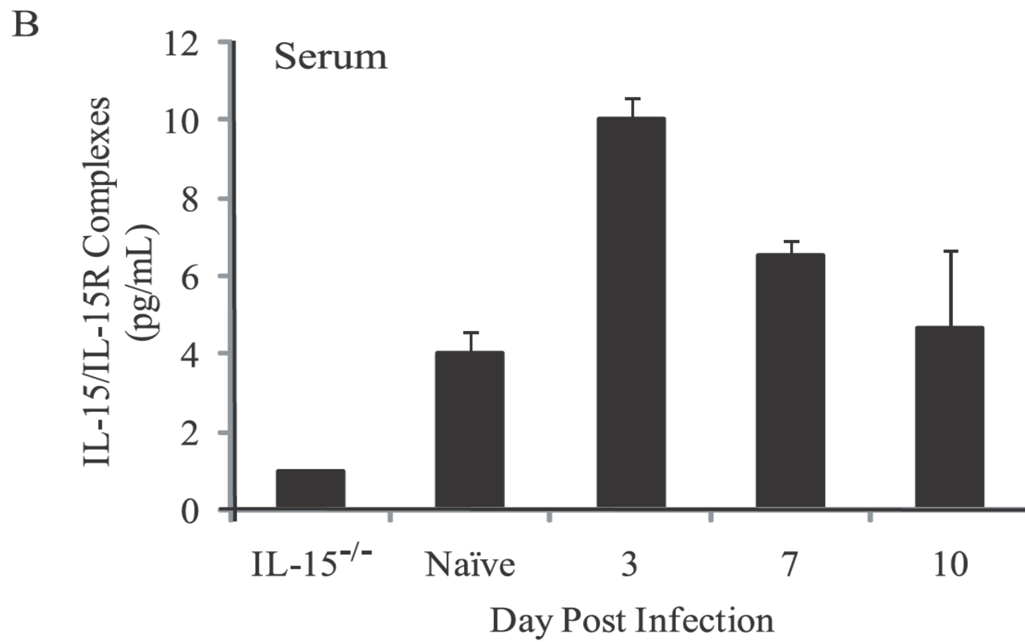
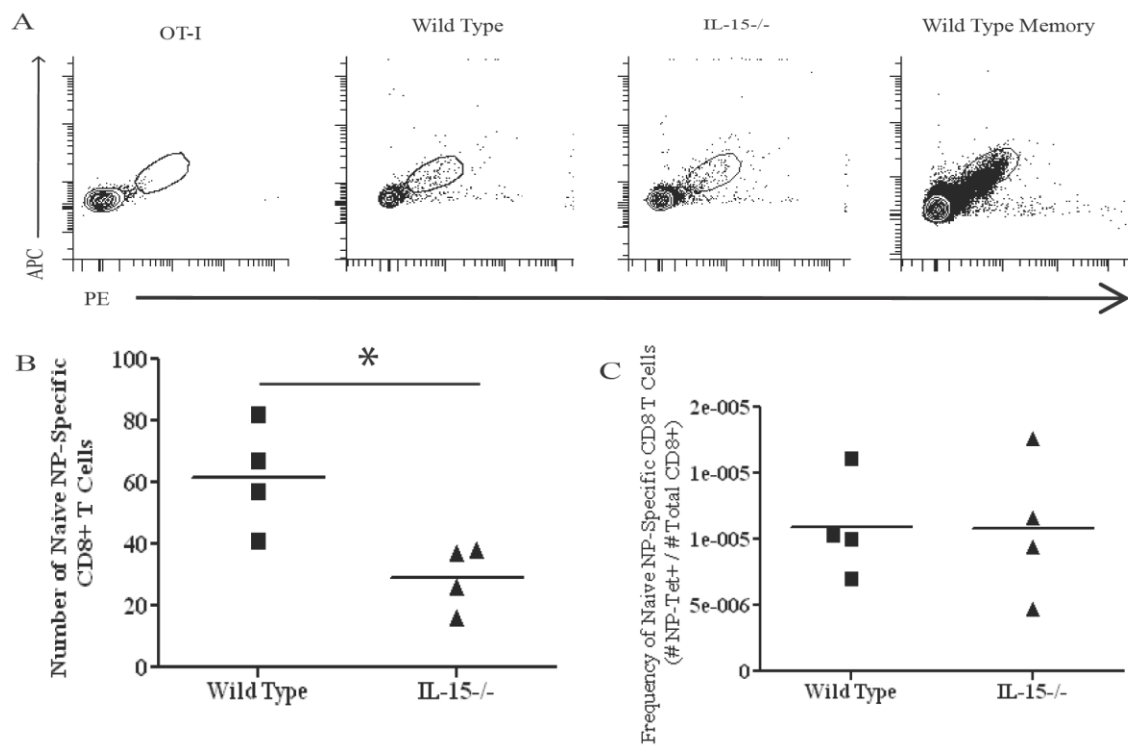

A
Percent NP Tet+ of CD8 T Cells
25
20
15
10
5
0
*
*
PBS
IL-15c
Lung
BAL
Spleen
MdLN
B
# NP Tet+ CD8 T Cells (x10³)
200
180
160
140
120
100
80
60
40
20
0
*
PBS
IL-15c
Lung
BAL
MdLN
600
500
400
300
200
100
0
Spleen
C
PFU/mg Lung Tissue (x10³)
1.5
1
.5
0
PBS
IL-15c
Memory
40
30
20
10
0
Naive

**Figure 2.6. Mucosal delivery of IL-15c during the effector phase of the anti-influenza CD8 T cell response augments the resultant Tmem pool.** Starting 10 days post x31 infection, animals received either PBS or IL-15c every other day for 10 days. Animals were rested for an additional 11 days until memory (day 31 p.i.) when cells from the indicated tissues were collected, counted, and analyzed for CD8 expression and NP-tetramer reactivity. Plotted are the mean frequencies (A) or mean numbers (B) of NP Tet+ CD8 T cells $\pm$ SEM (n=6 mice/group; *p=0.0224 (Lung), 0.0019 (BAL), and 0.0002, respectively). Data are representative of two independent experiments. (C) On day 45 post x31 infection, WT and IL-15$^{-/-}$ memory mice treated with PBS (circles) or IL-15c (squares) as in panel (A) and naïve (triangles) animals were challenged with PR8, and 6 days later lung viral titers were determined by plaque assay (n=3 mice/group).


A
Lung Lysate
IL-15/IL-15R Complexes (pg/Lung)
450
400
350
300
250
200
150
100
50
0
IL-15 -/-
Naïve
3
7
10
Day Post Infection


B
Serum
IL-15/IL-15R Complexes (pg/mL)
12
10
8
6
4
2
0
IL-15-/-
Naïve
3
7
10
Day Post Infection

**Supplemental Figure 2.1. Soluble IL-15/IL-15R complexes are released in response to influenza infection**. At the indicated time points p.i., lung homogenates (A) and serum (B) were collected from IL-15-/-, naïve wild type, and infected wild type animals and analyzed for the presence of IL-15/IL-15R complexes by ELISA as described in the Materials and methods section. Plotted are the means $\pm$ SEM (n=3 mice/group).


A
OT-I
Wild Type
IL-15-/-
Wild Type Memory
APC
PE
B
Number of Naive NP-Specific CD8+ T Cells
100
80
60
40
20
0
*
Wild Type
IL-15-/-
C
Frequency of Naive NP-Specific CD8 T Cells (# NP-Tet+ / # Total CD8+)
2e-005
1e-005
1e-005
5e-006
0
Wild Type
IL-15-/-

**Supplemental Figure 2.2. IL-15-/- mice have a reduced number but similar frequency of NP-specific naïve precursor CD8 T cells**. Total lymphocytes from spleen and all visible lymph nodes were taken from OT-I Rag2-/- mice, naïve wild type and IL-15-/- mice, and a memory wild type mouse (infected approximately 9 mo. previously). (A) Representative plots for lymphocytes previously gated on CD8+ and negative for CD4, CD19, and CD11c following pMHC class I tetramer enrichment with both PE- and APC-labeled H-2D(b) tetramers. (B and C) Graphical representation of the NP-specific naïve precursor number (B) and frequency (C) among CD8 T cells. Each symbol represents an individual mouse, and lines represent mean values ( *p=0.0179).

# CHAPTER 3

# IL-15 PARTICIPATES IN THE RESPIRATORY INNATE IMMUNE RESPONSE TO INFLUENZA VIRUS INFECTION[2]

[2] Verbist, K., D. Rose, C. Cole, M. Field, and K. Klonowski. 2012. Accepted by *PLoS ONE*. Reprinted here with permission of publisher.

### 3.1 Abstract

Following influenza infection, natural killer (NK) cells function as interim effectors by suppressing viral replication until CD8 T cells are activated, proliferate, and are mobilized within the respiratory tract. Thus, NK cells are an important first line of defense against influenza virus. Here, in a murine model of influenza, we show that virally-induced IL-15 facilitates the trafficking of NK cells into the lung airways. Blocking IL-15 delays NK cell entry to the site of infection and results in a disregulated control of early viral replication. By the same principle, viral control by NK cells can be therapeutically enhanced via intranasal administration of exogenous IL-15 in the early days post influenza infection. In addition to controlling early viral replication, this IL-15-induced mobilization of NK cells to the lung airways has important downstream consequences on adaptive responses. Primarily, an absence of responding NK cells reduces the immigration of influenza-specific CD8 T cells to the site of infection. Together this work suggests that local deposits of IL-15 in the lung airways regulate the coordinated innate and adaptive immune responses to influenza infection and may represent an important point of immune intervention.

### 3.2 Introduction

Influenza virus is a major human pathogen that causes substantial morbidity and mortality—approximately 36,000 deaths annually in the United States alone [237]. Combined with the severe economic burden imposed from seasonal influenza outbreaks and growing concerns over potential imminent influenza pandemics, there is considerable need for a firm understanding of the disease pathology, prevention strategies, and mechanisms of host defense against the virus [238].

Influenza virus is primarily transmitted via inhaled aerosols and results in an infection localized to the upper respiratory tract, with viral replication largely limited to epithelial cells [9]. Mechanisms by which the immune system eliminates influenza have been well studied and are known to involve the coordinated actions of the innate and adaptive immune systems. Namely, the cytolytic action of influenza-specific CD8 T cells has been shown to be the primary mediator of complete viral clearance, but important roles have also been described for CD4 T cells [51,60,239]. In addition to T cells, a crucial role has also been established for innate immune effectors including natural killer (NK) cells, which provide short-term control of viral replication prior to T cell activation [43]. NK cells become activated following the loss of inhibitory signals coupled with positive activating signals resulting in direct (via release of cytotoxic granules and interferon γ) or indirect (via activation of macrophages and dendritic cells) target cell lysis [34]. NK cells are vital in limiting influenza viral replication as depletion of NK cells dramatically increases morbidity and mortality in hamsters and mice [32], and in humans severe infections with the 2009 pandemic H1N1 virus positively correlated with reduced numbers of NK cells in the lungs [44]. Studies have indicated that the natural cytotoxicity receptors NKp44 and NKp46, which recognize hemagglutinin proteins of several different influenza strains [40,240] is one mechanism used by NK cells to protect against lethal viral challenge [38]. Secondarily, NK cells also aid in viral clearance indirectly through the production and secretion of cytokines which both amplifies local inflammation and recruits antigen-specific CD8 T cells to sites of inflammation [241]. Implicit in both of these functions is the ability of NK cells to accumulate within the

respiratory tract to contact infected cells and provide a source of chemotactic signals to recruit recently activated CD8 T cells.

Type I IFNs expressed within hours after viral infection have been documented to induce expression of the chemokines CXCL9 and 10 which function to recruit CXCR3 expressing NK cells to sites of infection [242]. However, Type I IFNs also modulate the expression of the common gamma chain cytokine interleukin 15 (IL-15) [141,214,243], which we recently reported to be temporally and locally increased following influenza infection [244]. This expression of IL-15 in the respiratory tract facilitates the recruitment of antigen-specific CD8 T cells to the respiratory tract. However, it is unclear whether the chemotactic properties of IL-15 uniquely affect migratory CD8 T cells or could be extended to other IL-15-sensitive immune cells. NK and NKT cells are nearly absent in IL-15$^{-/-}$ animals [102], highlighting the important role of IL-15 on NK cell development and homeostasis in the steady-state. Following viral infections, de novo production of IL-15 by dendritic cells results in the activation and proliferation of NK cells [119,245], and transient systemic stimulation of NK cells with soluble IL-15/IL-15Rα complexes also results in an accumulation of phenotypically and functionally mature NK cells [120,127]. In addition to these roles, IL-15 also can stimulate the migration of NK cells *in vitro* and enhances their adhesion to cultured endothelial cells [129]. We therefore hypothesized that virally induced IL-15 functionally assists in the migration of NK cells into the lung airways. We show here that an IL-15 deficiency results in a site-specific reduction in NK cells from the lung airway and an exacerbation of viral load at early time points post influenza infection. Additionally, exogenous IL-15 induces the specific migration of NK cells in vitro and in vivo. This IL-15-dependent enhanced mobilization of NK cells to the

lung airways correlates with decreased viral loads. Importantly, in the absence of NK cells, antigen-specific CD8 T cells fail to accumulate at the site of infection, providing a possible link between IL-15-mediated migratory effects of both the innate and adaptive immune responses to influenza infection and suggest therapeutic possibilities regarding the use of IL-15 to simultaneously regulate both arms of the immune system for improved responses to viral infection.

## 3.3 Materials and Methods

*Ethics Statement*

All animals were handled in strict accordance with good animal practice as defined by the American Association for Accreditation of Laboratory Animal Care as well as federal and state agencies. All animal work presented here was approved by Institutional Animal Care and Use Committee of University of Georgia (AUP No. A2009-6-114).

*Mice, Viruses, IL-15 Blocking, and NK Cell Depletion*

C57BL/6 mice were purchased from Charles River (Wilmington, MA) through the NCI program. Influenza A/HK-x31 (x31, H3N2) was generously donated by Dr. S. Mark Tompkins (University of Georgia, Athens, GA). Animals were infected intranasally (i.n.) with $10^3$ PFU HKx31 diluted in 50µL sterile PBS. IL-15 was blocked using 25µg anti-IL-15 mAb (clone AIO3) (eBioscience, San Diego, CA) administered daily via intraperitoneal injection (i.p.) in 200µL sterile PBS. IL-15 depletion was confirmed by reductions in frequencies of both NK and $CD44^{hi}$ $CD8^+$ T cell in antibody-treated animals compared to untreated animals 7 days after the initiation of mAb treatment. NK1.1+ cells were depleted via intravenous (i.v.) injections of 200uL PBS containing 200µg anti-

NK1.1/mouse (clone PK136) every other day (UCSF monoclonal antibody core facility, San Francisco, CA), and depletion of NK cells was verified using an anti-NKp46 mAb (Clone 21A9.4, eBioscience) throughout the experiment and 2 days after the last injection of the NK1.1 depleting mAb.

*Tissue Preparation and Flow Cytometry*

Lung airway-resident cells were harvested by bronchioalveolar lavage (BAL) with 3 consecutive washes of 1mL PBS. To isolate cells from the lung parenchyma, lungs were perfused with ~25mL PBS/heparin sodium solution, harvested, minced and incubated at 37°C for 30 minutes in 1.25mM EDTA. The tissue was subsequently incubated in collagenase diluted in RPMI (6mg/mL) at 37°C for 1 hour and passed through a 5μm cell strainer. Isolated cells were subjected to separation via density gradient centrifugation by resuspending cells in 47% Percoll underlain with 67% Percoll. The gradients were then centrifuged at 2800 rpm for 20 minutes, and lymphocytes at the interface were collected. Spleen and lymph nodes were harvested from animals, homogenized, and then passed through a cell strainer. Spleen homogenate was depleted of erythrocytes by incubation in tris-buffered ammonium chloride.

Single cell suspensions were stained with combinations of cocktails containing anti-CD3, NK1.1, NKp46, CD11c, CD11b, CD122, and CD132 (eBioscience, San Diego, CA) as indicated for 20 minutes at 4°C. Where indicated, cells were concurrently stained with or without biotinylated anti-IL-15Rα (R&D Systems, Minneapolis, MN) followed by 20 minute incubation with APC-conjugated Streptavidin at 4°C. Influenza nuclear protein (NP) MHC class I tetramer [H2-$D^b$/ASNENMETM] were generated by the National Institute of Allergy and Infectious Diseases Tetramer Facility (Emory

University, Atlanta, GA). Tetramer staining was conducted at room temperature for 1 hour concurrently with anti-CD3, NK1.1, NKp46, CD8, and CD44 (eBioscience, San Diego, CA). Stained cells were analyzed using a BD LSRII digital flow cytometer (BD Biosciences, San Jose, CA) and either BD FACSDiva or FlowJo software (Tree Star, Inc., Ashland, OR).

*Migration and Proliferation Assays*

IL-15 complexes (IL-15c) were generated on the day of use by incubating 1.5µg recombinant mIL-15 with 7µg IL-15Rα Fc-chimera (R&D Systems, Minneapolis, MN) at 37°C for 20 minutes followed by 4°C for at least 10 minutes. For Rα only controls, 7µg IL-15Rα Fc-chimera (R&D Systems, Minneapolis, MN) was incubated similarly to complexes without addition of the cytokine. Complexes or Rα alone were administered via passive inhalation into both nostrils using a micropipette delivering 36.25µL (for daily treatments) or 72.5µL (for one time treatments) of complexes in sterile PBS. For assessment of cell proliferation, animals received 2 mg of BrdU (Sigma-Aldrich, St. Louis, MO) administered i.p. in a 200µL volume of PBS. Cells were isolated from these animals 12 hours after treatment and stained with 20µL aminoactinomycin D (7-AAD; BD Pharmingen, San Jose, CA) for 20 minutes at 4°C. Cells were surface stained as previously described and stained intracellularly with FITC-labeled anti-Ki-67 and APC-labeled anti-BrdU monoclonal antibodies (BD Pharmingen, San Jose, CA) according to manufacturer's instructions.

In vitro migration assays were performed by placing bulk populations of lymphocytes containing predetermined numbers of NK cells (verified by FACs analysis) from the indicated tissues on the top insert of a 5µm chemotaxis transwell (Fisher

Scientific, Waltham, MA) in which the bottom well contained warm media alone or supplemented with 100ng/mL IL-15c. IL-15c was generated by incubating 100ng of IL-15 with 500 ng IL-15Rα Fc-chimeric protein at 37° for 20 minutes and 4° for 10 minutes. Plates containing transwells were then incubated at 37°C with $CO_2$ exchange, and 90 minutes after plating, cells were harvested from bottom chambers and the percent migration of NK cells was calculated as the ratio of the number of NK cells in the bottom chamber compared to the number of NK cells determined in the input sample.

*Plaque Assays*

Plaque assays were preformed as previously described [246]. Briefly, lungs from HK-x31 infected animals were collected and homogenized using a tissue lyser (Qiagen, Hilden, Germany). Monolayers of Madin-Darby kidney cells were incubated with 10-fold serial dilutions of 10% homogenate in dilution media (1xMEM, 1µg/mL TPCK-treated trypsin) for 1 hour at 37°C. Cells were washed with 1x sterile PBS and overlaid with MEM containing 1.2% Avicel microcrystalline cellulose (FMC BioPolymer, Philadelphia, PA), 0.04M HEPES, 0.02mM L-glutamine, 0.15% $NaHCO_3$ (w/v), and 1µg/mL TPCK-treated trypsin. After 72 hours, the overlay was removed, and the cells were washed with 1x sterile PBS, fixed by incubation with cold methanol/acteone (60:40%), and stained with crystal violet.

*Statistics*

Statistical significance was determined by Student's T test using Prism 5 software (GraphPad Software). Significance was determined to be any p-value where $p < 0.05$.

**3.4 Results**

*NK cells expressing the IL-15 receptor accumulate in the lung airways of influenza-infected animals.*

We and others have shown that following influenza infection, IL-15 message and protein is increased in the lung airways [149,244]. Because this IL-15 expression was rapidly induced by influenza infection and reached significant levels as early as day 3 post infection (p.i.) [244], we hypothesized that influenza-induced IL-15 expression may be an important mediator of NK cell responses to influenza infection. We therefore first sought to determine whether NK cells responding to influenza infection were capable of receiving signals from this locally produced IL-15. To this end, lymphocytes were isolated from the lung airways of influenza-infected animals via BAL, and $CD3^-$, $NK1.1^+$ NK cells were analyzed for the expression of IL-15 receptor components by flow cytometry. The IL-15 receptor is a heterotrimer, consisting of the common gamma chain (CD132), the shared IL-2/IL-15Rβ chain (CD122), and the specific IL-15Rα chain [83]. To date the majority of biological effects of IL-15 on NK cells, however, are mediated through the paired co-expression of CD122 and CD132 [247-249], while IL-15Rα is only required by accessory cells which present IL-15 to respondent cells, a mechanism referred to as trans-presentation [89]. However, some groups have suggested that IL-15Rα expression alone contains some signaling moieties which may participate in distinct biological functions [27]. Therefore it is important to establish the kinetics of IL-15 receptor component expression and IL-15 signaling potential on NK cells in our model.

NK cells are known to respond rapidly to influenza infection and continue accumulating in the lung airways through day 5 p.i. ([38] and data not shown). Since we

wished to specifically evaluate recent NK cell immigrants responding to the airway inflammation resulting from influenza infection, we restricted our analyses of NK cell kinetics to day 4 p.i.. NK cells were first detected in the BAL at day 2 post influenza infection (albeit at a low frequency, ~0.1-0.6% of lymphocytes, Figure 1A and Figure 2A) but were completely absent in control mock-infected animals (data not shown). Despite the low frequency of NK cells in the lung airway at this early time point, one fifth consistently expressed IL-15Rα. Additionally, 30-40% of them expressed CD122 and CD132 (Figure 1B). By day 3 p.i., when NK cells represented a much more discernible population (~2-3% of lymphocytes, Figure 1A & 2A), greater than 90% of these gated cells expressed CD122 and CD132, and expression levels of these receptors on a per-cell basis increased over time as indicated by a higher median fluorescence intensity by day 4 p.i. (Figure 1B) and consistent with evidence that expression of IL-15R components is induced by activating stimuli [250]. Expression of IL-15Rα however, was variable but consistently much lower that the expression levels of CD122 and CD132. This biased expression of CD122/132 receptor chains over IL-15Rα and enhanced IL-15 levels [19] following influenza infection, indicate that NK cells accumulating at the site of influenza infection are capable of responding to locally produced IL-15 via the trans-presentation pathway.

*IL-15 blockade results in reduced numbers of NK cells at the site of infection and impairs early viral control.*

Since co-expression of CD122 and CD132 render NK cells responsive to IL-15 signals, we next wished to determine whether virally induced IL-15 and subsequent signaling through these receptors affected the accumulation of these cells in the

respiratory tract. Because IL-15$^{-/-}$ mice exhibit a severe developmental defect in both NK and NKT cell lineages and are nearly devoid of these cell populations [102], we chose to use an anti-IL-15 blocking antibody to selectively deplete IL-15 concurrent with infection. Therefore, we monitored the influx of NK cells in animals receiving either PBS or anti-IL-15 mAb administered i.p. daily from day 0 through day 4 post influenza infection. In order to more accurately define bona fide NK cells, particularly in the lung airways harboring few NK cells at very early time points post influenza infection, we included NKp46 reactivity in our staining protocol and henceforth define NK cells as CD3$^{-}$ lymphocytes positive for both NK1.1 and NKp46. While NK cells accumulated in the lung airways of untreated mice as expected, by day 2 p.i. the overall frequency of NK cells in IL-15 blocked animals was reduced by half and remained this low through day 4 p.i. (Figure 2A). Concordantly, total numbers of NK cells in IL-15-blocked mice were partially reduced at day 2 p.i., and substantially diminished by days 3 and 4 p.i. (Figure 2A). In fact, whereas the numbers of NK cells continued to accumulate in the lung airways of control-treated animals through day 4 p.i., numbers of NK cells in the lung airways of treated animals plateaued by day 3 p.i. (Figure 2A). In the lung parenchyma of control-treated animals, NK cells also accumulated over time post infection, similar to those in the lung airways, but the frequencies of NK cells in this site remained unchanged in IL-15-blocked mice. Numbers of NK cells in the lung parenchyma of anti-IL-15 treated animals also remained similar to control animals with only a slight reduction at day 2 p.i. (Figure 2B). Importantly, while the numbers of NK cells were significantly reduced at the site of infection as a result of an IL-15 deficiency, anti-IL-15 treatment had little effect on the frequency or number of NK cells found in anatomical sites distal to the

site of infection such as the spleen (Figure 2C). In order to determine whether an absence of IL-15 in the lung airways resulted in the reduced frequencies and numbers of NK cells specifically, numbers of other populations of innate cells in the airways at these early time points post infection were analyzed. $CD11c^{-}CD11b^{+}$ cells (granulocytes) or $CD11c^{+}CD11b^{-}$ (dendritic cells) in the airways following infection were mostly unaffected by the IL-15 deficiency, with only a small reduction observed at day 4 p.i. (Figure 2D). In contrast, $NK1.1^{+}CD3^{+}$ (NKT cells) were markedly reduced in the lung airways of IL-15-blocked mice (Figure 2D), but overall, these cells represented a low proportion of the innate cells responding to influenza infection at these early time points following infection (Figure 1A). Together, these data indicated that short term blockade of IL-15 did not result in global defects in NK cell homeostasis or survival in peripheral tissues and the effects of IL-15 were largely specific to NK cells as blocking IL-15 selectively resulted in a significant loss of NK cells recovered from the site of infection.

To test whether this local reduction in NK cells impairs the control of viral replication, we performed plaque assays on control- and anti-IL-15 treated mice to quantify viral load in the lungs of animals with intact or diminished IL-15 and NK cell responses. Viral load was quantified on days 1-5 and 7-8 p.i. to specifically look at control during the time frame of NK cell entry and accumulation in the lung airways and the kinetics of subsequent viral clearance. In IL-15 blocked animals, differences in viral load were apparent as early as d2 p.i. where viral titers were about 3x higher through day 3 p.i. (Figure 2E); however, these animals seemed to regain control of viral replication by day 4 p.i., which perhaps corresponds with the early entry of cells of the adaptive immune response as anti-influenza specific CD8 T cells are first detectable in the lung

airways by d6 post infection by flow cytometry ([69] and data not shown). Thus, while viral elimination is not ultimately dependent on IL-15, early control of the virus is impaired in the absence of IL-15, which correlates with the arrival of a significant number of NK cells in the lung airways. We thus hypothesized that IL-15 was important for the migration of NK cells in the lung airways following influenza infection.

*Administration of exogenous IL-15 enhances the number of NK cells in the lung airways.*

We observed significant reductions in the numbers of NK cells in the lung airways of influenza-infected animals in which IL-15 was blocked at time points associated with their arrival at the site of infection, and failure of these cell populations to accumulate had implications in early viral control (Figure 2). In order to determine whether IL-15 might be an important signal for NK cells in the migration to and/or the proliferation within the site of infection, we chose to provide exogenous IL-15 in an attempt to enhance any IL-15-dependent NK cell migration and/or in situ proliferation within the lung airways of influenza-infected animals. To this end, either PBS or recombinant IL-15/IL-15Rα fusion protein complexes (IL-15c) were administered intranasally to mice three days following influenza infection. To ensure that any biological effects of the IL-15c could be attributed to activity of the cytokine (which is merely stabilized by complexing to IL-15Rα), a control group of mice received the IL-15Rα only. Concurrent with treatment, mice received an i.p. pulse of the thymidine analog BrdU to identify proliferating cells. Twelve hours post treatment, the frequency and total number of NK cells in the BAL was quantified as well as the percentage of these cells incorporating BrdU. Isolated cells were simultaneously stained with 7-AAD as an indicator of cell viability, as only cells with disrupted membranes stain positive for

this fluorescent dye. Importantly, neither the IL-15Rα alone nor the IL-15c affected cell viability, as cells isolated from animals receiving these treatments had similar percentages of 7-AAD$^+$ NK cells as those from PBS-treated mice (data not shown).

Upon introduction of IL-15c to the lung airways, the overall frequency of NK cells isolated from the BAL was significantly increased (Figure 3A and B), and the total number of NK cells isolated from this site was nearly three times that of PBS-treated control animals (Figure 3B). Interestingly, the percentage of NK cells expressing CD122, the IL-2/15 Rβ chain, was reduced in IL-15c-treated animals (Figure 3B), perhaps indicative of increased signaling through and subsequent internalization of this receptor complex by IL-15 responsive cells. Unlike T cells, which require large clonal bursts of proliferation to achieve effector status, the effector function of NK cells is more related to activation and mobilization to the site of inflammation [99]. Nevertheless, we wished to test whether the large increases in NK cell number in the lung airways following IL-15c administration could be attributed to IL-15-induced proliferation of NK cells at the site. To assess the potential role of proliferation in this observed increase in NK cell frequency and number in the BAL of treated mice, these NK cells were analyzed for BrdU incorporation. Concomitantly, cells isolated from the BAL were assessed for expression of the cell-cycle-specific protein Ki-67. Since BrdU is incorporated into the DNA of only cells in S phase whereas Ki-67 is expressed by cells in any stage of the cell cycle, it was unsurprising that BrdU$^+$ cells were only a fraction of Ki-67$^+$ cells (Figure 3A). Therefore, we considered only cells positive for both markers as cells undergoing proliferation at the time of treatment. Although the percentage of BrdU-incorporating cells was modestly increased in IL-15c-treated animals, the overall frequency of proliferating cells was low

in untreated (<10%) and treated (<12%) animals (Figure 3B). These data suggest that IL-15c may trigger the proliferation of NK cells, but proliferation of this cell population in the lung airways is, in general, low and not likely to be solely responsible for the total number of cells extracted from the lung airways. IL-15Rα alone had no significant impact on the accumulation or proliferation of this cell population (Figure 3A and B). Finally, differences in cell frequencies, numbers, CD122 expression, and BrdU incorporation were specific to the lung airways (the sight of treatment), as NK cells isolated from spleens were similar in control and IL-15c-treated animals (Figure 3C and data not shown). Together, these data demonstrate that exogenous IL-15 results in increased numbers of NK cells in the lung airways. Since this increase appeared to be independent of IL-15-mediated effects on cell survival, and proliferation was low, we hypothesized that IL-15 may be responsible for a substantial amount of migration of NK cells into the lung airways following influenza infection similar to its effects on CD8 T cells [244].

*NK cells migrate to IL-15c in vitro*

Intranasal administration of IL-15c resulted in increased numbers of NK cells in the lung airways that appeared to be due to increased migration into that site. Previous studies have indicated that IL-15 is indeed chemotactic for NK cells. In vitro checkerboard assays revealed that freshly isolated NK cells migrated to IL-15 gradients, and IL-15 stimulation increased LFA-1-dependent binding of NK cells to cultured endothelial cells [129]. IL-15 has also been shown to play a central role in the recruitment of $CD16^-$ human NK cells into the endometrium following ovulation [251]. To test the direct chemotactic potential of IL-15c for NK cells in our own system, we employed an

in vitro chemotaxis transwell assay. Bulk lymphocytes (or purified splenic NK cells, data not shown) from the BAL, lung, and spleen of mice collected three days after infection with influenza were placed in the top chamber of a transwell filter support with either media alone or media supplemented with IL-15c in the bottom chamber. After 90 minutes of incubation, IL-15c significantly enriched NK cells isolated from the lung and spleen (Figure 4A). Consistent with our findings with CD8 T cells [244], NK cells from the lung airways did not migrate to IL-15c, perhaps because this site represents the terminal destination for these cell populations. Unlike CD8 T cells, which lose expression of CD122 upon residence in the lung airways following influenza infection [244] [70], nearly 100% of the NK cells residing in the BAL of influenza-infected mice express CD122 (Figure 1A and B). Nonetheless, these data indicate that NK cells in the lung parenchyma or the general circulation (as represented by the spleen) migrate to IL-15 in vitro.

*Intranasal administration of IL-15c during the innate phase enhances early viral control.*

A temporal cessation in IL-15 bioavailability reduced the numbers of NK cells (Figure 2A) in the respiratory tract resulting in increased viral titers (Figure 2E), presumably due to impaired NK cell responses. Conversely, exogenous IL-15c promoted the migration of NK cells (Figure 4) and resulted in increased numbers of NK cells in the lung airways (Figure 3B). We therefore hypothesized that intranasal administration of IL-15c early after infection could be used to enhance the early innate immune response to influenza and augment viral control. To this end, influenza-infected animals received either PBS or IL-15c intranasally on days 1-4 p.i., a time frame corresponding to the migration of NK cells into the lung airways and limiting any confounding effects IL-15c

might have on adaptive immune cells entering the lung airways at later time points. Every other day, from day 2-8 p.i., whole lungs were collected and viral titers were quantified via plaque assay (Figure 5A). Early (d2) p.i., we observed no difference in viral load between PBS and IL-15c-treated mice, but as viral replication reached more significant levels at day 4 p.i., animals receiving the IL-15c had 2.4x less viral load than animals receiving only PBS (Figure 5B and C). Although viral titers dropped 10 fold in both groups of animals by day 6 p.i., as expected, surprisingly, there remained a greater than 2 fold significant difference in viral load (Figure 5B and D). Although both groups of animals cleared the influenza virus completely by day 8 p.i. (Figure 5B), our data suggest administration of IL-15c on days 1-4 p.i. can enhance early control of influenza virus by cells of the innate immune system.

*NK cell responses to influenza are requisite for the optimal accumulation of antigen-specific CD8 T cells at the site of infection.*

Because we have previously reported that IL-15 is important for the migration of influenza-specific CD8 T cells into the lung airways [244], it was possible that those observed effects were secondary to the recruitment of NK cells to the lung. To test whether the accumulation of NK cells in the lung was required for the subsequent immigration of influenza-specific CD8 T cells to the respiratory tract, influenza-infected animals were assayed for the accumulation of anti-influenza specific CD8 T cells in NK deficient animals, generated by administration of the αNK1.1 depleting mAb PK136 every other day (Figure 6A). Flow cytometric analyses of NKp46 expression of day 4 p.i. lymphocytes isolated from the lung, BAL, spleen, and mediastinal lymph nodes (MdLN) of αNK1.1-treated animals revealed robust depletion of NK cells as the number of $CD3^-$

NKp46$^+$ cells were reduced to less than one third of those observed in PBS-treated control animals (Figure 6B and data not shown). On days 6 and 8 p.i., the numbers of influenza-specific CD8 T cells in these same tissues were quantified through the identification of cells staining positive for a tetrameric reagent loaded with the immunodominant peptide derived from the influenza nucleoprotein (NP). Tetramer positive CD8 T cells could first be detected in the lung airways at day 6 p.i., and although numbers were low, they were somewhat reduced in the BAL of PK136-treated mice (Figure 6C). No reduction in influenza-specific CD8 T cell numbers could be observed in any other tissue. In fact, CD8 T cells seemed to accumulate in other tissues of animals depleted of NK1.1-expressing cells. By day 8 p.i., the frequency of influenza-specific CD8 T cells in the BAL of PK136-injected animals was less than half than that of control animals, and the total numbers were reduced nearly threefold (Figure 6D). Again, this effect was specific to the lung airways, since frequencies and numbers of NP- tetramer$^+$ CD8 T cells were unchanged in other tissues examined (Figure 6D). Therefore, the largely tissue-specific nature of the dependence of influenza-specific CD8 T cells on the presence of NK1.1-expressing cells in the lung airways partially indicates that lung-resident NK and possibly NKT cells are requisite for the subsequent migration of influenza-specific CD8 T cells into this site. These data suggest that IL-15-mediated migration of CD8 T cells to the lung airways may be, at least partially, an indirect effect of NK1.1$^+$ cells moving into the lung airway space in response to IL-15 and that subsequent tissue remodeling or production of an intermediate factor is responsible for the subsequent recruitment of the CD8 T cells. To our knowledge, these data for the first

time describe a role for IL-15 in linking the innate and adaptive responses to influenza infection necessitating the further inquiry of IL-15 as a potential vaccine adjuvant.

**3.5 Discussion**

Here, we have demonstrated that NK cells, which accumulate in the lung airways early after influenza infection, are dependent on IL-15 for this accumulation and subsequent ability to control viral load. NK cells in the lung airways express high levels of the common gamma chain (CD132) and CD122, receptors responsible for imparting IL-15 responsiveness. Since IL-15 is known to be produced in the lung airways following influenza infection, we investigated the role of IL-15 in NK cell responses to influenza. In the absence of IL-15, NK cell frequencies and numbers were significantly reduced in the BAL, resulting in impaired early control of influenza virus. Although numbers of $CD3^+$ $NK1.1^+$ cells were also substantially reduced following anti IL-15 treatment, it is unclear whether this cell population plays a relevant role in controlling primary infections with influenza. While NK cells are thought to be very important participants in the control of influenza replication, evidence for NKT cells playing a similar role is more controversial [252]. Models of NKT cell activation using αGal-Cer revealed enhanced innate responses to influenza and improved disease outcome [253], but challenge of $CD1d^{-/-}$ mice with influenza led to increased survival, implying an immuno-regulatory role for NKT cells in this model [254]. Whether or not these cells are critically involved in viral clearance, in our model, they represent a very small percentage (<1%) of the lymphocytes in the lung airways in the first five days following infection. Therefore, we believe that an abrogation of virally-induced IL-15 in the lung airways most dramatically affects $CD3^-$, NK1.1, NKp46 double positive NK cells responding rapidly to infection.

By the same principle, exogenous IL-15 could be used therapeutically to increase NK cell populations in the BAL—primarily as a result of IL-15-induced migration—to enhance viral control. Therefore, these combined data indicate that influenza-induced IL-15 is an important signal for the migration of NK cells to the lung airways where they help limit viral replication.

IL-15 is produced by a variety of cell types within and outside of the immune system, including dendritic cells, monocytes and macrophages, stromal cells, endothelial cells, and epithelial cells [82], and pathogenic stimuli are known to induce this expression above constitutive levels [140,243,255,256]. Although dendritic cells are known to be an important source of IL-15 at day 6 post influenza infection [149], it is unclear whether DCs or other cell types produce IL-15 to facilitate specific immune responses to influenza. For example, lung epithelial cells constitutively express IL-15 and IL-15Rα [223], and neutrophils and macrophages can be a major source of IL-15 that is produced during a variety of lung inflammatory diseases including sarcoidosis, tuberculosis, bronchitis, and asthma [257]. Following influenza infection, the IL-15-producing cell type(s) is/are still unknown, but influenza-induced expression of IL-15 is clearly an important regulator of the NK and CD8 T cell responses to this virus [19].

Not only might there be different cellular sources of IL-15 at different times after infection, but IL-15 signaling in NK cells could also be regulated by the context in which IL-15 is presented. At least one isoform of IL-15 is known to enter the cell secretory pathway and could therefore be released as a soluble molecule [258,259]. These data lend the idea that soluble IL-15 could bind heterotrimeric receptors on NK cells. However, IL-15 is also known to be transpresented to NK cells, and indeed, this mode of signaling

appears to be most important for IL-15-mediated NK cell survival [116]. While IL-15Rα is dispensable for NK cell survival, IL-15Rα is thought to be an important component in signaling the migration of $CD16^-$ human NK cells into the endometrium, since this particular NK cell population expresses higher levels of this receptor component than $CD16^+$ NK cells that do not migrate to IL-15 [251]. In our studies, IL-15Rα was only expressed on a relatively low proportion of NK cells in the lung airways of influenza-infected animals (Figure 1). In contrast, CD122 signaling appears to be important for NK cell migration to IL-15 as this receptor chain is down regulated on NK cells exposed to IL-15c in vivo (Figure 3). Thus, we consider it likely that signaling through IL-15Rα is not essential to IL-15-mediated of migration of NK cells to the lung airways. Therefore, future work is needed to identify the IL-15-producing cell population(s) and the mechanism of migration to this cytokine in order to target this response for eventual applications in influenza vaccines and treatments.

A significant finding of our work is that NK cells immigrating into the lung airways are partially required for substantial trafficking of influenza-specific effector CD8 T cells to this location. We found that NK cells are necessary for the optimal accumulation of antigen-specific CD8 T cells, important effectors of eventual viral clearance [60,239], at the site of infection, since depletion of NK1.1-expressing cells resulted in a significant decrease in the number of influenza-specific CD8 T cells in the BAL. A subpopulation of CD8 T cells has been shown to express NK1.1 upon activation [260], and while these cells were detected in the lung parenchyma of influenza-infected C57Bl/6 mice, they comprised less than 1% of the NP-tetramer+ CD8 T cells at this site alone and were never detected in the lung airways. This and the fact that the reduction of

activated, influenza-specific CD8 T cells was observed only in the lung airways, and only at later time points post infection lead us to believe that direct depletion of NK1.1-expressing CD8 T cells cannot account for this reduction in treated mice. These data strongly suggest that NK1.1$^+$ cells in the lung airways are requisite for the subsequent accumulation of influenza-specific CD8 T cells in the BAL and implicate IL-15-mediated NK cell migration into the lung airways as a link between the innate and adaptive responses to influenza infection. We currently favor a model in which NK cells migrate directly to influenza-induced IL-15 in the lung airways, and, CD8 T cell trafficking, in turn, occurs to both IL-15 directly and to chemotactic factors produced by NK cells already present at the site. A positive feedback loop in which NK cells responding to IL-15 induce further expression of IL-15 by dendritic cells for the stimulation of CD8 T cells is an additional possibility [261]. Moreover, IL-15 has also been shown to modulate chemokine and chemokine receptor expression by NK cells and T cells [131,178,262]. As IL-15 deficiencies also cause reductions in CD8 T cell accumulation in the BAL [19], chemotactic potential of IL-15 for NK cells presented here provides a possible link between IL-15-mediated effects of both the innate and adaptive immune responses to influenza infection. Future work will attempt to tease out the roles of direct and indirect migration of both NK cells and CD8 T cells to IL-15 following influenza infection.

Regardless of mechanism, IL-15-induced trafficking of lymphocytes is an important part of our overall understanding of influenza immunobiology. The studies presented here emphasize the importance of IL-15 in mediating the innate response to influenza via the trafficking of NK cells, as viral titers were not as efficiently controlled early after infection in IL-15-blocked animals.  These and previous studies also suggest

that exogenous IL-15c could be used to modulate the immune response at both the innate and adaptive phases. Overall, we believe that IL-15 may be in important point of eventual immune intervention for treatment of primary infection and for adjuvanting vaccines.

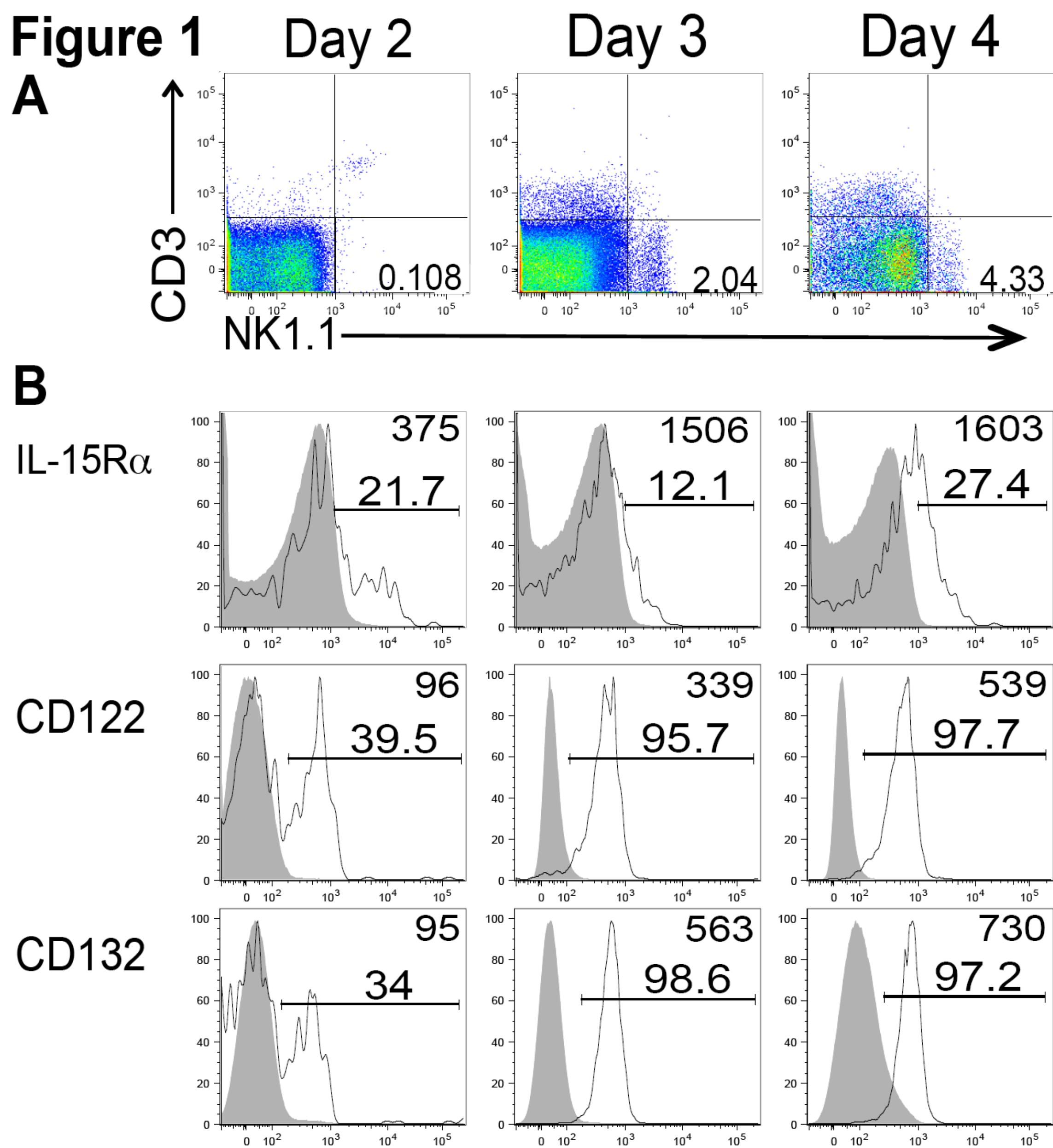


Figure 1
A
Day 2
Day 3
Day 4
CD3
NK1.1
0.108
2.04
4.33
B
IL-15Rα
CD122
CD132
375
21.7
1506
12.1
1603
27.4
96
39.5
339
95.7
539
97.7
95
34
563
98.6
730
97.2

**Figure 3.1. NK cells expressing IL-15 receptor accumulate in the lung airways of influenza-infected animals**. Lymphocytes were isolated from the BAL of animals infected with $10^3$ pfu HKx31i.n. at the indicated time points post infection. (A) Representative flow plots of NK 1.1$^+$, CD3$^-$ cells accumulating in the BAL over time. (B) Expression of IL-15Rα, CD122, and CD132 on NK cells is shown compared to either incubation with the secondary streptavidin alone (for IL-15Rα) or a fluorescence minus one (FMO) control (for CD122 and CD132 expression) (shaded histograms) for each time point p.i. (n=3 mice). Number in the upper right hand corner represents median fluorescence intensity. Data are representative of two independent experiments.

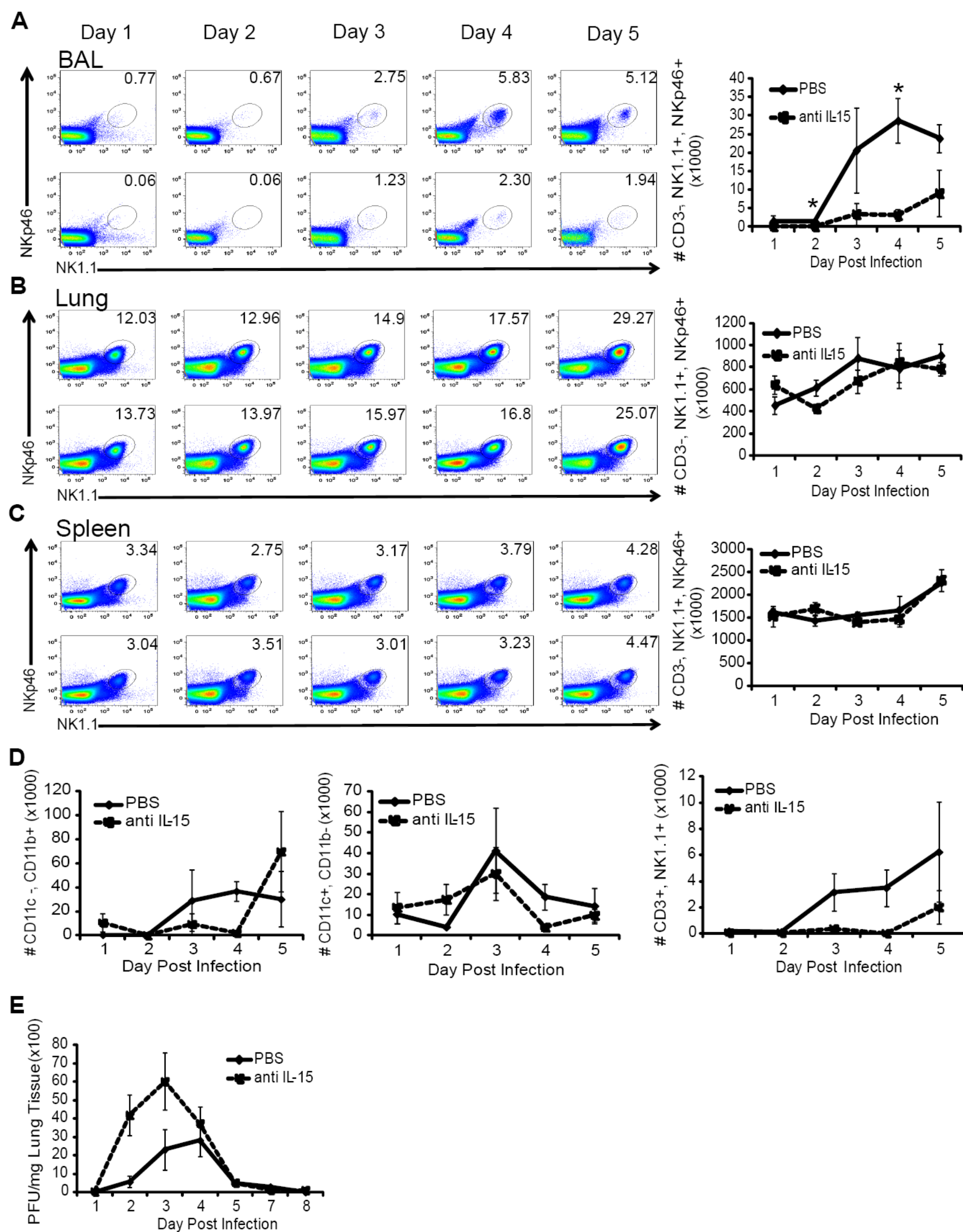

A
Day 1
Day 2
Day 3
Day 4
Day 5
BAL
0.77
0.67
2.75
5.83
5.12
0.06
0.06
1.23
2.30
1.94
NKp46
NK1.1
# CD3-, NK1.1+, NKp46+ (x1000)
PBS
anti IL15
Day Post Infection
B
Lung
12.03
12.96
14.9
17.57
29.27
13.73
13.97
15.97
16.8
25.07
C
Spleen
3.34
2.75
3.17
3.79
4.28
3.04
3.51
3.01
3.23
4.47
D
# CD11c -, CD11b+ (x1000)
# CD11c+, CD11b- (x1000)
# CD3+, NK1.1+ (x1000)
E
PFU/mg Lung Tissue (x100)
anti IL-15

**Figure 3.2. Absence of IL-15 selectively reduces the accumulation of NK cells at the site of infection, which impairs early viral control.** Representative flow plots depicting the mean frequency and graphs depicting the mean number $\pm$ SEM of $CD3^-$, $NK1.1^+$ $NKp46^+$ NK cells in PBS control (solid lines) or αIL-15-treated (dashed lines) mice recovered from the BAL (A), lung (B), and spleen (C) is plotted over time following i.n. infection with $10^3$ pfu HKx31 (n=3 mice/group; *p=0.032 and 0.0145). Data are representative of three independent experiments. (D) Mean number of $CD11c^-$ $CD11b^+$ cells, $CD11c^+$ $CD11b^-$ cells, and $CD3^+$ $NK1.1^+$ cells from BAL are shown $\pm$ SEM in PBS control (solid lines) or αIL-15-treated (dashed lines) mice (n=3 mice/group). (E) At the indicated times p.i. with $10^3$ pfu HKx31, lung viral titers from PBS and αIL-15-treated mice were determined by plaque assay. Mean viral titer is plotted $\pm$ SEM (n=2 mice/group on days 1 and 5 p.i. or 3 mice/group for remaining time points; day 2 p=0.0503). Data are representative of two independent experiments.

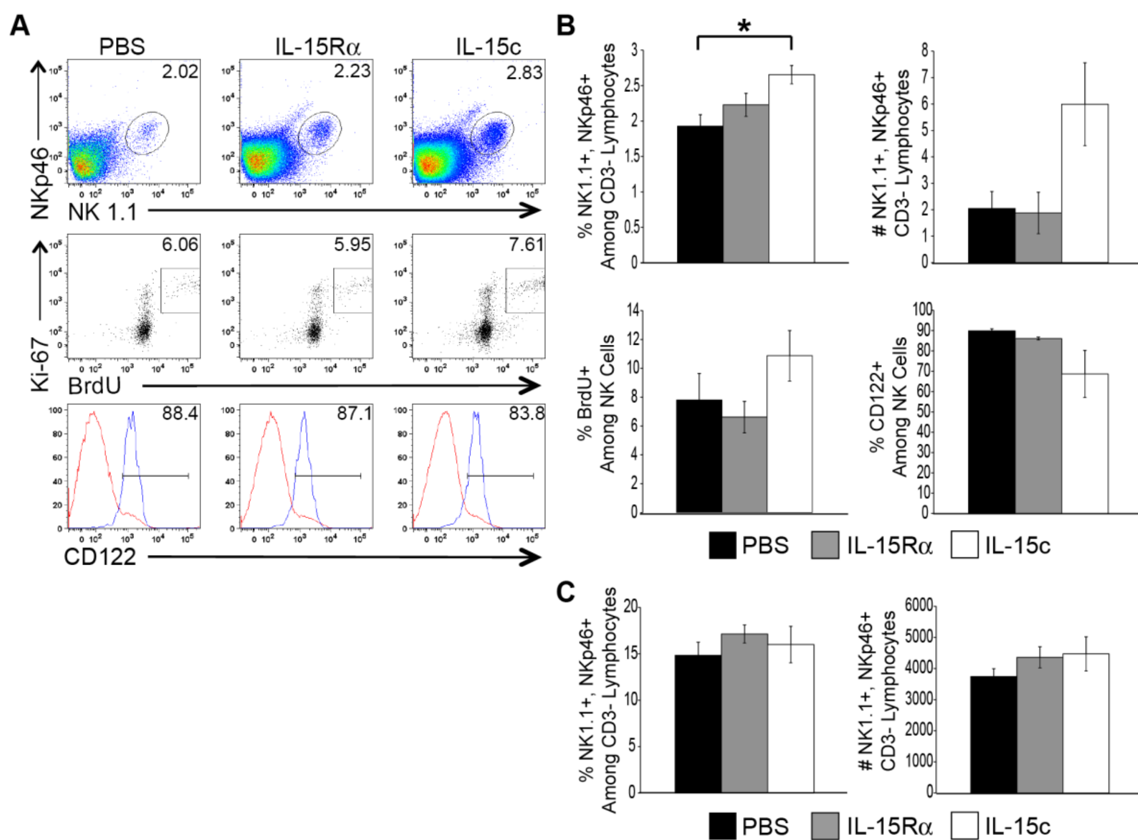

A
PBS
IL-15Rα
IL-15c
2.02
2.23
2.83
NKp46
NK 1.1
6.06
5.95
7.61
Ki-67
BrdU
88.4
87.1
83.8
CD122
B
% NK1.1+, NKp46+ Among CD3- Lymphocytes
# NK1.1+, NKp46+ CD3- Lymphocytes
% BrdU+ Among NK Cells
% CD122+ Among NK Cells
PBS
IL-15Rα
IL-15c
C
% NK1.1+, NKp46+ Among CD3- Lymphocytes
# NK1.1+, NKp46+ CD3- Lymphocytes
PBS
IL-15Rα
IL-15c

**Figure 3.3. Administration of IL-15c i.n. increases the number of NK cells recovered from the lung airways.** On day 3 p.i. with $10^3$ pfu HKx31, mice were administered either PBS vehicle control (black bars), IL-15Rα alone (shaded bars) or IL-15c (open bars) intranasally. Twelve hours post treatment, $CD3^-$ lymphocytes were analyzed for NK1.1 and NKp46 expression. These NK cells were quantified and analyzed for CD122 expression, BrdU incorporation, and Ki-67 staining. Representative flow plots are depicted in panel (A), and graphical representations of mean frequencies and numbers of NK cells ± SEM, as well as percentage of these cells positive for CD122 and BrdU are depicted in panel (B) (n=3 mice/group; *=p<0.025). (C) Mean frequencies and numbers of NK cells in the spleen are plotted ± SEM (n=3 mice/group). Data are representative of two independent experiments.

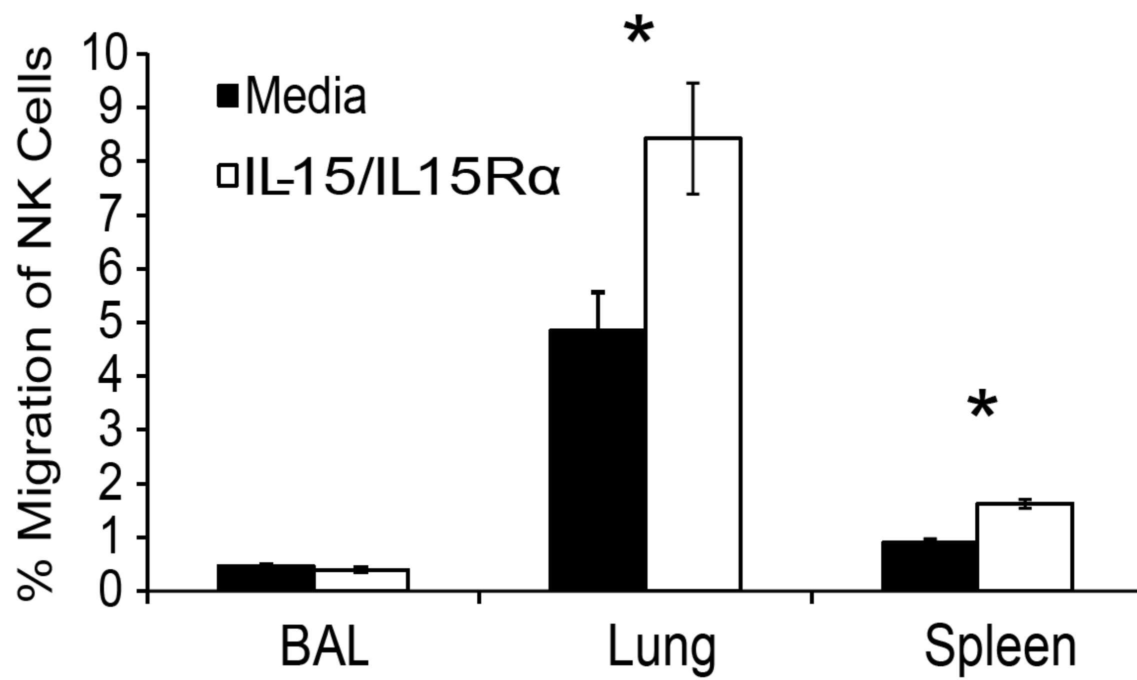

% Migration of NK Cells
10
9
8
7
6
5
4
3
2
1
0
Media
IL-15/IL15Rα
*
*
BAL
Lung
Spleen

**Figure 3.4. IL-15 is chemotactic for NK cells in vitro.** Three days following infection with $10^3$ pfu HKx31 i.n., $1x10^6$ bulk lymphocytes from the pooled BAL, lung, and spleen of 8 mice were placed in the top chamber of a transwell with the bottom chamber containing 500 µL either media alone (black bars) or supplemented with 100 ng IL-15c (open bars). Mean percent migration of $CD3^-$, $NK1.1^+$ NK cells is depicted $\pm$ SEM (n=3 replicates/group; *p=0.046 and 0.003). Data are representative of three independent experiments. Significant differences in migration to media alone or media containing IL-15c are indicated by stars (*p<0.05).

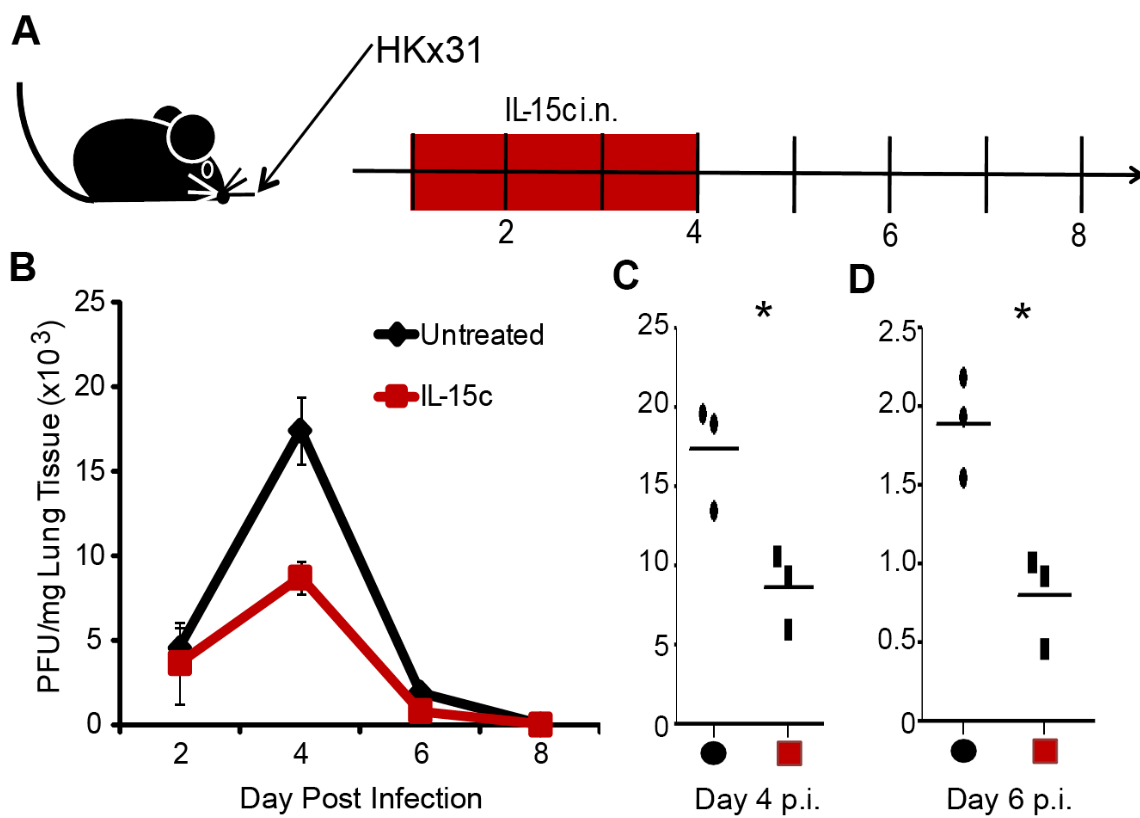

A
HKx31
IL-15c i.n.
2
4
6
8
B
PFU/mg Lung Tissue (x10³)
Untreated
IL-15c
Day Post Infection
C
Day 4 p.i.
D
Day 6 p.i.

**Figure 3.5. Intranasal administration of IL-15c during the innate phase of the immune response against influenza improves early control of viral control.** (A) On days 1-4 post infection with $10^3$ pfu HKx31 i.n., animals received either PBS vehicle control (Black circles) or IL-15c (red squares) i.n.. (B. Whole lungs were collected and analyzed via plaque assay for viral titer on days 2, 4, 6, and 8 p.i.. Significant differences between control and IL-15c-treated mice were observed on days 4 (C) and 6 (D) p.i. (*p=0.022 and 0.013).

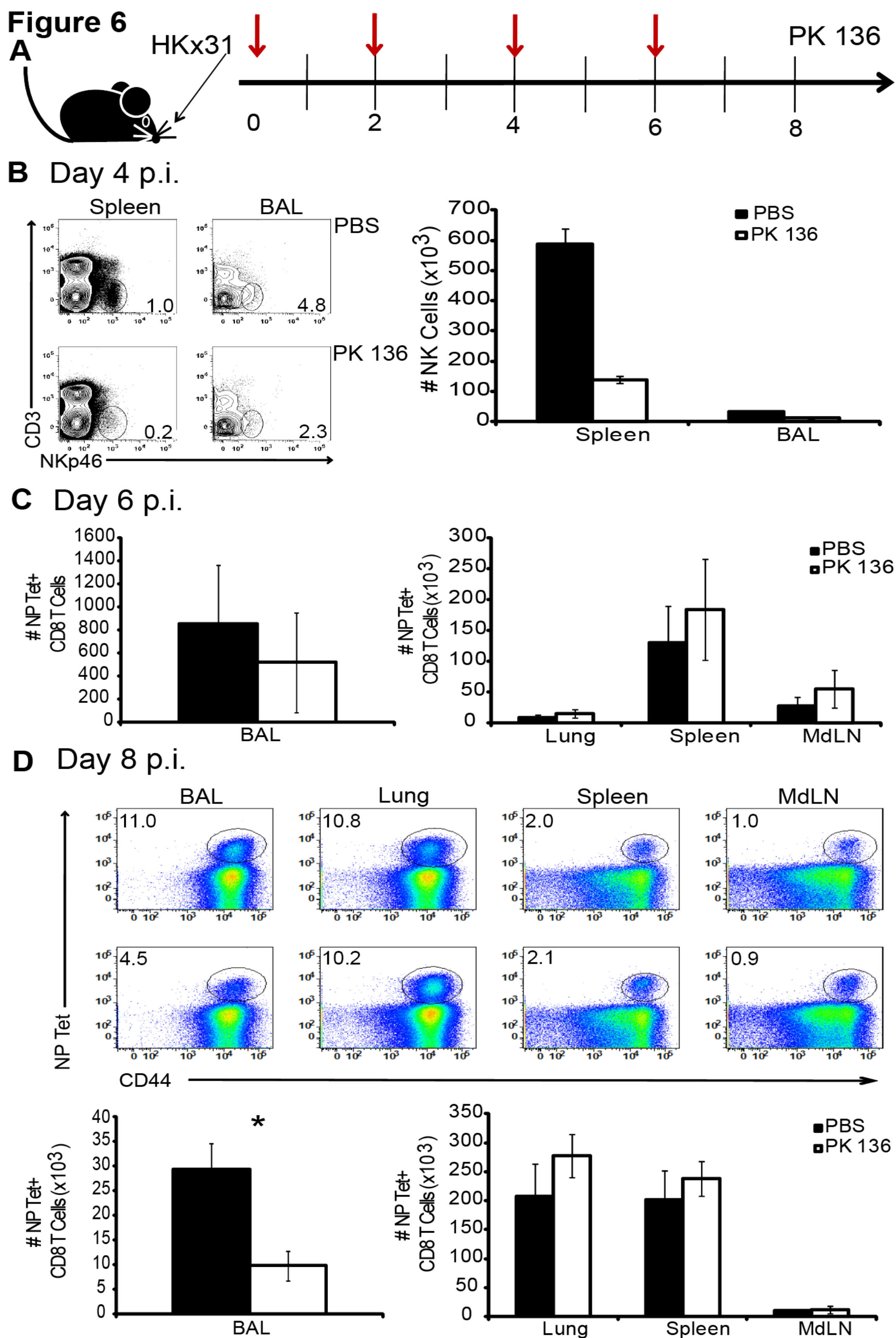


Figure 6
A
HKx31
PK 136
0
2
4
6
8
B Day 4 p.i.
Spleen
BAL
PBS
PK 136
1.0
4.8
0.2
2.3
CD3
NKp46
# NK Cells (x10^3)
PBS
PK 136
Spleen
BAL
C Day 6 p.i.
# NP Tet+ CD8 T Cells
BAL
# NP Tet+ CD8 T Cells (x10^3)
Lung
Spleen
MdLN
D Day 8 p.i.
BAL
Lung
Spleen
MdLN
11.0
10.8
2.0
1.0
4.5
10.2
2.1
0.9
NP Tet
CD44
# NP Tet+ CD8 T Cells (x10^3)
*
BAL
Lung
Spleen
MdLN

**Figure 3.6. NK1.1+ NK cells are partially required for the subsequent accumulation of influenza-specific CD8 T cell accumulation at the site of infection.** Beginning on the day of infection, mice received i.v. injections of either PBS vehicle control or αNK1.1 (PK136) every other day until day 6 p.i. (red arrows), and indicated tissues were collected on days 4, 6, and 8 p.i. (A). Mean number NK cells cells on day 4 p.i. in untreated (black bars) and PK136-treated (open bars) are shown ± SEM (n=3 mice/group) (B). Mean number of NP-Tet$^+$ CD8 T cells in the BAL, lung, and spleen were quantified and depicted ± SEM on day 6 (C) and day 8 (D) p.i. (n= 3 mice/group). Stars indicate statistical significance (*p=0.009). Data are representative of three independent experiments.

# CHAPTER 4

# CUTTING EDGE: IL-15 INDEPENDENT MAINTENANCE OF MUCOSALLY GENERATED MEMORY CD8 T CELLS[3]

[3] Verbist, K., M. Field, and K. Klonowski. 2011. *The Journal of Immunology: Cutting Edge*. 186:6667-6671. Reprinted here with permission of publisher.

## 4.1 Abstract

Effective vaccines against intracellular pathogens rely on the generation and maintenance of CD8 memory T cells (Tmem). Hitherto, evidence has indicated that CD8 Tmem use the common gamma chain cytokine IL-15 for their steady-state maintenance in the absence of antigen. This evidence, however, has been amassed predominantly from models of acute, systemic infections. Given that the route of infection can have significant impact on the quantity and quality of the resultant Tmem, reliance on limited models of infection may restrict our understanding of long-term CD8 Tmem survival. Here, we show IL-15-independent generation, maintenance, and function of CD8 Tmem following respiratory infection with influenza virus. Importantly, we demonstrate that alternating between mucosal and systemic deliveries of the identical virus prompts this change in IL-15 dependence, necessitating a reevaluation of the current model of CD8 Tmem maintenance.

## 4.2 Introduction

An effective host defense following reinfection with an intracellular pathogen relies on the generation and maintenance of memory CD8 T cell (Tmem) populations. Therefore, there has been considerable investigation into the signals that govern these processes as their exploitation could aid in vaccine development. The current model of Tmem development dictates that following pathogen clearance a small proportion of effector CD8 T cells (Teff) survive the contraction phase of the immune response and emerge as a stable Tmem pool that is maintained in the absence of Ag via sustained cytokine signaling [263]. IL-7 and IL-15 are members of the common gamma chain (γc)

family of cytokines which retain the steady-state numbers of Tmem, although their contributions to the maintenance of CD8 Tmem populations are not necessarily redundant [148,164].

While there is considerable evidence substantiating the role of γc cytokines in CD8 Tmem development and maintenance, there remain additional contexts in which to explore these roles. The landmark studies implicating IL-15 in the maintenance of CD8 Tmem were conducted in models of acute, systemic infection where intravenous infection of IL-15$^{-/-}$ mice with vesicular stomatitis (VSV) or lymphocytic choriomeningitis (LCMV) viruses resulted in the ~50% reduction of Ag-specific CD8 Tmem compared to WT mice, with Tmem attrition exacerbated over time [71,182,183]. Alterations in type or route of infection, however, can impact the CD8 Tmem pool. Qualitatively different CD8 Tmem is generated in response to the identical Ag delivered by either the mucosal or systemic route [186], and these different Tmem populations may require distinct homeostatic signals for their proliferation and survival.

The majority of pathogens causing human and animal diseases enter the host via a mucosal route, and unlike their systemically derived counterparts, the longevity of mucosal CD8 Tmem is limited [69,77]. Importantly, in the case of influenza infection, protection from challenge with heterosubtypic viruses is highly correlated with the retention and survival of a pool of Tmem in the airways [72]. We hypothesized that limited availability of IL-15 or the loss of IL-15 responsiveness by airway-resident Tmem was responsible for this attrition. We demonstrate, however, that loss of IL-15 neither prevents the generation of Tmem nor accelerates the loss of mucosally-generated, influenza-specific CD8 Tmem. Moreover, altering the route of infection correspondingly

alters the requirement for IL-15 in CD8 T cell development and homeostasis. Together our data demonstrate that both IL-15-dependent and independent Tmem pools exist and CD8 T cells primed in the mucosa require distinct signals for their long-term maintenance.

**4.3 Materials and Methods**

*Mice, viruses, and infection*

Age and sex-matched C57BL/6 and IL-15$^{-/-}$ mice were purchased from NCI (Bethesda, MD) and Taconic Farms (Germantown, NY). Drs. S. Mark Tompkins (University of Georgia, Athens, GA) and Leo Lefrançois (University of Connecticut, Farmington, CT) generously provided the influenza viruses A/HK-x31(x31, H3N2) and A/PR/8 (PR8, H1N1) and vesicular stomatitis virus (VSV), respectively. Animals were infected intranasally (i.n.) with either $10^3$ pfu x31, $5 \times 10^3$ pfu of PR8 or $10^4$ pfu of VSV and intravenously (i.v.) with $10^4$ pfu VSV, as indicated.

*Tissue preparation and flow cytometry*

Single-cell suspensions were obtained from spleens, lymph nodes, and peripheral blood and depleted of erythrocytes using Tris-buffered NH4Cl. Airway-resident cells were harvested via bronochoalveolar lavage (BAL) by gavaging with 1ml PBS 3-5x. Lung parenchyma lymphocytes were isolated following perfusion and enzymatic digestion with 1.25 mM EDTA and 6 mg/mL collagenase at 37°C and percoll gradient centrifugation.

MHC class I tetramers reactive against the influenza nucleoprotein (NP) (H-2D(b)/ASNENMETM) and VSV NP (H-2K(b)/RGYVYQGL) were generated by the NIAID Tetramer Facility (Emory University). Tetramer staining was conducted at RT 1

hr with other mAbs indicated and purchased from eBiosciences (San Diego, CA), BD Pharmingen (San Jose, CA), or BioLegend (San Diego, CA). Stained cells were analyzed using a BD LSR II digital flow cytometer and BD FACs Diva software.

*Plaque and in vivo CTL assays*

For plaque assays, lungs from x31-immune WT and IL-15$^{-/-}$ mice challenged with PR8 7d previously were lysed in 1ml 1x MEM+1μg/ml TPCK-treated trypsin. MDCK cells were incubated with serially diluted homogenate for 1 hr at 37°C. Inoculums were removed and monolayers were washed and overlaid with MEM containing 1.2% Avicel microcystalline cellulose (FMC BioPolymer, Philadelphia, PA), 0.04M HEPES, 0.02 mM L-glutamine, 0.15% NaHCO3, and 1μg/ml TPCK-trypsin. 72 hrs post infection (p.i.), monolayers were fixed and stained with crystal violet.

For *in vivo* CTL assays, half the splenic targets were pulsed with 10 μM influenza NP peptide (ProImmune) for 1 hr at 37°C and the remainder unpulsed. Following labeling with 10 μM and 1 μM CFSE respectively, 1x107 of targets were injected 50:50 i.v. into recipient mice. 14 hrs later, target number and intensity of CFSE staining was determined by flow cytometry. Percentage of target killing was determined by 100-((% of NP-pulsed targets in infected recipients / % unpulsed targets in infected recipients) / (% NP-pulsed targets in naïve recipients / % unpulsed targets in naïve recipients) x 100).

*Statistics*

Unpaired two-tailed student's T test was applied using Prism Graphpad software.

P values are indicated in the figure legend where statistical significance was found.

## 4.4 Results and Discussion

*IL-15 is dispensable for the generation and maintenance of influenza-specific CD8 Tmem.*

Following intranasal (i.n.) infection with influenza virus, a population of Ag specific CD8 T cells are activated, migrate to the lung airways, and differentiate in situ into CD8 Tmem where, if maintained in adequate number, confer subsequent protection to heterologous infections [72,77,244]. Unfortunately, in the months that follow influenza infection, this population declines, despite the fact that a stable reservoir of splenic CD8 Tmem continues to migrate to the lung airways to maintain steady-state numbers [74]. These data would suggest that the respiratory tract fails to support the long-term maintenance of these CD8 Tmem originally positioned to respond quickly to reinfection. Since IL-15 is well characterized for its role in maintaining CD8 Tmem in other models of acute viral infection [148,182,183], we hypothesized that the limited longevity of the protective CD8 Tmem in the lung airways following a mucosal influenza infection is due to dysregulation of IL-15 signaling at this site.

We first determined whether IL-15 is required for the generation of influenzaspecific CD8 Tmem. by infecting C57Bl/6 wild type (WT) and IL-15$^{-/-}$ mice with influenza A/HKx31 (x31) and monitoring the specific CD8 T cell response to influenza nucleoprotein (NP) via MHC class I tetramers. Because IL-15$^{-/-}$ mice have only half the number of total CD8 T cells and NP-specific, naïve precursors as WT animals [102], we monitored alterations in the overall frequency of these influenza-specific CD8 T cells in the peripheral blood of WT and IL-15$^{-/-}$ mice through day 40 p.i. (Fig 1). The frequency of NP-specific CD8 T cells in the circulation of IL-15$^{-/-}$ mice remained similar

to WT animals throughout the course of the response (Fig 1B). Additionally, the overall kinetics of CD11ahi (likely representing CD8 T cells responding to influenza epitopes other than NP) CD8 T cell responses to influenza were similar in WT and IL-15$^{-/-}$ mice (Fig. 1 A and data not shown). Thus, in contrast to previous studies in which IL-15 was required for the generation of VSV-specific CD8 Tmem [148], we found IL-15 to be dispensable for the generation of influenza-specific CD8 Tmem.

While IL-15 has been implicated in the development of CD8 Tmem, other studies have found it dispensable for generation but essential for the maintenance of CD8 Tmem [182,183]. Therefore, it was possible that following full differentiation and trafficking to specific sites influenza-specific Tmem would gradually become more dependent on IL-15 for survival signals. To test this possibility, we collected lymphocytes from the lung airways (via broncheoalveolar lavage [BAL]), lung parenchyma, spleen, and lung draining mediastinal lymph nodes (MdLN) of WT and IL-15$^{-/-}$ mice at an early memory time point (day 31) and later, (day 115) p.i.. While the overall frequency of NP-specific Tmem decreased between days 31 and 115 p.i. in WT and IL-15$^{-/-}$ animals, both groups harbored a similar frequency of NP-specific CD8 T cells in all tissues and at all time points examined (Fig 2 A-B). Surprisingly, not only did the IL-15 deficiency fail to exacerbate the loss of CD8 Tmem from the lung airways, the frequency of splenic CD8 Tmem recovered was unaltered in the absence of IL-15. While Ag-specific CD8 T cells isolated from BAL express low levels of CD122 (1-5%), which is required to receive survival signals from transpresented IL-15 (15), 40-60% of splenic anti-influenza CD8 Tmem are CD122+ throughout the time course analyzed (data not shown). These data indicate that IL-15 does not contribute to the homeostatic maintenance of differentiated

influenza-specific CD8 Tmem at either the site of infection or in the periphery.

*The homeostatic proliferation of influenza NP-specific CD8 Tmem in WT and IL-15$^{-/-}$ mice is equivalent.*

Whereas IL-15 is known to be important for the homeostatic proliferation of CD8 Tmem, other γc cytokines such as IL-7 are more important for providing pro-survival signals to CD8 Tmem [264]. To eliminate the possibility that the equivalent accumulation of influenza-specific CD8 Tmem in WT and IL-15$^{-/-}$ mice was due to enhanced compensatory survival signals in the absence of IL-15, we examined the expression of Ki-67 in NPtetramer+ CD8 Tmem cells isolated from IL-15$^{-/-}$ mice for lack of cell cycle entry. Thirty-two days p.i. the percentage of influenza-specific CD8 Tmem actively proceeding through the cell cycle was modestly reduced in the lymphoid tissues of IL-15$^{-/-}$ mice suggesting that compensatory signals may sustain the survival of anti-influenza CD8 Tmem in lymphoid tissue early after infection when IL-15 is absent. However, this reduction was resolved by day 115 p.i. (Fig. 2 C). Thus, in contrast to models of systemic viral infection wherein CD8 Tmem turn-over was severely impaired over time [148,182,265], a similar proportion of influenza-specific CD8 Tmem populations undergo homeostatic proliferation in both WT and IL-15$^{-/-}$ animals as late as 115 days p.i. suggesting that IL-15 independent signals regulate CD8 Tmem homeostasis in response to respiratory infection.

*The differentiation of influenza-specific CD8 Tmem subsets is unaltered in IL-15$^{-/-}$ mice.*

In the linear model of Tmem development, transitioning CD8 Teff can elect one of two fates delineated by the differential expression of the killer-lectin-like receptor G-1 (KLRG-1) and the IL-7 Rα chain (CD127), which denote KLRG-1hiCD127lo short-lived

effector cells (SLECs) and KLRG-1loCD127hi memory precursor effector cells (MPECs) [264]. IL-15 has been shown to be particularly important for promoting the survival of SLECs during the contraction phase of an Ag-specific CD8 T cell response [174]. Consistent with these findings, frequencies of NP-specific MPECs were slightly elevated in the tissues of IL-15$^{-/-}$ animals at day 32 p.i. (data not shown). However, by d115 post influenza infection, frequencies of MPECs were equivalent in IL-15$^{-/-}$ and WT mice (Fig 3 A), indicating that, even though SLECs are rapidly lost in the absence of IL-15 signaling, MPECs are preserved as they transition into a steady-state memory population. Moreover, CD8 Tmem are phenotypically heterogenous and may be sub-classified as either CD62Llo extra lymphoid tissue-homing effector memory (Tem) or CD62Lhi lymphoid-homing central memory (Tcm) [67]. To test whether an IL-15 deficiency differentially affected a specific Tmem subset, we monitored the expression of CD62L on influenza-specific CD8 T cells in both WT and IL-15$^{-/-}$ mice over time. The kinetics of CD62L expression on the NP-specific CD8 T cells in the blood (data not shown) and tissues of both IL-15$^{-/-}$ and WT animals was similar at d115 p.i. (Fig. 3 B), demonstrating that the sustained ratio of influenza-specific Tcm and Tem generated in the presence or absence of IL-15 is equivalent. Recent work, however, has demonstrated that in respiratory models of infection the activation markers CD27 and CD43 provide a better indication of the capacity of CD8 Tmem to participate in secondary responses than traditional Tcm and Tem phenotyping [266]. Therefore, we monitored the frequency of CD27hiCD43lo CD8 Tmem in WT and IL-15$^{-/-}$ mice to test whether this subpopulation of CD8 Tmem required IL-15 signaling for their maintenance in different tissues. At day

115 p.i., frequencies of CD27hiCD43lo Tmem were similar in WT and IL-15$^{-/-}$ animals in all tissues examined (Fig 3C). Thus, although the possibility exists that an IL-15-independent subset of CD8 Tmem expands to compensate for the loss of an IL-15-dependent CD8 Tmem subset, using three different Tmem subset phenotypes we could observe no requirement for IL-15 in maintaining distinct CD8 Tmem populations following influenza infection.

*CD8 Tmem generated in IL-15$^{-/-}$ mice is fully functional*

True immunological memory is defined by the ability of Ag-specific CD8 Tmem to rapidly recognize and control an infection after a secondary encounter. IL-15 deficiency impaired a CD127hiCD62Llo subset of secondary memory CD8 T cells induced by *Listeria* or Sendai infections [267]. Thus, it was possible that, although similar frequencies of influenza-specific CD8 Tmem were maintained in WT and IL-15$^{-/-}$ mice, an IL-15 deficiency functionally impaired the memory population. To test this possibility, x31- immune WT and IL-15$^{-/-}$ or naïve WT mice were challenged with a lethal dose of the heterosubtypic H1N1 influenza A/PR/8/34 (PR/8) virus that shares the conserved NP protein with x31. One week post challenge, the presence of virus in the lung was determined by plaque assay. While naïve animals averaged 750 pfu/mg lung tissue, the lungs of both WT and IL-15$^{-/-}$ memory mice were completely devoid of virus (Fig. 2 C). The functionality of NP-specific CD8 Tmem generated in the absence of IL-15 was also tested via an *in vivo* cytotoxic T lymphocyte assay. Equal numbers of naïve splenocytes pulsed either with or without influenza NP-peptide and differentially labeled with CFSE were injected into WT and IL-15$^{-/-}$ mice 60 days after infection with x31. Fourteen hrs post transfer, spleens of recipient animals were harvested and the percentage

of specific killing was determined. Both WT and IL-15$^{-/-}$ influenza-immune recipient mice killed ~30% of the peptide pulsed targets (Fig. 2 D). Together, these data indicate that the functional quality of the CD8 Tmem is preserved independent of IL-15.

*Route of infection alters requirements for IL-15 by resultant CD8 Tmem.*

While the frequency of VSV and LCMV-specific CD8 Tmem decayed over time in IL-15$^{-/-}$ mice, we failed to observe any accelerated loss of influenza-specific in any site. A major difference between all of these studies is the route of infection, which can alter CD8 T cell responses and lineage programming. Thus, to directly test the hypothesis that the route of the infection results in a differential requirement of Ag-specific CD8 Tmem cells for IL-15, WT and IL-15$^{-/-}$ mice were systemically (i.v.) or mucosally (i.n.) infected with VSV. On day 30 p.i., both lymphoid and non-lymphoid tissues were examined for reactivity with the VSV NP-tetramer. As observed previously [148], the frequency of Nspecific CD8 Tmem cells in IL-15$^{-/-}$ mice was reduced by >50% in all tissues after systemic infection (Fig. 4). In contrast, however, WT and IL-15$^{-/-}$ animals mucosally infected with the identical virus contained an equivalent frequency of N-specific CD8 Tmem in all sites examined despite a lower magnitude of CD8 Tmem overall (Fig. 4). Thus, altering the route of infection with the same virus concordantly altered the requirement of IL-15 for the generation and maintenance of Ag-specific CD8 Tmem cells.

We were surprised to find an equal preservation of CD8 Tmem in the secondary lymphoid tissues of IL-15$^{-/-}$ mice infected i.n. with either influenza or VSV. One might speculate that altering infection route favors the development of either Tem or Tcm, which could be differentially dependent on IL-15 for survival. Comparing systemic to

intranasal VSV infection, the ratio of central: effector splenic N-specific CD8 Tmem shifted from 20:80 to 40:60 (data not shown). While the overall alteration in Tmem subsets could be due to differences in Ag load [268], IL-15$^{-/-}$ mice generated ratios of Tmem subsets equivalent to WT regardless of the route of infection. While IL-7 can redundantly substitute for IL-15, we failed to observe any differences in CD127 expression of Tmem [165,175]. We also failed to observe any differences in CD122 expression on circulating Tmem due to route of infection (data not shown). Therefore, IL-15-independent Tmem generated after mucosal infection are not the result of alterations in the development of a particular Tmem subset or CD122 expression but are more likely due to a unique priming environment which bestows a homeostatic program on the Tmem that is IL-15-independent.

Another possibility is that the harsh, regulatory environment of the lung airways renders this site incapable of sustaining CD8 Tmem long-term. Mucosal immune responses require extensive regulation of immune activation to prevent immunopathology. Thus, the mucosal environment stringently regulates the type, level, and duration of cytokines and chemokines elicited by mucosal infections in order to activate, recruit, and ultimately sustain (perhaps at set numerical thresholds) the appropriate lymphocytes at these sites. Our experiments contrasting the differential requirement for IL-15 in systemic vs mucosally administered VSV illustrates this phenomenon. While systemically there may be a need to maintain large numbers of VSV-specific CD8 Tmem (to prevent pathogen dissemination or reinfection), the regulatory mechanisms in place in the lung could be sufficient to inhibit the maintenance of memory cell pools beyond a certain threshold, regardless of IL-15 availability. Thus, while

adjuvanting IL-15 could prolong CD8 Tmem responses to systemic infections, such regimens may have little benefit in sustaining differentiated respiratory-derived CD8 Tmem.

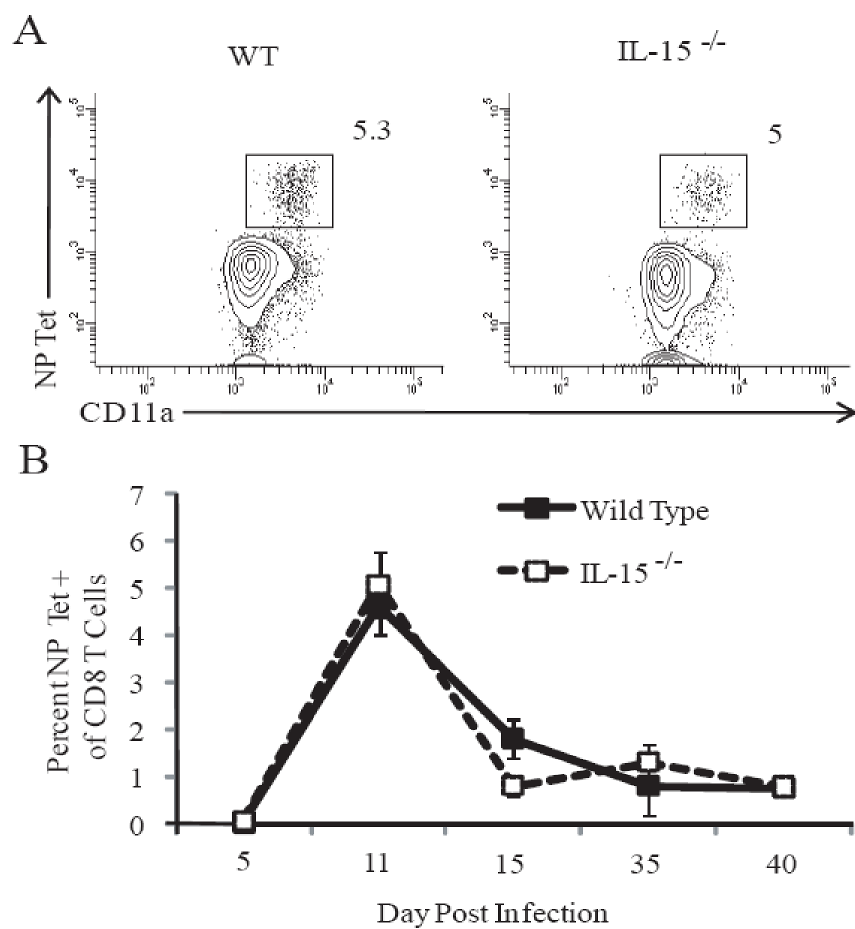

A
WT
IL-15 -/-
5.3
5
NP Tet
CD11a
B
Wild Type
IL-15 -/-
Percent NP Tet + of CD8 T Cells
Day Post Infection
0
1
2
3
4
5
6
7
5
11
15
35
40

**Figure 4.1. IL-15 is dispensable for the generation of influenza NP-specific CD8 Tmem.** At the indicated time points post influenza infection, CD8+ PBLs were analyzed for NP tetramer reactivity. (A) Representative flow plots for WT and IL-15$^{-/-}$ mice at day 11 p.i. (B) Mean frequencies of NP-Tet+ CD8 T cells among total CD8 T cells are plotted over time $\pm$ SEM for WT (solid line) and IL-15$^{-/-}$ (dashed line) mice (n=6 mice/group). Data are representative of four independent experiments.

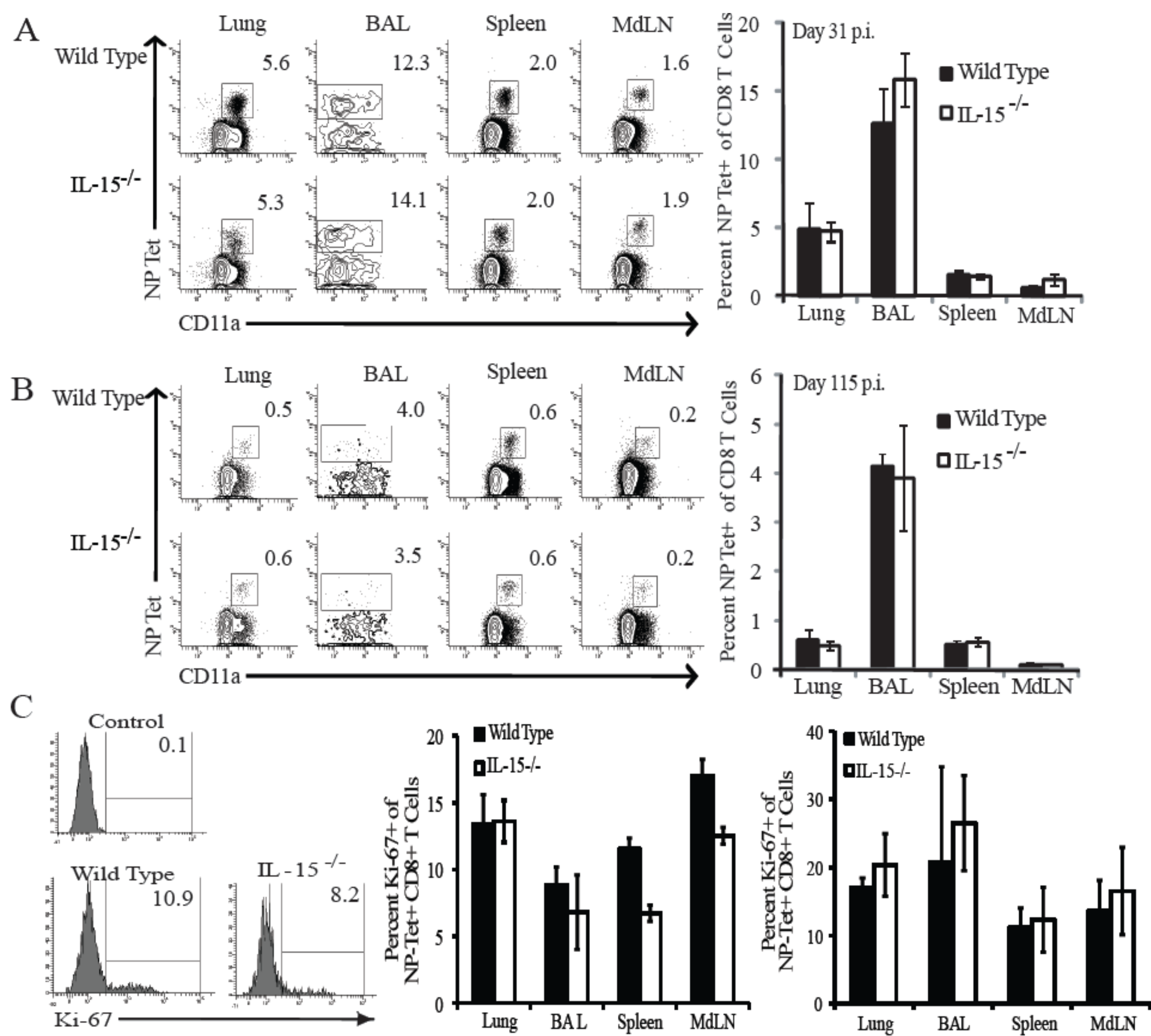

A
Lung
BAL
Spleen
MdLN
Wild Type
IL-15-/-
5.6
12.3
2.0
1.6
5.3
14.1
2.0
1.9
NP Tet
CD11a
Day 31 p.i.
Wild Type
IL-15 -/-
Percent NP Tet+ of CD8 T Cells
B
Day 115 p.i.
0.5
4.0
0.6
0.2
0.6
3.5
0.6
0.2
C
Control
0.1
Wild Type
10.9
IL-15 -/-
8.2
Ki-67
Percent Ki-67+ of NP-Tet+ CD8+ T Cells
IL-15-/-

**Figure 4.2. IL-15 is not required for the homeostatic maintenance of influenza-specific CD8 Tmem.** At day 31 (A) and 115 (B) post x31 infection, CD8 T cells were isolated from the specified tissues and analyzed for tetramer reactivity and CD11a expression. Representative flow plots for the individual tissues from WT and IL-15$^{-/-}$ mice are shown. The mean percent NP-Tet+ of total CD8+ T cells are plotted for WT (shaded bars) and IL-15$^{-/-}$ (open bars) $\pm$ SEM on day 31 p.i. (n=3-4 mice/group; data are representative of three independent experiments) and on day 115 p.i. (n=5 mice/group). (C) Representative flow plots for intracellular Ki-67 staining in NP-Tet+ CD8 T cells from spleens of WT and IL-15$^{-/-}$ mice at day 32 p.i.. Control represents fluorescence minus one stain. The mean percent Ki-67+ among NP-Tet+ CD8 T cells are plotted for WT (shaded) and IL- 15$^{-/-}$ (open bars) mice $\pm$ SEM on days 32 and 115 p.i. (n=at least 4 mice/group).

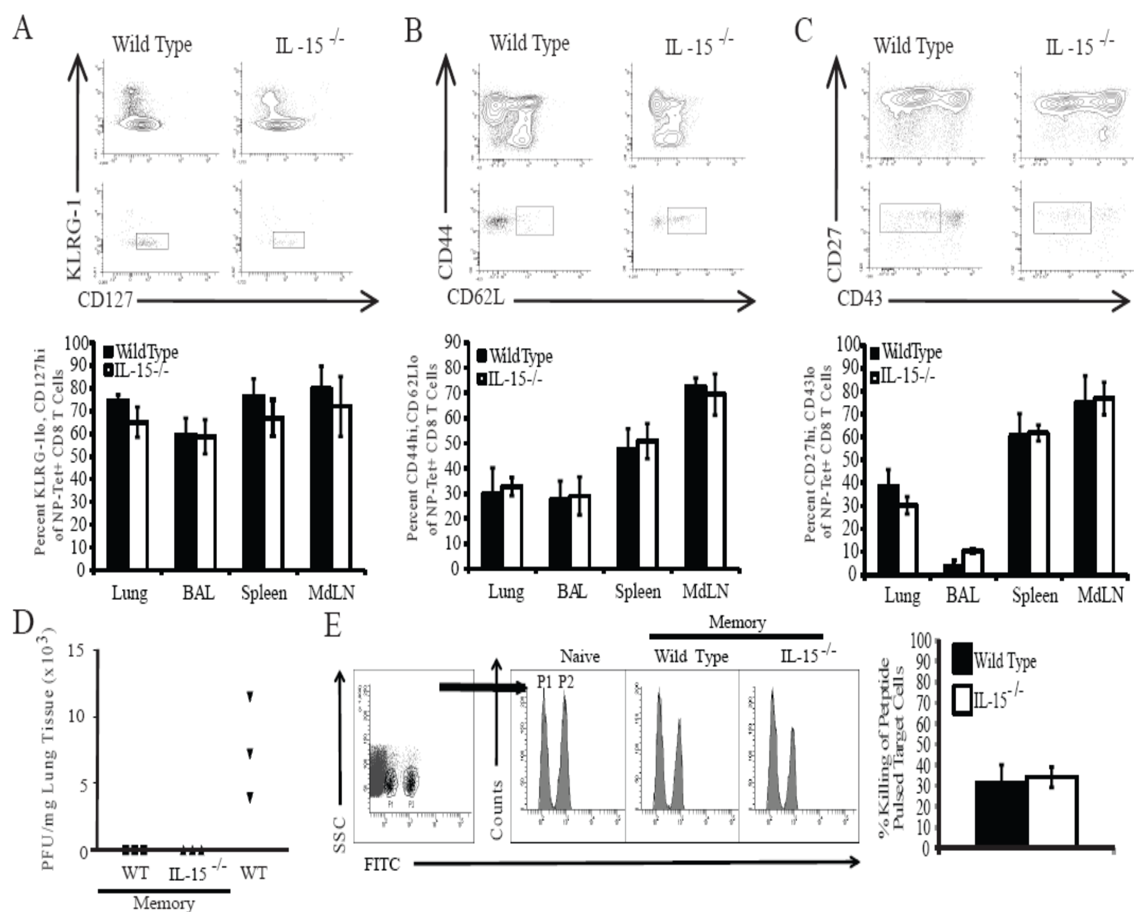
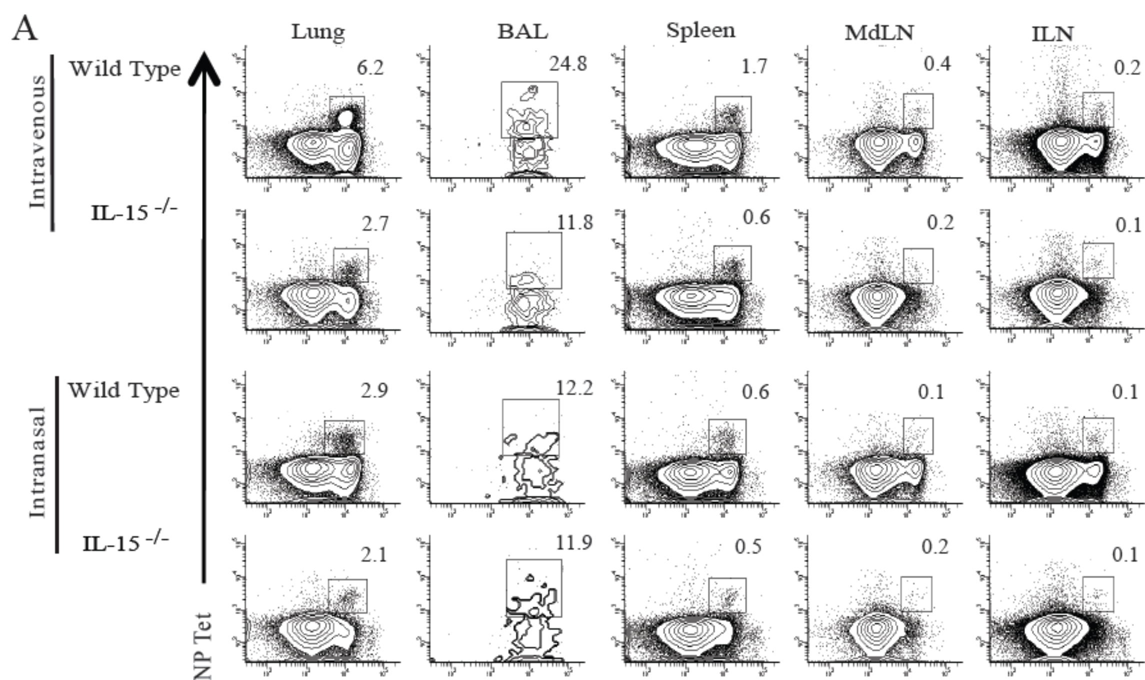

A
Wild Type
IL -15 -/-
KLRG-1
CD127
Percent KLRG-1lo, CD127hi of NP-Tet+ CD8 T Cells
WildType
IL-15-/-
Lung
BAL
Spleen
MdLN
B
Wild Type
IL -15 -/-
CD44
CD62L
Percent CD44hi,CD62Llo of NP-Tet+ CD8 T Cells
WildType
IL-15-/-
Lung
BAL
Spleen
MdLN
C
Wild Type
IL -15 -/-
CD27
CD43
Percent CD27hi, CD43lo of NP-Tet+ CD8 T Cells
WildType
IL-15-/-
Lung
BAL
Spleen
MdLN
D
PFU/mg Lung Tissue (x10^3)
WT
IL-15 -/-
WT
Memory
E
Memory
Naive
Wild Type
IL-15 -/-
P1 P2
SSC
Counts
FITC
% Killing of Peptide Pulsed Target Cells
Wild Type
IL-15 -/-

**Figure 4.3. Neither the function nor the maintenance of specific influenza NP-reactive CD8 Tmem subsets is altered in IL-15$^{-/-}$ mice.** NP-Tet+ CD8 T cells from the indicated tissues were analyzed for CD127 and KLRG-1 (A), CD44 and CD62L (B), and CD27 and CD43 (C) expression on days 32 and 115 post x31 infection. Flow plots represent total CD8 T cells (top row) and NP-Tet+ CD8 T cells (bottom row) in lungs from WT and IL-15$^{-/-}$ mice at day 115 p.i.. Mean frequencies among total NP-Tet+ CD8+ T cells are plotted $\pm$ SEM for WT (shaded bars) and IL-15$^{-/-}$ (open bars) mice (n=4-6 mice/group). (D) Viral titers from the lungs of WT (squares) and IL-15$^{-/-}$ (triangles) memory or WT naïve (inverted triangles) mice as determined by plaque assay (n=3 mice/group). Data are representative of two independent experiments. (E) Representative flow plots for *in vivo* killing of unpulsed (P1) and influenza NP-pulsed (P2) target cells in naïve WT or WT and IL-15$^{-/-}$ memory mice 60 days p.i.. CFSE+ populations were gated as indicated. The mean percent specific killing (right) is depicted for WT (shaded bar) and IL-15$^{-/-}$ (open bar) $\pm$ SEM (n=3 mice/group).


A
Lung
BAL
Spleen
MdLN
ILN
Intravenous
Wild Type
IL-15 -/-
Intranasal
Wild Type
IL-15 -/-
6.2
24.8
1.7
0.4
0.2
2.7
11.8
0.6
0.2
0.1
2.9
12.2
0.6
0.1
0.1
2.1
11.9
0.5
0.2
0.1
NP Tet
CD44

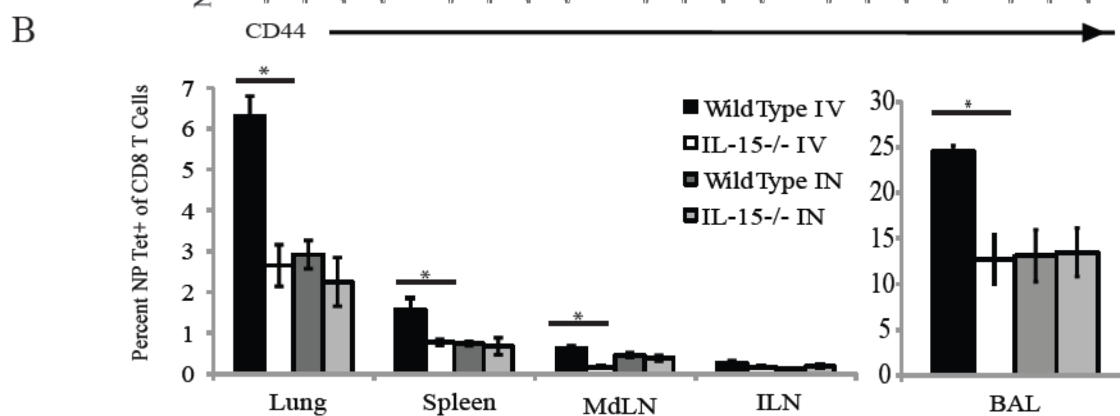
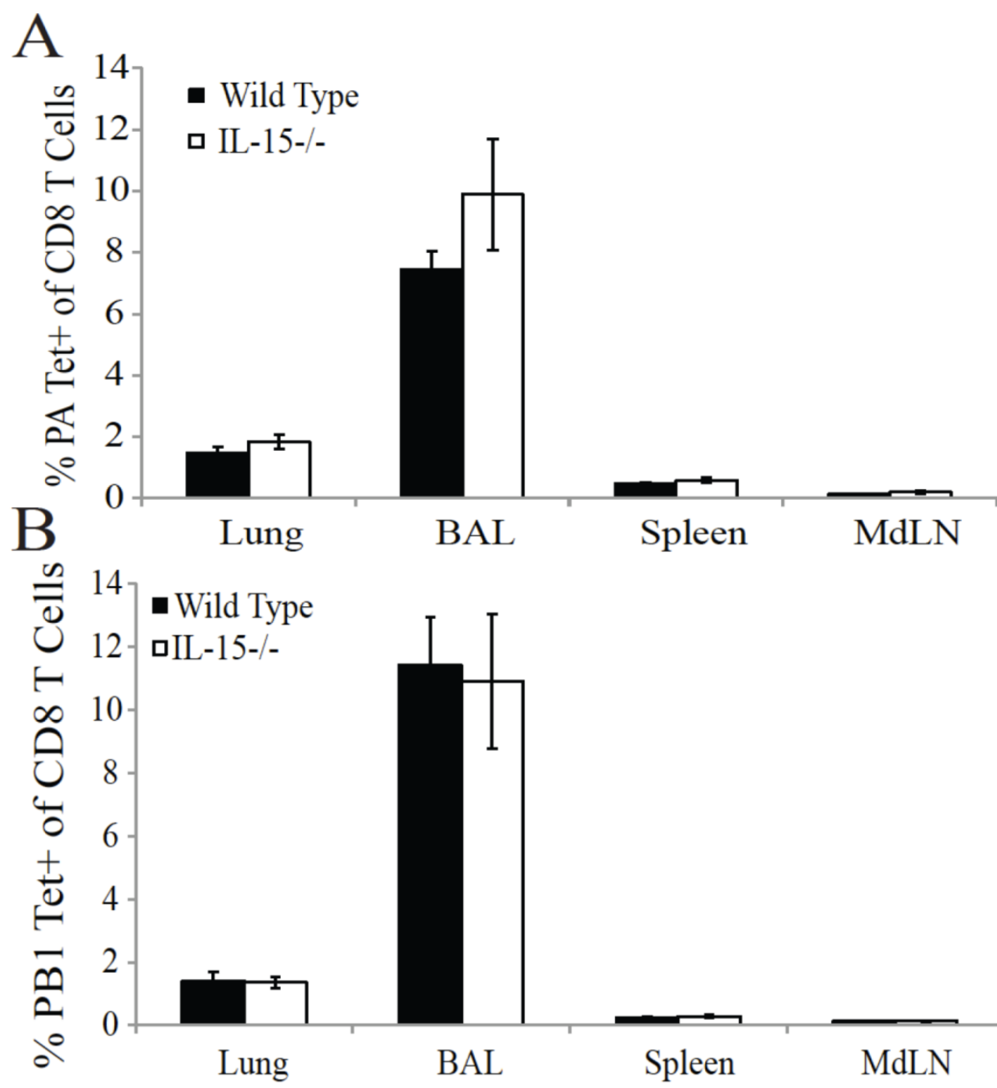

B
Percent NP Tet+ of CD8 T Cells
WildType IV
IL-15-/- IV
WildType IN
IL-15-/- IN
Lung
Spleen
MdLN
ILN
BAL
*

**Figure 4.4. Differential requirement for IL-15 in the development and maintenance of CD8 Tmem generated by a systemic vs. mucosal infection.** The mean percent VSV-NP-Tet+ among CD8 T cells in the indicated tissues (A) are plotted (B) for systemically ($10^4$ pfu VSV i.v.) infected WT (black bars) or IL-$15^{-/-}$ (open bars) and mucosally ($10^4$ pfu VSV i.n.) infected WT (darkly shaded bars) or IL-$15^{-/-}$ (lightly shaded bars) are plotted $\pm$ SEM (n=4-5 mice/group) on day 30 p.i.. Data are representative of two independent experiments.


A
% PA Tet+ of CD8 T Cells
Wild Type
IL-15-/-
14
12
10
8
6
4
2
0
Lung
BAL
Spleen
MdLN
B
% PB1 Tet+ of CD8 T Cells
Wild Type
IL-15-/-
14
12
10
8
6
4
2
0
Lung
BAL
Spleen
MdLN

**Supplemental Figure 4.1. Influenza-specific CD8 Tmem exhibit IL-15 independence regardless of Ag specificity.** At day 35 post x31 infection, CD8 T cells were isolated from the specified tissues and analyzed for tetramer reactivity with either PA (H2D(b) SSLENFRAYV) or PB1 (H2K(b) SSYRRPVGI) and CD11a expression. The mean percent PA Tet+ (A) or PB1 Tet+ (B) among total CD8 T cells are plotted for WT (shaded bars) and IL-15$^{-/-}$ (open bars) $\pm$ SEM (n=5 mice/group).

# CHAPTER 5

# CONCLUSIONS AND FUTURE DIRECTIONS

The coordinated actions of the innate and adaptive immune responses are essential for the clearance of influenza infection. Each arm of the immune system contains multiple cell types with unique and over-lapping effector mechanisms that participate in viral control, and these cell types are in continuous communication with each other. This variety, redundancy, and interdependence yield a very complex web of responses to influenza. Despite this intricacy, two cell populations have emerged as being especially important in immune responses to influenza infection. In the innate responses, NK cells provide control of early viral replication, and once adaptive responses are initiated, influenza-specific CD8 T cells are critical for the clearance of the virus. Because infection and replication of the virus is limited to the upper respiratory tract, these lymphocyte populations must migrate from peripheral anatomical sites—where they develop and proliferate—to the site of infection. At the commencement of this work, it was widely assumed that various cytokines and chemokines upregulated in the lung airways in response to infection provide the primary chemotactic signals for these cell types, but the nature of these signals was mostly unexplored. Mechanisms of lymphocyte trafficking into the lung airways are probably various, redundant, and in need of continued investigation because such understanding would greatly enhance vaccine and therapeutic strategies that enhance the effector functions of these cells.

We have demonstrated in this dissertation that local increases in the homeostatic cytokine IL-15 provide important chemotactic signals for both NK cell and influenza-specific CD8 T cell populations to enter the site of infection and control viral replication there. Initial investigations revealed that influenza infection induces the expression of IL-

15, resulting in dramatic upregulation of transcript levels, as measured by QRT-PCR. Protein levels in the lung and in the serum are also increased as measured by ELISA. This data has also been confirmed by independent studies in other labs [149]. Although McGill *et al*. identifies lung-resident dendritic cells as an important source of IL-15 at day 6 post infection [149], our data demonstrate that levels are significantly increased as early as day 3 post infection. The source of this early pool of IL-15 is as yet unidentified. As mentioned in the “Discussion” section of chapter 3, IL-15 is produced by a variety of cell types within and outside of the immune system, including macrophages, endothelial cells, and epithelial cells. Thus, influenza-infected epithelial cells themselves may be an important early source of IL-15, and DCs and other APCs may be a later source. IL-15 not only mediates the migration of NK and CD8 T cells into the lung airways, but IL-15 is known to also alter the activation status and enhance and survival and proliferation of these cell types. Therefore, either the source or context of IL-15 signaling must differentially dictate the eventual functional outcome. It is well-described that IL-15 transpresented by IL-15Rα-expressing dendritic cells promote the development of NK cells and the survival and proliferation of CD8 T cells [83]. IL-15 signaling, however, can proceed through a variety of receptor chain combinations and pathways. Expression of IL-15Rα by the NK and CD8 T cells themselves is completely dispensable for IL-15 to induce survival and proliferation in these cell types; yet, both cell types constitutively express this receptor chain. There is no currently known function for IL-15Rα on mature NK and CD8 T cells. It is tempting to speculate that soluble IL-15 signaling through the Rα chain results in cell migration whereas transpresentation of the cytokine results in survival or proliferation. It is also known that IL-15 bound to the Rα can be cleaved from

the presenting cell surface and released as a soluble complex. Thus, IL-15 in a soluble form (either alone or complexed with Rα) might induce migration. Clearly, further research is needed in this area, as modulation of different functions will require the manipulation of different signaling pathways.

It must also be considered that migration to IL-15 may not be direct chemotaxis. Indeed, our own studies presented here provide evidence of indirect migration to IL-15. Although both NK cells and CD8 T cells migrate to IL-15 in chemotaxis assays, our studies in which depletion of NK cells result in reduced influenza-specific CD8 T cells at the site of infection (Fig 4.6) support a model of indirect migration of CD8 T cells to IL-15 via the influx of NK cells migrating directly to the IL-15 induced by infection. The early peak expression of IL-15 (~d3 p.i.) correlates with the influx of NK cells into the site of infection (Fig 2.1 and Fig 3.2), and NK cells migrate very strongly to IL-15 in vitro even when enriched (unpublished observations). CD8 T cells, which enter the site of infection later (days 6-8 p.i.) may respond to IL-15 directly, may respond to signals such as chemokines produced by the NK cells, may have altered chemokine receptor expression upon IL-15 signaling, or (more likely) may respond to a combination of redundant signals in order to migrate into the lung airways (Fig 5.1). Because IL-15 is a potent proinflammatory cytokine often implicated in chronic inflammation and autoimmune disorders, identification of the source and mechanism of IL-15-induced trafficking will be essential in order to target this cytokine with any degree of control.

An important finding in this work is that IL-15 administration can be used to enhance immune responses to influenza. Introduction of IL-15 complexes to the lung airways via intranasal administration resulted in augmented frequencies and numbers of

NK cells and influenza-specific CD8 T cells found in the BAL. These experiments, while important in demonstrating the in vivo capacity of IL-15 to induce the migration of lymphocytes looked only at short-term effects—12 hours post treatment. We went on to show, however, that continued administration of the cytokine resulted in enhanced viral control, presumably due to continued migration of either NK cells or CD8 T cells. At first glance, a therapeutic target that requires continuous intervention seems counter-intuitive. The introduction of plasmids and recombinant viruses as vaccine platforms, however, has made this concept much more feasible. As noted in the introduction, a vaccinia-virus-based vaccine for influenza was engineered to express IL-15, and this vaccine platform induced cross-neutralizing antibody responses and robust cellular immune responses that conferred cross-clade protection to mice challenged with different H5N1 viruses [207]. Vaccines with integrated IL-15 have been created and tested against smallpox [208], hepatitis B [209], and HSV-1 [210], with promising results. Although it can be imagined that plasmid or viral DNA vectors may provide the means to deliver continuous IL-15 for immune intervention, our data suggest that with regards to influenza infection, it is optimal to have IL-15 at only the site of infection. Site-specific continuous delivery of an immunotherapy is a hurdle that, by in large, experimental medicine has not yet jumped.

Despite these difficulties, our results suggest that even transient administration of IL-15 intranasally can have longer lasting benefits. When animals received IL-15c every other day for 2 weeks, 25 days later, treated animals still harbored elevated frequencies and numbers of influenza-specific CD8 T cells in the lung airways. These animals cleared a lethal heterosubtypic challenge faster than vehicle control-treated animals. These data not only imply that IL-15 may be exploited therapeutically, but these data also highlight

the importance of the memory CD8 T cells generated at the site of infection in providing heterosubtypic immunity. It has long been recognized that protection against heterosubtypic challenges declines as the population of memory CD8 T cells in the BAL declines, but due to the technical difficulties in adoptively transferring this memory population from the BAL of an immunized mouse to the BAL of a naïve mouse, the model that this memory population is sufficient for protection has never been formally tested. Our observation that numerically augmenting the memory population in the BAL confers faster viral clearance to a heterosubtypic challenge provides strong evidence supporting the idea that these cells exclusively mediate the heterosubtypic immunity observed in these models. Unfortunately, it is unclear how long-lasting these effects of IL-15 treatment are. We surmise that this population will eventually decline, as is observed in untreated animals. Identifying the cause of this numerical decline, then, will be an essential step in creating a T cell-mediated heterosubtypic vaccine.

Because IL-15 is such a well-characterized and important homeostatic signal for antigen-specific CD8 T cells, we hypothesized that either limited IL-15 availability in the BAL or limited abilities of CD8 T cells in the BAL to receive IL-15 signals could account for their limited survival there. Indeed, influenza-specific CD8 T cells in the BAL lose expression of CD122, and our analysis of IL-15 expression in the lung airways indicated that levels are increased upon infection but decline by about day 10 p.i.. While these early data supported our original hypothesis, detailed analyses of the antigen-specific CD8 T cells maintained at memory in IL-15 deficient mice were indistinguishable in response kinetics and phenotype to those in wild type mice. That is, we observed no requirement for IL-15 in the maintenance of CD8 Tmem in the BAL or

in the periphery following influenza infection. As an aside, the reduced migration of effectors into the BAL of IL-15$^{-/-}$ mice had little impact on the eventual memory pool, implying that it is the IL-15-dependent CD8 T cell pool in the BAL of WT mice declines, leaving the IL-15-independent pool at the steady state. Interestingly, this IL-15 independent pool does not provide protection against heterosubtypic challenge. Follow up experiments in the VSV model further support the idea that mucosally-primed CD8 T cells require survival signals distinct from those required by systemically-primed CD8 T cells.

What results in different cytokine requirements following different routes of infection is still an open question. It is well-established that levels and types of soluble and cellular effectors of the innate immune system are very different in mucosal versus systemic sites. It could be that innate responses at mucosal sites such as mucins and collectins, IgM, complement, and phagocytic cells are much more effective at preventing and/or controlling viral infection. Lower infection efficiency at a mucosal site compared to a systemic infection would result in a lower antigenic load, a factor known to influence the magnitude and quality of antiviral CD8 T cell responses [263]. This is not the only explanatory model, however. The types of APCs found in mucosal versus systemic sites are known to be different, and the discrete patterns of costimulatory molecules and cytokine milieus might impart different transcriptional programs in the CD8 T cells primed in different anatomical locales. Future research into this area will be very important in considering optimal vaccination strategies, especially as the popularity of mucosal vaccines (such as flu mist) rises.

Overall, we have demonstrated that influenza infection induces the local production of IL-15, which is important for the migration of NK cells, and later, CD8 T cells into the site of infection where they mediate viral clearance. We have also demonstrated that IL-15 is not a requisite signal for the long-term survival of mucosally-generated CD8 Tmem. While further research is required to comprehensively address how lymphocytes migrate into the lung airways and how they are maintained there following influenza infection, we have made important inroads toward answering the two questions raised in the introduction to this dissertation, two questions which predicated much of our research. We have demonstrated that IL-15 is an important signal for lymphocyte migration, which in large part answers our first question. We have also eliminated IL-15, hitherto a prime candidate, from consideration as a mechanism for the long-term maintenance of mucosally-generated CD8 Tmem in the lung airways, which will serve as a useful groundwork for future attempts to answer the second question. We are confident that these findings represent significant steps forward in the ongoing project of improving strategies for influenza prevention and treatment.

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