THE ROLE OF CHEMOKINES AND CHEMOKINE RECEPTORS IN IL-15 MEDIATED LYMPHOCYTE MIGRATION FOLLOWING INFLUENZA INFECTION

by

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(Under the Direction of Kimberly Klonowski)

ABSTRACT

A CD8 T cell based vaccine has been proposed as an alternative to the current influenza vaccine. The success of such a vaccine requires a better understanding of the overall CD8 T cell response in the respiratory tract and how it can be optimally generated and sustained. Previous work from our lab described a role for IL-15 in the migration of influenza-specific effector CD8 T cells (Teffs) to the lung airways. However, the mechanisms by which IL-15 mediates this migration is unclear. The research presented here shows that infected epithelial cells are a significant source of IL-15 mRNA. We also show that IL-15 deficiency affects chemokine and chemokine receptor expression on migrating Teffs. Gaining a greater understanding of the underlying mechanisms of IL-15 induced Teff migration will be an important step in the development of an optimal T cell mediated influenza vaccine.

INDEX WORDS: Influenza, CD8 T cells, Migration, Chemokines, Chemokine Receptors, IL-15

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A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2013

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May 2013

DEDICATION

I would like to dedicate this work to my parents, for their unwavering support, love, and encouragement. You've raised me with the belief that I can accomplish anything with hard work and faith, and everything I do accomplish is a testament to that lesson.

ACKNOWLEDGEMENTS

First, I would like to sincerely thank Dr. Kim Klonowski for giving me the opportunity to work in her lab. The amazing guidance, mentoring, and encouragement I received along the way has left a lasting impression and won't soon be forgotten. I would also like to thank my committee members Dr. Rick Tarleton and Dr. S. Mark Tompkins. My experience at the University of Georgia was largely shaped by those accompanying me on this journey, and I am honored to have shared it you. To my lab mates Kat Verbist, Hillary Shane, Brett Marshall, Dave Rose, Josh Cole, Jeffrey Tran, and Lina Tibavinsky: thank you so much for all the fun times we shared, I couldn't imagine a better lab environment to have called home. I couldn't have done it without your help and insights. I think we finally got the model right too. To my friends, new and old: thank you for the good times and reminding me to smile and take time to appreciate the journey.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Influenza A viruses are negative sense RNA viruses belonging to the *Orthomyxoviridae* family, and are the causative agent of the flu. Influenza infection shares a number of characteristic symptoms with milder respiratory infections but has the capacity to cause severe disease pathogenesis in children, the elderly, and the immuno-compromised. Three to five million people are infected with influenza every year causing up to 500,000 deaths worldwide [1-3]. Influenza is a consistent public health concern due to the danger of pandemic outbreaks. One such pandemic, the Spanish Flu of 1918, is attributable to approximately 40 million deaths, decimating up to an estimated five percent of the world's population at the time [4]. There have been three more pandemics since: the 1957 Asian Flu, the 1967 Hong Kong Flu, and the 2009 Swine Flu. These pandemics were overall less fatal than the Spanish Flu of 1918 but are still attributable to a combined 2-3 million deaths [4]. Influenza pandemics occur as a result of genetic reassortment in the hemagglutinin (HA) and neuraminidase (NA) surface proteins as brought about by antigenic shift. Antigenic drift also causes changes in viral proteins as a result of selective pressure from the immune response. These unforeseen changes in the antigenic determinants increase susceptibility of at risk populations to more severe infections.

In addition to the associated mortality of influenza infection, there is also a significant economic burden [5]. Millions of people miss numerous days of work a year as a result of

acquiring seasonal influenza infections. Due to the danger of pandemics and the economic burden associated with flu, significant resources are allocated towards the formulation and reformulation of yearly vaccines for the prevention of influenza. Reformulation of the vaccine is required to account for changes in the antigenic determinants of the circulating seasonal strains of influenza. These factors dictate a need for the greater understanding of the underlying mechanisms behind influenza infection, and the host immune response to infection so that effective, less cumbersome, vaccines and other immunological therapies will be possible in the future.

Current influenza vaccines confer protection against the virus through the generation of an antibody response against viral HA, but fail as the virus undergoes any type of genetic reassortment. These vaccines are tri- or quad-valent, protecting against two influenza type A subtypes and one or two influenza type B subtypes. They are quite successful at establishing homosubtypic protection against defined virus subtypes in immunocompetent individuals. However, gradual changes in viral surface glycoproteins make it necessary to reformulate and revaccinate almost yearly [6]. A significant amount of research has shifted towards identifying regions of viral targets that remain invariant yet induce protective responses. It has been shown that memory CD8 T cells residing in the lung airways following an influenza infection are able to confer heterosubtypic immunity [7]. In animal models, these memory CD8 T cells have the ability to recognize conserved internal viral proteins regardless of HA and NA surface protein expression [7]. Therefore considerable interest remains in the prospects of a CD8 T cell mediated vaccine that would provide a longer-term solution than our current yearly vaccines. However, as the population of memory CD8 T cells declines in number so does the animal's ability to maintain heterosubtypic immunity. In order to

successfully arrive at that endpoint a better understanding of the mechanisms behind CD8 T cell migration to the sites of infection, as well as maintenance of the memory pool at these sites are necessary.

Infected individuals typically transmit influenza via respiratory drop inhalation from coughing or sneezing. These viral particles may be spread throughout a population even before symptoms are outwardly visible. Once an individual contracts an influenza infection, the virus will replicate primarily in the epithelial cells causing injury along the nasal, tracheal, and bronchial tracts [2, 8]. Most infections can be resolved naturally in immunocompetent hosts through the coordinated interactions between the innate and adaptive arms of the immune system, with virus finally being eliminated by CD8 T cells [9].

Influenza viruses are characterized by their expression of surface hemagglutinin (HA) and neuraminidase (NA) proteins. The different subtypes of influenza are defined by their varying expression of these surface proteins (e.g. H1N1, H5N1, H3N2). Viral HA recognizes and attaches to host sialic acids expressed on proteins on the surface of epithelial cells of the upper respiratory tract [10]. This interaction is one of the first steps towards infection and the release of viral particles into the host cell cytoplasm. NA on the other hand is responsible for the enzymatic removal of sialic acids from the host cell surface in order to allow viral particles to continue binding to other host cells. NA proteins are frequently the target of current antiviral drugs, with one of the most popularly used being the NA inhibitor Oseltamivir [10].

Critical to the recovery of a naïve host from a primary influenza infection is the CD8 T cell response. The successful clearance of virus has been shown to be delayed in animals deficient in either CD8 T cells or CD4 T cells individually [11, 12]. Additionally,

immunodeficient animals lacking both T cell types succumb to influenza infection [3, 6]. Normally, low numbers of naïve pathogen specific CD8 T cells circulate through the primary lymphoid tissues via the blood and lymph. Intermittent interactions with antigen presenting cells (APCs) is part of the normal surveillance process for antigens. When CD8 T cells encounter their cognate antigen presented in the context of Major Histocompatibility Complexes I (MHC I) on APCs, binding and activation occurs. This binding initiates a signaling cascade resulting in the clonal expansion and gain of effector function of identical effector CD8 T cells (Teffs) recognizing the antigen. Chemokine receptors as well as other adhesion molecules direct circulating Teffs to the sites of infection. Once the Teffs reach the inflamed tissues, increasing chemokine gradients result in the firm adhesion and arrest of these cells on the surface of endothelial cells, followed by transmigration through the endothelial layer to contact virally infected cells. When these Teffs encounter antigen presented on the surface of infected cells they are able to exert their effector functions such as the release of TNF-α and perforins, mediating the clearance of virus. Upon successful viral clearance the Teffs will undergo a contraction phase whereby ~95% of respondent cells will die by apoptosis. The remaining cells are now termed memory T cells, and have a much longer lifespan as well as the capability of rapidly responding to a secondary infection with the identical antigen [13].

The ability of Teffs to reach the site of infection from the circulation is critical in the control and clearance of virus. Migration of lymphocytes to sites of inflammation is a process that has historically been attributed to the expression of chemokines and their receptors. Our lab has recently described a similar role for the cytokine IL-15 in directing the migration of influenza-specific effector CD8 T cells to the sites of infection[14]. However, the underlying

mechanisms of this migration remain unclear. In particular, we are interested in whether IL-15 mediated chemotaxis occurs through direct attraction of CD8 T cells, the indirect modulation of chemokines and chemokine receptors, or the indirect alterations of chemotactic signals via a secondary cell type affected directly by IL-15. The work presented in this thesis will help better define these mechanisms, primarily through the determination of the source of IL-15, as well as if IL-15 indirectly modulates the expression of chemokines and chemokine receptors on respondent influenza-specific effector CD8 T cells. Ultimately, gaining a greater understanding of the underlying mechanisms of IL-15 induced CD8 T cell migration will be an important step in the development of an optimal T cell mediated influenza vaccine.

1.2 Immune Response to Influenza Infection

The Innate Immune Response

Innate immunity can be defined as the non-specific first line of defense that is initiated rapidly after infection and provides many of the signals necessary for a successful subsequent adaptive immune response to occur. The innate immune system functions to prevent the infection of respiratory epithelial cells as well as modulate the response of a number of cellular components aimed at controlling viral replication. This response consists of a coordinated effort between a number of unique cells and proteins found throughout the body. After influenza initially enters the respiratory tract, mucins on the surface of ciliated epithelial cells function to inhibit viral infection by providing decoy sialic acid receptors to which the virus binds [10]. Mucins are also believed to induce viral agglutination resulting in clearance via mucociliary mechanisms to the gastrointestinal tract. Mucin composition

includes the presence of collectins, that when bound to influenza virus are able to neutralize it, restricting replication and preventing excess inflammation [15]. In addition to mucins, defensins produced by neutrophils, and secreted IgM produced by B1 B cells function to neutralize the virus as well [10, 16].

If the influenza virus is able to evade this first barrier line of defense the virus targets cells of the epithelial layer of the upper respiratory tract [10, 17]. Upon successful binding of HA to sialic acid residues on the surface of epithelial cells, the virus invades the host cell by endocytosis and begins to replicate. Toll-like receptors (TLRs) and pattern recognition receptors (PRRs) detect viral RNA following the invasion of viral particles to the inner epithelial layer. Epithelial cells respond by initiating the synthesis and release of cytokines, chemokines, and other antiviral mediators [18]. Of the infiltrating cells, a large number of neutrophils are recruited to the lung and lung airways through the production of IL-8 by the epithelial cells during the first few days post infection. These neutrophils, along with macrophages, have been shown to phagocytize virus-infected apoptotic cells, but their role in viral clearance and pathogenesis is not yet fully defined [10, 19] As epithelial cells die from viral infection, they release factors (only partially characterized to date) that promote phagocytosis and the recruitment of macrophages, dendritic cells, and T cells [19, 20].

Viral replication begins to become controlled following the production of type I interferons (IFN-α and IFN-β) by the infected epithelial cells and monocytes/macrophages. Type I IFNs also augment the antiviral response and work in conjunction with Natural Killer cells to control the spread of virus. For anti-influenza responses IFN signaling occurs; primarily either through the engagement of TLR3 to dsRNA or TLR7 to ssRNA in endosomal compartments, or via cytoplasmic RNA recognition by RIG-I helicase [21]. Once

IFN genes are transcribed, a cellular and humoral response follows [22]. The Type I IFN-induced humoral response includes the production of all classes of IgG, long-term antibody production, promotion of isotype switching, as well as displaying natural adjuvant activity [23]. The cellular responses modulated by IFN gene transcription include the induction of NK cell cytotoxicity and the promotion of CD8 over CD4 T cell responses, as well as enhancing CD8 T cell IFN-γ expression [24]

Natural Killer (NK) cells are one of the critical cells mediating the innate immune response. NK cells are found primarily in the spleen and liver but circulate throughout both lymphoid and non-lymphoid tissues [25]. They are capable of directly lysing virus-infected cells, producing a plethora of cytokines including IFN-γ and TNF-α, as well as initiating the recruitment and activation of other effector cell types [25-27]. NK cells have the ability to produce the chemokines CCL1, CCL3, CCL4, CCL5, CCL22, CXCL8, and XCL1 following *in vitro* activation [28]. The production of these chemokines attracts B cells, T cells, and additional NK cells among other cell types to the immediate vicinity of activated NK cells. Functional NK cell responses are also critical in the clearance of influenza as increased mortality was observed in mice whose NK cells had been depleted during influenza infection [29, 30] NK cells also aid the priming of CD8 T cells through the secretion of cytokines and crosstalk with dendritic cells. This crosstalk results in the activation of additional NK and dendritic cells, as well playing a role in prolonging their survival. Taken together, these actions figuratively bridge the gap between the innate and adaptive arms of the immune system, although there is no clear delineation where one begins and the other ends [31].

The Adaptive Immune Response

Dendritic cells (DCs) and other antigen presenting cells present in the lung interstitium and intraepithelial layers constantly monitor for antigens in the surrounding environment [32]. Following the recognition of antigens, these DCs then exit the respiratory epithelium and travel to either the surrounding lymph nodes (mediastinal and cervical) via the afferent lymphatics or to the spleen via the blood [3]. In the lymph nodes, these DCs that have phagocytosed viral antigens process and present amino acid peptide sequences on their surface in the context of Major Histocompatibility Complexes I or II that are recognized by CD8 and CD4 T cells respectively [32].

The T cell mediated adaptive response begins to form 3-4 days post infection but doesn't exert its effects until around days 5-7 post infection following migration into the lungs, with full viral clearance occurring by day 10 post infection in mice [33-35]. Binding of the T cell receptor to its cognate antigen initiates a sequence of signaling events resulting in activation, proliferation of effector functions, and changes in trafficking molecules. The migration of influenza-specific Teffs is mediated by a number of adhesion molecules including selectins, integrins, and chemokines, all of which will be expounded upon in forthcoming sections. Increased Teff cell numbers are maintained in the lung and lung airways even after viral clearance occurs and can be attributable to the influx of cells to these sites prior to the contraction phase.

The mechanisms behind CD8 T cell migration to the respiratory tract have yet to be completely elucidated but it is believed that chemokines, a subfamily of chemoattractive cytokines, play a prominent role and will be further discussed in the upcoming sections. Teffs are capable of clearing influenza infected cells through the release of perforin and

granzymes, perforins permeabilize the cell membrane allowing granzymes to enter the cell and induce apoptosis [20]. Following the clearance of virus-infected cells and the subsequent removal of pro-inflammatory signals, the recently proliferated population of Teffs undergoes contraction by apoptosis [9, 14].

CD8 T cells that have survived the contraction phase can be divided into two subsets, memory precursor effector cells (MPECs) and short-lived effector cells (SLECs) [36]. These subsets differ in their expression of a number of surface markers including the IL-7Rα (IL-7 receptor alpha chain), CD62L (L-selectin), CCR7, and KLRG-1 (killer cell lectin-like receptor G1) [36-39]. MPECs are defined as KLRG-1^{lo} IL-$7R\alpha^{hi}$, whereas SLECs express these markers in the opposite manner, KLRG-1^{hi} IL-$7R\alpha^{lo}$. The expression of IL-7Rα as one of the main parameters for defining each subset can be attributed to the importance of IL-7 in the survival and renewal of memory CD8 T cells [40]. The inability of IL-$7R\alpha^{lo}$ cells to receive IL-7 signaling limits their life span to a much shorter duration than cells that can use IL-7 to extend survival time. As a result, MPECs have the capacity to ultimately become long-lived memory CD8 T cells, whereas SLECs typically do not.

The proportion of MPECs to SLECs may be skewed depending on the type of infection. Acute infections typically have a greater frequency of MPECs to SLECs, but chronic or persistent viral infections skew the frequencies in favor of SLECs [36]. These cell types also differ in their maintenance and survival requirements while still maintaining similar effector functions [38]. Although MPECs express high levels of IL-7Rα, the cytokine IL-15 has been shown to have a compensatory role in the survival of these MPECs in the absence of IL-7 [41]. SLECs rely primarily on IL-15 for survival following differentiation due to an inability to respond to IL-7. Experiments evaluating SLEC and MPEC survival in

IL-15 $^{-/-}$ and IL-15R$^{-/-}$ mice clearly demonstrate that in the absence of the cytokine or its signaling, the vast majority of the SLEC population (>90%) diminish rapidly [36, 42]. These examples outline a critical role for IL-15 in the generation and maintenance of the SLEC subset of memory CD8 T cells. This role of IL-15 and its other major functions will be discussed further in subsequent sections.

1.3 Interleukin-15 (IL-15)

Structure Of The Cytokine And Its Receptors

IL-15 is a 14-15 kDa glycoprotein that belongs to the common gamma chain (γc) family of cytokines. Along with IL-15 members of this family include IL-2, IL-4, IL-7, IL-9, and IL-21. These cytokines all share some overlapping signaling and function with one another through their shared usage of the γc [43]. IL-15 was first identified and described by both the Waldmann and Grabstein groups individually in 1994 [44, 45]. These groups reported similarly that IL-15 has the ability to stimulate proliferation of an IL-2 dependent lymphocyte cell line, and that there is shared interaction between both cytokines and the IL-2Rβ [44, 45]. IL-15 has historically been implicated in NK cell development, lymphocyte homeostasis, proliferation of activated T cells and the maintenance of memory CD8 T cells[46].

IL-15 mRNA is constitutively expressed in a number of tissues including skeletal muscles, lungs, and the placenta [47]. It is also expressed on a number of unique cell types including monocytes/ macrophages, dendritic cells, and epithelial cells [48]. However, IL-15 production and expression is tightly regulated at the transcriptional and translational steps, as well as in the trafficking and secretion of the assembled protein [47]. Following the initial

discovery of IL-15, and the observations that IL-15 and IL-2 share many structural characteristics, it was believed that the two cytokines act in a redundant fashion. At first glance this conclusion is validated in that both cytokines have the ability to induce the proliferation or stimulation of various T cell subsets *in vitro* [49]. As research on the cytokines progressed it became much more evident that each cytokine plays a distinct role in the immune system and the response to antigen *in vivo*.

Structurally IL-15 is most similar to IL-2, sharing not only the common γc but also the IL-2Rβ (CD122) which are both expressed on the surface of T cells [49, 50]. Each respective cytokine has a unique receptor α chain, CD123 for IL-15 and CD25 for IL-2, which confers selectivity onto each cytokine's heterotrimeric receptor bundle [51-53]. IL-15 differs somewhat from IL-2 in that the interaction between IL-15 and its receptor subunits can occur through one of two mechanisms. High affinity IL-2 binding occurs when the cytokine interacts with a complex of IL-2Rα, IL-2Rβ and the γc on the same T cell [53]. IL-15 also has the ability to be presented in trans, that is IL-15 bound to IL-15Rα on the surface of DCs or monocytes and recognized by IL-2Rβ/γc on an adjacent CD8 T cell or NK cell [54, 55]. Recently, IL-2 has also been shown to be capable of trans-presentation by CD25 (IL-2Rα), however this binding has a much lower affinity than the traditionally described binding motif and fails to transduce a signal [56]. Differences in cytokine expression, mechanisms of receptor binding and distribution of each cytokine's private receptor α chain account for the non-redundant roles of IL-2 and IL-15 *in vivo*.

Since its discovery in 1994, the role of IL-15 in the immune response as well as normal homeostatic functions has been expanding. As previously described, IL-15's capacity to induce proliferation in an IL-2 dependent cell line led much of the early research

to be focused on IL-15's ability to act as an IL-2 mimic [44]. Unique roles have become much more evident as research into the topic expands. Currently, IL-15 has been implicated as being a critical component in the inhibition of IL-2 induced activation induced cell death (AICD), the development and maintenance of NK, NK-T, and memory CD8 T cells, as well as playing a key role in a number of autoimmune disorders [46, 57-60]. In addition to these known functions, our lab has also recently discovered a role for IL-15 in the migration of influenza-specific effector CD8 T cells to the lung and lung airways [14].

IL-15 in Lymphocyte Biology

NK cells and CD8 T cells share somewhat homologous functions although both lie on different sides of the innate/adaptive boundary. Each cell type arises from a common lymphoid progenitor and is capable of mediating anti-viral responses through the release of cytokines such as IFNγ. They kill virally infected cells via the release of perforin and granzymes following their migration to the sites of infection. NK cells and CD8 T cells are also both intimately reliant on γc cytokine signaling, especially IL-15, for their development, proliferation, and survival [47, 61].

Both IL-15 and the IL-15Rα are critical in the development and proliferation of NK cells. Experiments using mice deficient in IL-15 show an absence of NK cells in the spleen and liver, but the administration of exogenous IL-15 for 1 week reverses this defect [60]. Similarly, experiments using IL-15Rα$^{-/-}$ mice show an absence in splenic NK cells along with other multiple defects in innate immune effector cells including diminished frequencies of intestinal intraepithelial lymphocytes [61]. These findings assert that IL-15 and its Rα are indispensible to NK cell development, and this role is nonredundant, with no other cytokine

being able to compensate for the absence of IL-15. IL-15 also plays a critical role in the homeostasis and survival of NK cells by inducing the gene transcription of the anti-apoptotic molecule Bcl-2 [62]. The early NK cell response to antigenic offense is critical in shaping not only the innate response but the adaptive response as well. NK cells produce a number of cytokines, including IFNγ, along with chemokines that help to orchestrate effector migration and recruitment.

Similar to NK cells, CD8 T cells are also heavily reliant on IL-15 throughout their lifespan. IL-15 affects CD8 T cell development, activation, and proliferation, as well as migration and survival [50]. However, IL-15 signaling has been most studied in the maintenance and survival of memory CD8 T cells, more so than other T cell subsets. Memory CD8 T cell numbers are significantly diminished in IL-15 $^{-/-}$ and IL-15Rα $^{-/-}$ mice, with frequencies only reaching roughly half of those of wildtype mice [61]. Binding of IL-15Rα to IL-15 and the remaining receptor components initiates a signaling cascade through the JAK-STAT pathway. Upon activation and phosphorylation of Janus kinases 1 and 3 (Jak1 and Jak3), the signal transducer and activator of transcription proteins 3 and 5 (STAT3 and STAT5) become tyrosine-phosphorylated [49]. This tyrosine-phosphorylation causes STAT3 and STAT5 to form either homo- or heterodimers and translocate directly to the nucleus where they bind to their DNA regulatory elements and participate in the activation of gene expression [63]. This transcriptional activation is important in immune inflammatory responses as well as T-cell differentiation and expansion. More recently, IL-15Rα-mediated signal transduction has also been shown to induce Bcl-2 and Bcl-x_L upregulation, both of which are anti-apoptotic molecules and play a key role in the survival of NK and memory CD8 T cells [47, 64]

The IL-15 mediated induction of transcription ultimately results in the production of a variety of pro-inflammatory factors including IL-8, TNF, and IL-1β [43, 49, 65]. Although IL-15 is considered a pleiotropic cytokine, it can also act in a pro-inflammatory manner and relies on transcriptional regulation to prevent overly harmful inflammatory and autoimmune disorders. Dysregulation of IL-15 has been implicated in the disease etiology of a number of inflammatory bowel diseases, rheumatoid arthritis, and psoriasis among other autoimmune disorders [48, 66, 67]. All of these disorders display an overexpression of either IL-15 mRNA or IL-15 protein, which in turn augments NK and CD8 T cell infiltration in each respective disease locale. The pathogenic effects of IL-15 in these disorders can be attributed to the enhanced stimulation of proliferation and activation of T cells, production of pro-inflammatory cytokines, and the recruitment of other inflammatory cells [48].

1.4 Leukocyte Migration: Role of Chemokines and IL-15

The migration of effector cells to the site of infection is one of the critical steps in viral clearance. The ability of circulating NK and CD8 T cells to mediate an anti-viral response is nullified if the cells fail to migrate to the inflamed tissues and contact infected cells. The process by which leukocytes migrate to the sites of infection has been historically and primarily attributed to chemokines. Chemokines are a diverse subfamily of chemoattractive cytokines whose receptors are selectively expressed on the surface of lymphocytes during development, steady-state trafficking, and in response to inflammation. These receptors bind to their respective chemokine ligands on endothelial cells and through this interaction of surface proteins and those of other surface proteins a complex process of binding, arrest and adhesion occurs. This sequence of events ultimately leads to the

transendothelial migration or extravasation of the leukocytes. IL-15, a common γc cytokine previously discussed, has also recently been implicated in the migration of effector CD8 T cells [14]. The effects of these molecules in respect to cellular migration will be explored in greater detail in the following sections.

Leukocyte Migration

Although the individual molecules regulating the process of leukocyte migration and extravasation may differ, the steps and key players are similar regardless of the type of leukocyte involved. Initially, circulating leukocytes must adhere to the endothelial layer of blood vessels near the target tissue. This reversible adhesion is mediated by the selectin family of proteins, which is comprised of L-selectin, P-selectin, and E-selectin. L-selectin (CD62L) is expressed on the surface of naïve T cells along with CCR7, but is internalized once these T cells have become activated. E- and P-selectin are both expressed on the surface of activated endothelial cells, with the expression of each respective selectin dependent on the specific inflammatory stimulus [68]. The binding of selectins to their conjugate ligands alone is not enough to mediate the arrest of migrating leukocytes, but it does slow them down. This initial rolling step is critical in the upcoming steps of leukocyte extravasation and transmigration to the infected tissues.

As the activated leukocytes migrate through the blood vessel and roll over endothelial cells, selectins bind to their ligands and the overall velocity of leukocytes decreases [69]. These slower moving leukocytes are now more susceptible to chemokine signaling via increased interaction time. Chemokines play a critical role in the interaction between adhesive molecules on the surface of endothelial cells and chemokine receptors on the surface of

leukocytes. Prior to leukocytes approaching the site of infection, chemokines are expressed in an increasing gradient, peaking at the site of inflammation. This gradient increases the probability that the chemokine will successfully bind its receptor on the leukocyte surface and arrest will occur near the infected tissue site. After successful chemokine/chemokine receptor binding occurs, integrins on the surface of the leukocyte undergo conformational changes that significantly increase their affinity to relevant Immunoglobulin (Ig) superfamily ligands on the surface of endothelial cells [70]. Activated leukocytes upregulate the expression of the β1 and β2 integrins LFA-1 and VLA-4 as they conversely downregulate L-selectin and CCR7 [71]. The binding of activated integrin to their ligands results in the firm adhesion of the leukocyte to the endothelial cell. These actions lead to the arrest of the leukocyte on the surface of the endothelial cell which is now capable of transmigrating to the site of infection in order to exert its effector functions. Transmigration or diapedisis primarily occurs through one of two pathways, either paracellularly or transcellularly, with each causing some degree of cytoskeletal rearrangement [70]. Paracellular transmigration occurs when leukocytes migrate through the tightly regulated junctions between adjacent endothelial cells. Transcellular migration occurs when leukocytes migrate directly through an individual endothelial cell. Leukocytes that migrate transcellularly must also traverse a second layer in the basement membrane prior to accessing infected tissue sites. T cells have been shown to be able to use either method of transmigration, although the exact physiological factors controlling the types of migration are an area of ongoing research [72, 73].

Homeostatic and Inflammatory Chemokines (Figure 1)

The first chemokines were identified in the late 1980s and have since rapidly expanded to over fifty family members, with more than twenty receptors being identified to date [74, 75]. Chemokines are small proteins (8-14 kD) that are grouped into subfamilies based on the arrangement of two N-terminal cysteine residues [75]. These subfamilies are CXC, CC, (X)C, and CX3C, with C representing a cysteine residue, and the X representing any other amino acid [76]. Chemokine receptors are class A G-protein coupled receptors coupled with the Gαi class of heterotrimeric G-proteins [77]. Chemokines have the ability to function in both an autocrine (on the producing cell) or paracrine (on adjacent cells) manner. Chemokines are commonly classified based on their functionality as either inflammatory or homeostatic, with neither being mutually exclusive. The type of infection as well as the inflammatory stimuli received are the primary determinants of whether a chemokine will act in a homeostatic or inflammatory capacity. Inflammatory chemokines are those that upon receiving any type of inflammatory stimulus are upregulated, and are mainly involved in the recruitment of leukocytes to inflamed tissues. Alternatively, homeostatic chemokines are constitutively expressed and function primarily to mediate the normal positioning or migration and homing of various cell types [75, 77, 78].

For the purposes of this thesis, I will focus primarily on inflammatory chemokines and their receptors, however it would be remiss to overlook the importance of homeostatic chemokines, especially in the homing of lymphocytes to specific tissues. A primary example of this importance is seen in CCR7. The expression of CCL19 and CCL21, both, which bind the chemokine receptor CCR7, plays an essential role in the homing of lymphocytes and dendritic cells to secondary lymphoid organs [76, 79]. Naïve T lymphocytes as well as

specific subsets of memory T cells that express CCR7 are reliant on the interaction of the receptor and its ligands in order to successfully enter lymph nodes so that they may encounter antigen-presenting cells. Mice genetically deficient in CCR7 show significantly reduced numbers of naïve T cells in the lymph nodes [79-81]. Similarly CCL17 and CCL22, the ligands to CCR4, play a role in the normal T cell homing to the skin. These ligands are expressed on different cell types, with CCL17 being expressed on the surface of endothelial cells, and CCL22 expressed by dendritic cells. However, they function coordinately to successfully guide T cells expressing CCR4 to the skin tissues. It has been shown that mice genetically deficient in CCR4 still maintain the ability to migrate to the skin tissues, suggesting that there may be other chemokines or receptors acting in a redundant capacity [82, 83]. Chemokine promiscuity is a common theme between both the homeostatic and inflammatory chemokines, and may help to insure that signaling and trafficking capability is maintained even in the absence of the primary targets.

Inflammatory chemokine expression by leukocytes and tissue cells is upregulated following activation signals received during pathological conditions which include Type I IFNs, IL-1β, IL-6, TNF-α and TLRs [84]. The genes for inflammatory chemokines are almost all clustered on chromosomes 4 and 17, with the genes for homeostatic chemokines distributed throughout the genome [75]. This localization along with a greater number of chemokines being classified as inflammatory than homeostatic is believed to have arisen as a result of evolutionary selective pressures requiring species to respond to a variety of unique pathogens [85]. Inflammatory chemokines maintain the ability to bind a large variety of cell types, imparting their effects on cells of both the innate or adaptive immune system.

Following a viral infection, two waves of chemokines are produced. The first wave shapes the early innate response, although the precise signals mediating this response are still unclear. However, it is believed that signals generated as part of the innate immune response, such as the production of Type I interferons, can regulate the expression of some chemokines. As the cell-mediated response begins to shape, a second wave of chemokines is produced by myeloid and plasmacytoid dendritic cells, serving to attract more effector cells to the site of infection [86]. The chemokines recruited by each wave are unique and can be dependent on the type of viral infection. During an influenza infection the expression of CCL2, CCL5, and CXCL10 is upregulated in the lungs up to day 3 post-infection, thus helping to shape the innate response by the recruitment of neutrophils to the sites of infection [87]. As the adaptive response begins to shape and exert its effects CCL3, CCL4, CCL5, and CXCL10 are upregulated in the lungs through day 10 post-infection, mediating the recruitment of effector T cell subsets [88]. There remains overlap between the expression of specific chemokines during both phases of the immune response, but it was observed that levels of expression varied throughout the course of infection and based on the specific strains of virus [2]. Chemokine receptors echo similar trends to those of chemokines in differential expression as T cells undergo the various stages of differentiation. Importantly, even different effector subsets express unique sets of chemokine receptors. For example; Th1 T cells predominantly express CCR5 and CXCR3 and Th2 T cells express CCR3 and CCR4 [89].

Role of IL-15 in Lymphocyte Trafficking

In addition to its important previously described roles, IL-15 has also been shown to be a potent T cell chemoattractant [90]. For example, in the autoimmune disorder Rheumatoid Arthritis, synovial fluid containing high levels of IL-15 attracts activated T cells, macrophages, and synoviocytes, all of which contribute to the disease pathogenesis [91]. Our lab has previously demonstrated that IL-15 modulates the migration of influenza-specific effector CD8 T cells to the lung airways following infection and that provision of IL-15 complexed to the soluble IL-15Rα restores the deficient accumulation of these CD8 T cells in IL-$15^{-/-}$ mice [14]. More recently, our lab has also described a role for virally induced IL-15 expression in the trafficking of NK cells into the lung airways following influenza infection [59]. From these findings, the importance of IL-15 induced migration of NK and T cells becomes evident. This method of lymphocyte migration is likely very important in the arrival of other immune effectors to the sites of infection as well.

IL-15 has been shown to induce the production mRNA in chemokines and chemokine receptors in resting T cells. Low levels of CXCL10, CCL2, CCL3, CCL4, and CCL5 mRNA is detectable in resting T cells, but upon stimulation with recombinant human IL-15 these expression levels were induced over sixfold greater as measured by a ribonuclease protection assay [92]. The CC chemokine receptors CCR1, CCR2, CCR4, and CCR5 mRNA were expressed at low levels on resting T cells, were was similarly upregulated by greater than fivefold in response to stimulation with IL-15 [92]. Curiously, IL-15 stimulation failed to modulate any mRNA induction in the CXC family of chemokine receptors on resting T cells. In a similar set of experiments it was reported that stimulation of neutrophils with IL-15 induces the production of CXCL8 (IL-8), a potent chemokine responsible for neutrophil

recruitment [93]. Currently, the role of IL-15 mediated chemokine/receptor production in infection models is not well studied, but will be addressed at length in this thesis in respect to influenza infection.

The mechanisms behind IL-15 mediated chemoattraction and migration of effector CD8 T cell remains to be fully understood. IL-15 may potentially be modulating these cells through a number of methods: (1) direct chemotaxis where the Teffs migrate directly to IL-15, (2) indirectly by modulating the expression of chemokines and chemokine receptors, or (3) via an additional indirect method whereby IL-15 imparts its effects on a secondary cell (such as an NK cell) which subsequently regulates the migration of effector CD8 T cells.. The work presented in this thesis aims to address the contribution of the first two possibilities and will ultimately improve our overall understanding of the mechanisms by which IL-15 mediates its chemoattractive functionality.

Chemokine	Other Names (Human)	Category	Gene Symbol		Other Names (Mouse)	Receptor	
			Human	Mouse[a]		Agonistic	Antagonistic
CXC Subfamily							
CXCL1	GROα, MGSA	I, ELR	*CXCL1*	*Cxcl3*[b]	Gm1960	CXCR2	
CXCL2	GROβ	I, ELR	*CXCL2*	*Cxcl2*	MIP-2	CXCR2	
CXCL3	GROγ	I, ELR	*CXCL3*	*Cxcl1*[b]	KC	CXCR2	
CXCL4	PF4	Pt, non-ELR	*PF4*	–		CXCR3-B	
CXCL4L1	PF4V1	Pt, non-ELR	*PF4V1*	*Pf4*[b]		CXCR3-B	
CXCL5	ENA78	I, ELR	*CXCL5*	–		CXCR2	
CXCL6	GCP2	I, ELR	*CXCL6*	*Cxcl5*[b]	LIX	CXCR1, CXCR2	
CXCL7	NAP-2	Pt, I, ELR	*PPBP*	*Ppbp*		CXCR1, CXCR2	
CXCL8	IL-8	I, ELR	*IL-8*	–		CXCR1, CXCR2	
CXCL9	MIG	I, non-ELR	*CXCL9*	*Cxcl9*		CXCR3	CCR3
CXCL10	IP-10	I, non-ELR	*CXCL10*	*Cxcl10*		CXCR3	CCR3
CXCL11	I-TAC	I, non-ELR	*CXCL11*	*Cxcl11*		CXCR3, CXCR7	CCR3, CCR5
CXCL12	SDF-1	H, non-ELR	*CXCL12*	*Cxcl12*		CXCR4, CXCR7	
CXCL13	BLC, BCA-1	H, non-ELR	*CXCL13*	*Cxcl13*		CXCR5, CXCR3	
CXCL14	BRAK	H, non-ELR	*CXCL14*	*Cxcl14*		unknown	
–	–	U, non-ELR	–	*Cxcl15*	Lungkine, Weche	unknown	
CXCL16	SR-PSOX	I	*CXCL16*	*Cxcl16*		CXCR6	
CXCL17	DMC	U	*CXCL17*	*Cxcl17*		unknown	
CC Subfamily							
CCL1	I-309	I	*CCL1*	*Ccl1*	TCA-3	CCR8	
CCL2	MCP-1	I	*CCL2*	*Ccl2*	JE	CCR2	
CCL3	MIP-1α, LD78α	I	*CCL3*	*Ccl3*		CCR1, CCR5	
CCL3L1	LD78β	I	*CCL3L1*	–		CCR1, CCR3, CCR5	
CCL3L3	LD78β	I	*CCL3L3*	–			
CCL4	MIP-1β	I	*CCL4*	*Ccl4*		CCR5	
CCL4L1	AT744.2	I	*CCL4L1*	–			
CCL4L2	–	I	*CCL4L2*	–			
CCL5	RANTES	I, Pt	*CCL5*		Ccl5	CCR1, CCR3, CCR5	
CCL7	MCP-3	I	*CCL7*	*Ccl7*	MARC	CCR1, CCR2, CCR3	CCR5
CCL8	MCP-2	I	*CCL8*	–		CCR1, CCR2, CCR5	
–	–	I		*Ccl8*[b]		CCR8 (mouse)	
CCL11	Eotaxin	D	*CCL11*	*Ccl11*		CCR3, CCR5	CXCR3, CCR2
–	–	I	–	*Ccl12*	MCP-5		
CCL13	MCP-4	I	*CCL13*	–		CCR2, CCR3	
CCL14	HCC-1	P	*CCL14*	–		CCR1, CCR3, CCR5	
CCL15	HCC-2, Leukotactin-1	P	*CCL15*	*Ccl9*[b]	CCF18, MIP-1γ	CCR1, CCR3	
CCL16	LEC, HCC-4	U	*CCL16*	–		CCR1, CCR2, CCR5, CCR8, H4	
CCL17	TARC	D	*CCL17*	*Ccl17*	ABCD-2	CCR4	
CCL18	PARC, DC-CK1	H	*CCL18*	–		PITPNM3	CCR3
CCL19	MIP-3β, ELC	H	*CCL19*	*Ccl19*		CCR7	
CCL20	MIP-3α, LARC	D	*CCL20*	*Ccl20*		CCR6	
CCL21	SLC, 6Ckine	H	*CCL21*	*Ccl21a*		CCR7	CXCR3 (mouse)
–	–	H	–	*Ccl21b*		CCR7	
–	–	H	–	*Ccl21c*		CCR7	
CCL22	MDC	D	*CCL22*	*Ccl22*	ABCD-1	CCR4	
CCL23	MPIF-1	P	*CCL23*	*Ccl6*[b]	C10	CCR1, FPRL-1	
CCL24	Eotaxin-2, MPIF-2	H	*CCL24*	*Ccl24*		CCR3	
CCL25	TECK	H	*CCL25*	*Ccl25*		CCR9	
CCL26	Eotaxin-3	I	*CCL26*	(*Ccl26*)		CCR3, CX3CR1	CCR1, CCR2, CCR5
CCL27	CTACK, ILC	H	*CCL27*	*Ccl27a*		CCR10	
-	-	H	-	*Ccl27b*			
CCL28	MEC	H	*CCL28*	*Ccl28*		CCR10, CCR3	
XC Subfamily							
XCL1	Lymphotactin, ATAC, SCM-1α	D	*XCL1*	*Xcl1*	lymphotactin	XCR1	
XCL2	SCM-1β	D	*XCL2*	–		XCR1	
CX3C Subfamily							
CX3CL1	Fractalkine	D	*CX3CL1*	*Cx3cl1*	fractalkine, neurotactin	CX3CR1	

Figure 1.1 Human and Mouse Chemokines and Chemokine Receptors (Adapted from Zlotnik and Yoshie, 2012 [75])

Abbreviations: I, inflammatory chemokines; H, homeostatic chemokines; D, dual chemokines; P, plasma chemokines; Pt, platelet chemokines; U, unknown. Chemokine and chemokine receptors evaluated in this thesis are highlighted in gray.

CHAPTER 2

THE ROLE OF CHEMOKINES AND CHEMOKINE RECEPTORS IN IL-15 MEDIATED MIGRATION OF ANTI-INFLUENZA EFFECTOR T CELLS TO THE LUNG AIRWAYS

2.1 Introduction

Influenza A viruses are negative sense RNA viruses belonging to the *Orthomyxoviridae* family. The influenza virus has the capacity to cause severe disease pathogenesis in children, the elderly, and the immunocompromised. Three to five million people are infected with influenza every year causing up to 500,000 deaths [1-3]. Pandemic outbreaks of influenza have occurred in the past causing significant casualties, including 40 million dead after the Spanish Flu of 1918 [3]. Influenza primarily infects the upper respiratory tract, causing fever, reduced pulmonary capacity, and inflammation of the bronchoalveolar tract. Influenza viruses are characterized by their expression of hemagglutinin and neuraminidase surface proteins, and these proteins are targeted by current vaccine strategies by eliciting an antibody response. A more ideal vaccine would provide cross-subtype or heterosubtypic immunity. Unfortunately, these surface proteins are subject to antigenic shift and drift, requiring vaccines to be reformulated yearly [6]. This causes a significant economic burden due to the costs associated with doing so, along with costs for treatment. CD8 T cells recognize peptide sequences to internal influenza proteins, including the nucleoprotein (NP), that are typically conserved across subtypes [6]. A CD8 T cell-mediated vaccine against a conserved internal protein would be a better long-term solution in

that yearly vaccinations would not be required [94]. However, a number of complicating factors exist that hinder the formulation of such a vaccine.

Naïve CD8 T cells recognize viral antigens in the context of MHC Class I on antigen presenting cells such as dendritic cells [9, 65]. Upon antigen recognition, a signaling cascade proceeds resulting in the synthesis of cytokines, effector molecules, and the proliferation of effector cells. This proliferation peaks by day 10 post infection and is followed by a contraction phase to return the immune system to homeostasis [3, 9]. The remaining population of antigen-specific cells are termed memory cells. These memory CD8 T cells have the capacity for massive clonal proliferation after secondary challenge, clearing the virus with faster kinetics than the primary infection. These differentiated memory CD8 T cells found in the lung airways are critical for providing heterosubtypic immunity, however, this population isn't maintained long-term like other memory populations outside of the lung airways. Therefore, understanding how to augment the survival and migration of this lung airway-specific memory pool and prolong the duration of heterosubtypic immunity could be an important aspect of developing a CD8 T cell mediated vaccine.

IL-15 is a 14-15 kDa cytokine belonging to the common gamma chain family. Along with IL-15, this group also contains IL-2, IL-4, IL-7, IL-9, and IL-21. The receptors for all of these cytokines share the gamma chain (CD132) along with their own unique receptor components [43, 49]. The IL-15 receptor is most similar to IL-2 in structure sharing the IL-2Rβ (CD122), but with a unique IL-15Rα (CD123) [58]. Binding of IL-15 to its receptor subunits often occurs through a process called trans-presentation. During trans-presentation IL-15 is bound to the IL-15Rα on the presenting cell and binds to CD122 and the γc (CD132) subunits on CD8 and NK cells. Signaling associated with CD122 and CD132 occurs through

Janus Kinases 1 and 3 (JAK1 and JAK3) and the signal transducer and activator of transcription proteins 3 and 5 (STAT3 and STAT5) [49, 58]. Upon IL-15 binding to its receptor subunits, JAKs become activated through autophosphorylation. This allows the STAT protein to bind to the phosphorylated JAKs, which also becomes phosphorylated, and translocates to the cell nucleus. Upon translocation, STAT then binds DNA and promotes the transcription of a number of genes, including the anti-apoptotic protein BCL-2 [43, 49]. IL-15 is constitutively expressed and produced by monocytes, dendritic cells, and epithelial cells [47]. Production of IL-15 increases upon exposure to inflammatory stimuli including Type I IFNs [47]. While IL-2 has been shown to be critical for the proliferation and survival of effector T cells, IL-15 has been primarily implicated in the maintenance of memory CD8 T cells as well as the development and maintenance of NK cells [14, 46, 49]. In addition to the described canonical roles of IL-15, it has also more recently been implicated in the migration of influenza-specific effector CD8 T cells [14, 95]. The mechanism by which IL-15 mediates lymphocyte migration remains unclear. This novel function is more traditionally attributed to the increased expression of chemokines, another subfamily of cytokines. IL-15 has also been shown to modulate chemokine receptor expression on human T lymphocytes as well [96].

Chemokines are strong chemoattractants that recruit various subsets of leukocytes to sites of inflammation and tissue damage in order to mediate specific effector functions [74, 97-99]. Chemokines are classified into homeostatic or inflammatory subgroups based on distinct physiological features [97, 100, 101]. Homeostatic chemokines are constitutively expressed on a variety cell types, and expression is often restricted to certain tissues. Homeostatic chemokines are involved in controlling the migration of cells during normal

processes of tissue maintenance or development and play important roles in regulating lymphocyte trafficking and functional compartmentalization of lymphoid organs [101]. Inflammatory chemokines show high levels of induced expression in response to inflammatory stimuli such as IL-1, TNF-α, and LPS [102, 103]. Upon stimulation these chemokines attract inflammatory cells [104]. Cognate chemokine receptors on the surface of patrolling or activated lymphocytes bind to chemokine ligands attached to the surface of endothelial cells within blood vessels. Chemokine/chemokine receptor binding also results in the activation of integrins on the surface of lymphocytes. Integrin activation is the result of a conformational change which allows for high affinity binding to their ligands expressed on the endothelial surface. This high affinity binding mediates firm adhesion of the lymphocyte to the endothelial cell, followed by transmigration across the endothelials to sites inflammation/infection. As lymphocytes roll through blood vessels they encounter an increasing gradient of chemokines [88, 99, 104-106]. This gradient increases the frequency by which chemokine receptors encounter their cognate ligands, and is an important component of lymphocyte recruitment.

The T cell mediated clearance of influenza virus relies on Teffs to recognize and bind epitopes on infected cells as well as kill virally infected cells. These lung airway derived Teffs that become Tmems have the ability to provide heterosubtypic immunity to conserved viral epitopes, which provides broader protection. IL-15 has been shown to play a role in the migration of Teffs to the lung airways of influenza-infected animals, but the mechanism behind this migration remains to be elucidated. The migration of lymphocytes has traditionally been attributed to the expression of chemokines and their receptors. It is not known whether IL-15 can act as an inflammatory stimulus to induce chemokine expression,

but significant levels of IL-15 can be detected as early as three days post infection [14]. Moreover, the cell type(s) responsible for this production of IL-15 have yet to be identified. Determining the cellular sources of IL-15 after influenza-infection will give us a better understanding of where respondent lymphocytes are primarily encountering IL-15, so that the consequences of these interactions may be further characterized. Therefore, we hypothesize that lung endothelial and epithelial cells are responsible for the production of IL-15 in response to influenza infection, and we will test this using an *in vitro* cell culture system. We further hypothesize that IL-15 modulates the expression of chemokine receptors on influenza-specific CD8 T cells. We will test this hypothesis by comparing chemokine receptor expression on Teffs from wildtype and IL-15 deficient animals which have defective Teff airway migration. Understanding the cell types responsible for IL-15 production in influenza infection, as well as how chemokines and chemokine receptors are modulated will be important contributions in our overall understanding of the mechanisms by which IL-15 mediates its chemoattractive functionality.

2.2 Materials and Methods

Mice, viruses, and infection

Wildtype C57BL/6 mice were purchased from Charles River through the NCI program. IL-15$^{-/-}$ mice were originally provided by Dr. Leo Lefrancois (University of Connecticut, Farmington CT), bred and maintained in our animal colony at the University of Georgia. Anesthetized animals were infected intranasally with 10^3 pfu of the H3N2 Influenza A/ Hong Kong x31 (x31) virus in 50 µl sterile PBS. The x31 virus was generously provided by Dr. S. Mark Tompkins (University of Georgia, Athens, GA).

Isolation of serum and lymphocytes

Serum was obtained after blood was collected retro-orbitally in BD Microtainer tubes with a serum separator additive. Blood was allowed to congeal at RT for 30 minutes prior to centrifugation. The supernatant containing the serum was removed and stored at -20°C until use.

Lymphocytes from the lung airways were collected via bronchioalveolar lavage (BAL) fluid by injecting and collecting 1mL PBS three times from the lung airways. In the indicated experiments, BAL fluid was separated from the cellular component via centrifugation, resuspended in 200µl PBS, and stored at -20°C until used.

Collected spleens and lymph nodes were homogenized by grinding between frosted microscope slides and passed through Nitex filters. Spleens (and BAL where necessary) were depleted of erythrocytes using Tris-buffered Ammonium Chloride. Lymphocytes were isolated from the lung parenchyma by first mincing the excised tissues and incubating them in a 1.25 mM EDTA solution for 30 minutes, followed by incubation in a 6 mg/mL collagenase solution for 60 minutes at 37°C with agitation. Samples were then passaged through 40µm cell strainers and resuspended in a 44% Percoll solution that was underlayed with a 67% solution creating a density gradient. Isolated lymphocytes were extracted from the interface following centrifugation and resuspended in RPMI-1640. All lymphocyte samples were stored at 4°C until antibody staining.

Detection of chemokines and chemokine receptors

Serum and BAL fluid were evaluated for chemokine expression using the Millipore Milliplex map multiplex panel for CXCL9, CXCL10, CCL2, CCL3, CCL4, and CCL5.

Serum and BAL were run separately to insure proper calibration and detection of chemokines. 25µl of isolated serum and 50µl of BAL fluid were used in the assays and processed according to manufacturers instructions. The Milliplex assays were read on a Bio-Rad BioPlex 2200, and the results are reported as a relative concentration. Samples were run in duplicate, and the average of these duplicates was used to determine sample concentrations. Values are reported as the mean ± standard deviation.

Flow cytometry

Isolated lymphocytes were stained at room temperature for 1 hour with monoclonal antibodies from R&D Systems (CXCR3, CXCR6, and CCR2), eBiosciences (CCR5, CD44, and CD8α), and MBL (CX3CR1). Cells were also jointly stained for the influenza nucleoprotein (NP) reactivity using an MHC Class I tetramer (H-2D(b)/ASNENMETM) generated by the NIAID Tetramer facility (Emory University). Stained cells were analyzed using a BD LSR II digital flow cytometer using the BD FACs Diva software. Results were analyzed primarily using the Treestar FlowJo software.

Cell cultures and infections

Murine lung epithelial (MLE-15) cells were originally obtained from Dr. Jeffrey Whitsett (University of Cincinnati) and stored in liquid nitrogen until thawed and used. MLE-15s were grown as monolayers in HITES media (RPMI-1640; Insulin, Transferrin, and Selenite; 10 mg/mL Transferrin; 100 µM Hydrocortisone; 100 µM β- Estradiol; 1M HEPES; and 200 mM L-Glutamine) and supplemented with an antibiotic/ antimycotic additive consisting of ampicillin, streptomycin, and amphotericin. Murine lung endothelial (1G11)

cells were obtained from Alberto Montovani (Farmacolighe Mario Megri, Milan, Italy) and stored in liquid nitrogen until thawed and used. 1G11 cells were grown as monolayers in DMEM (Invitrogen) supplemented with 100 μg/ml endothelial cell growth additive and heparin. 1G11 media also contained 20% FCS, 100 μg nonessential amino acids, 2 mM sodium pyruvate and 50 U of penicillin. All cells were grown in a 37°C incubator with 5% CO_2.

MLE-15 and 1G11 cells were infected at 0.5 MOI as calculated based on the number of viral particles necessary to infect half of all cells growing in a monolayer on a 12-well plate. The total number of cells per well was calculated based on average cell size and area of each well. Prior to infection, viability of confluent cells was assessed via Trypan blue staining. Cells were infected for a duration of 1 hour with infection media. Following this duration, infection media was aspirated, cells washed with PBS or HBSS, and cultured in 1-2 mL of each cell line's respective media.

Quantitative RT-PCR

Total RNA was extracted using a Qiagen RNeasy Plus Mini Kit. Briefly, samples are first lysed and homogenized and RNA binds to the RNEasy silica membrane. Contaminants are then washed away and RNA is eluted. RNA concentrations were quantified using a Nanodrop 2000, and all concentrations were normalized prior to reverse transcription. An ABI High Capacity cDNA Reverse Transcription kit was used to convert purified 10 μl RNA to cDNA. 2 μl cDNA was evaluated for induction of IL-15 mRNA by quantitative PCR using the ABI TaqMan Gene Expression system. Quantitative real-time RT-PCR was performed using IL15-FAM (#mm00434210_m1) and 18s-VIC (#4319413E) were combined

in a mastermix and added to all samples, which were run in triplicate (ABI). Thermal cycling began with a 30 minute incubation at 48°C, followed by 40 (15 second) denaturation cycles at 95°C for a total of 10 minutes, concluding with a 60°C annealing step for 1 minute. Samples were normalized against 18s RNA induction, and expressed relative to mock infected cells. Results are expressed as a Relative Quantification (RQ) value, as calculated by the ΔΔCt method.

IL-15/IL-15Rα Complex ELISA

The eBioscience Ready-Set-Go Mouse IL-15/IL-15Rα Complex ELISA was used to measure secreted/ soluble IL-15/IL-15Rα complexes in the supernatants of MLE-15 and 1G11 cell cultures. Supernatants were centrifuged at maximum microcentrifuge speed and resuspended in 200 µl of media and used in duplicate. ELISA plates were incubated with samples at room temperature for 2.5 hours and processed according to manufacturers instructions. Detection of IL-15c occurred via binding of biotin-conjugated antibody that was detected on the Vmax microplate after treatment with an Avidin-HRP enzyme. Plates were read on Molecular Diagnostics Vmax microplate reader at 450 nm and analyzed with Softmax software. Results are expressed as a concentration in pg/mL.

Adoptive transfers and in vivo migration assay

Congenically mismatched CD45.1 (recipients) and CD45.2 (donors) C57BL/6 animals were infected with 10^3 pfu x31 in 50µl PBS intranasally. On day 12 post infection donor mice were sacrificed and spleens were harvested for CD8 enrichment using a Dynal Mouse CD8 Negative Isolation kit (Invitrogen). CD8 T cells from donor animals were

enriched to >90% purity, and divided into three treatment groups. Treatment groups consisted of incubating with either media supplemented with 10% FCS (two treatment groups) or 20ng/mL pertussis toxin (Sigma-Aldrich) with media supplemented with 10% FCS (one treatment group) for 1.5 hours at 37°C. Following incubation donor cells were washed, recounted, and suspended at a concentration of $\sim 6 \times 10^6$ per 200µl treatment group. Donor cells were injected intravenously into infection-matched CD45.2 recipient animals. Five hours post transfer, animals receiving donor cells incubated with one treatment group of media and FCS received intranasal administration of 36.25µl PBS. The other two treatment groups (media + FCS or Ptx with media + FCS) received intranasal administration of 36.25 µl IL-15 complexes. IL-15 complexes were generated by incubating 1.5µg recombinant murine IL-15 and 7g IL-15Rα Fc-chimera (both purchased from R&D Systems) for 20 minutes at 37°C and placed on ice until intranasal administration. Recipient animals were sacrificed 12 hours post-administration of PBS or IL-15c, and the BAL, lung, and spleen were harvested. Following tissue processing, cells were extracellularly stained as previously described, with the addition of the CD45.1 and CD45.2 antibodies to discriminate host (CD45.1) versus donor populations (CD45.2).

Statistics

An unpaired two-tailed student's T-test was used to determine significance of the data. Significance was evaluated using Microsoft Excel, and defined as P values less than 0.05 or as indicated in the figure legend.

2.3 Results

Infection with influenza induces the upregulation of IL-15 mRNA in murine lung epithelial cells.

IL-15 is constitutively expressed in a number of tissues including skeletal muscles, lungs, and the placenta [50]. It is also expressed on a number of unique cell types including monocytes/macrophages, dendritic cells, and epithelial cells [48, 50]. IL-15 production and expression is tightly regulated at the transcriptional and translational steps but has been shown to be increased in response to type I IFNs [107]. Following influenza infection, one of the primary types of proinflammatory molecules produced are type I IFNs [84, 108]. IL-15 mRNA gene expression is also upregulated in infected wildtype animals compared to naïve animals [14]. Similarly, IL-15/IL-15Rα complex (IL-15c) concentrations are higher in influenza-infected wildtype mice lung lysates and serum compared to naïve and IL-$15^{-/-}$ infected animals [14].

To determine the cell types contributing to this upregulation of IL-15 mRNA and the increased concentrations of IL-15c we evaluated murine lung epithelial (MLE-15) cells and murine lung endothelial (1G11) cells for their ability to express IL-15 in response to influenza infection. To that end, MLE-15 cells were incubated with x31 virus in HITES media at an MOI of 0.5 for 1 hour. Following infection, media was aspirated, cells washed with PBS and supplemented with fresh media, and cultured for certain periods of time. Infected cells showed at least a three-fold greater induction of IL-15 mRNA compared to mock infected cells at 12, 24, 36, and 72 hours post infection (Figure 2A).

The secreted IL-15 protein is difficult to detect individually, but when complexed to its Rα subunit is detectable at a 10-100 fold increase over the cytokine alone [109]. Although

induction of IL-15 mRNA was evident throughout the course of infection, soluble IL-15c failed to be detected via ELISA at the same time points evaluated for IL-15 mRNA expression (Figure 2B). 1G11 mouse lung endothelial cells were also evaluated for IL-15 mRNA induction and production of IL-15/IL-15Rα complexes following x31 infection, but failed to produce a detectable response (data not shown).

IL-15 mediates influenza-specific lymphocyte migration through chemokine signaling in the lung airways

Our lab has previously described a role for IL-15 in the migration of influenza-specific CD8 T cells to the infected lung airways [14]. In our efforts to elucidate the mechanisms by which IL-15 mediates Teff migration to the lung airways we wished to evaluate the contributions of chemokine receptors to this Teff migration, as it is quite possible that IL-15 is either directly or indirectly impacting chemokine receptor expression. Chemokine receptors are a class of G-protein coupled receptors coupled with the Gαi class of the heterotrimeric G proteins [75]. Treatment of T cells with *Bordetella pertussis* toxin (Ptx) disables this signaling cascade, rendering this pathway functionless [76].

Thus, to test influenza-specific Teff migration to the lung airways without the chemokine receptor pathway, we compared the ability of adoptively transferred CD8 T cells pretreated with Ptx to migrate to the lung airways in response to respiratory delivery of IL-15c. Purified splenic CD8 T cells from CD45.2+ animals infected 12 days prior with x31 were incubated for 1.5 hours in either media alone or media with Ptx. Following incubation ~6 $x10^6$ Teff were adoptively transferred into CD45.1+ congenic recipients that were also 12 days post x31 infection. Five hours post-transfer, recipient mice received either PBS or IL-

15c intranasally to determine whether IL-15 induced migration of influenza-specific Teffs relies on chemokine receptor signaling to support migration to the lung airways (experimental layout and gating strategy in Figure 3A and 3B). We evaluated the BAL, lung, and spleen for the frequency of CD45.2+ donor populations at 12 hours post IL-15c treatment (Figure 3C). In the BAL, treatment with IL-15c increases the frequency of donor NP-tet+ populations compared to PBS treatment alone [14]. However, the frequency of donor cells recovered from the lung airways of animals that received adoptively transferred CD8 T cells treated with Ptx and given IL-15c was similar to those that received no IL-15c (Figure 3C, left). These findings support the hypothesis that IL-15 induced migration of Teffs likely involves the chemokine/chemokine receptor pathway. Unlike the BAL, the migration of influenza-specific NP-tet+ T cells to the spleen and the lung was unaffected by Ptx treatment (Figure 3C, middle and right).

Contribution of CXCR3, CCR5, and CX3CR1 on IL-15 induced migration of Teffs to the lung airways.

Lymphocyte migration is mediated by chemokine receptor expression on the surface of lymphocytes and binding to their respective ligands on endothelial cells. This interaction causes conformational changes in integrin molecules also on the surface of lymphocytes, thus activating them, and allowing these lymphocytes to firmly adhere to the endothelium. Lymphocytes are then capable of transmigrating through the endothelium to the sites of viral infection where they are able exert their antiviral effector functions. We sought to determine how chemokine receptor expression on respondent CD8 T cells was affected by IL-15 deficiency. Wildtype and IL-$15^{-/-}$ animals were infected with 10^3 pfu x31 virus and

chemokine receptor expression of our selected panel was evaluated days 8-12 post infection in the BAL, lungs, spleen, and the mediastinal (mLN, lung draining) and inguinal lymph nodes (iLN). The panel we chose to evaluate was determined after a literature review identified chemokine receptors implicated in the migration of Teffs, as well as those that were modulated during viral infection models.

We first evaluated CXCR3, a chemokine receptor that has been shown to be critical in the migration of effector T cells along with NK cells and plasmacytoid dendritic cells in a number of infection models including influenza, malaria, and HIV [100]. IL-15 deficient animals consistently express lower frequencies of CXCR3+ Teffs in both total CD8 T cells (left) as well as influenza-specific CD8 T cells (right) in the lung airways (Figure 4). This finding was replicated in all of the tissues tested aside from the lungs, where expression was roughly equal among wildtype and IL-15$^{-/-}$ animals. With influenza infection localizing to the upper respiratory tract and the lung airways, the lung airways represents the terminal site of lymphocyte migration. As a result, chemokine expression is downregulated upon reaching the lung and may account for the low expression levels and lack of differences in expression among wildtype and IL-15$^{-/-}$ animals.

We next chose to assess the expression of CCR5, a chemokine receptor primarily implicated in the migration of memory CD8 T cells as well as steady state effector CD8 T cell migration to the lung interstitium [102, 110]. During an x31 influenza infection CCR5 was expressed in greater frequencies in IL-15$^{-/-}$ animals relative to wildtype animals in certain tissues (Figure 5). CCR5 is expressed on influenza-specific Teffs isolated from the lung airways in both total CD8 T cells (day 10 only) and influenza-specific CD8 T cells throughout the course of infection (Figure 5). Expression of CCR5 was relatively similar

between wildtype and IL-15$^{-/-}$ animals in the other tissues evaluated, although IL-15$^{-/-}$ animals did express significantly higher frequencies in the spleen at day 10.

Likewise, IL-15$^{-/-}$ animals preferentially expressed CX3CR1, a chemokine receptor previously shown to attract monocytes, NK cells, and subsets of T cells in peripheral tissues and lymphoid organs [111]. Although not significant, IL-15$^{-/-}$ animals consistently expressed higher frequencies than their wildtype counterparts in the majority of tissues and time-points tested (Figure 6). Overall CX3CR1 expression on CD8 T cells is much lower than other chemokine receptors, but CX3CR1 expression remains one of the pathways by which CD8 T cells reach their target destinations as has been previously shown in a Rheumatoid Arthritis model [112]. This preferential expression of CCR5 and CX3CR1 by IL-15$^{-/-}$ animals may occur as a result of a lack of target cells to consume these resources. IL-15$^{-/-}$ animals have significantly lower numbers of both NK and CD8 T cells, both of which normally make use of these chemokine receptors for directed migration. The absence of these cells may indirectly cause higher frequency of expression among these chemokine receptors.

Our largest differences in chemokine receptor expression were among CXCR3+ and CCR5+ antigen-specific CD8 T cells. We think this is relevant because the migration of Teffs to the inflamed lungs in influenza infection is dependent on CXCR3 signaling. Similarly, CCR5 has also been shown to play a role in the Teff migration to the inflamed lung following influenza infection [102]. To evaluate whether the preferential expression of CCR5 in IL-15$^{-/-}$ animals resulted in a greater number of influenza-specific cells we compared CCR5+ NP-positive populations of wildtype and IL-15$^{-/-}$ animals at 8 and 10 days post infection (Figure 7). We also compared the overall cell numbers of CXCR3+ cells to serve as representative data when chemokine receptor expression is greater in wildtype

animals. First, the cell counts clearly show diminished numbers of overall CD8 T cells as well as tetramer-positive cells in all tissues of the IL-15$^{-/-}$ animals, as is consistent with published data [60, 61]. Regardless, we can still assess cell numbers because although the frequency of CCR5+ cells is greater in the IL-15$^{-/-}$ animals, the overall number of cells expressing CCR5 (hashed portion of bars) remains comparable or greater in the wildtype animals. The total number of CXCR3+ cells (gray portion of bars) is even more exaggerated in wildtype animals, as they not only have a significantly higher level of expression, but more overall cells as well. This is seen most clearly in the BAL, spleen and mediastinal lymph nodes at day 8 post influenza infection where the number of CXCR3+ wildtype cells is greater as both a frequency and numerically than in IL-15$^{-/-}$ animals. This lymphocyte deficit in the IL-15$^{-/-}$ animals can, in part, account for the magnitude of these specific changes.

CCR2 and CXCR6 expression is unaffected in IL-15$^{-/-}$ animals during influenza infection.

CCR2, a chemokine receptor expressed on the surface of monocytes/macrophages, dendritic cells, and T cells has been shown to be important in the early recruitment of macrophages during an influenza infection [87]. Previous work has also shown that following influenza infection CCR2 mRNA is upregulated, suggesting that trafficking of lymphocytes to the lungs is at least partially dependent on CCR2 [88] . We investigated whether the expression of CCR2 in an influenza infection was altered by IL-15 deficiency. CCR2 was expressed at similar frequencies in wildtype and IL-15$^{-/-}$ animals throughout the course of the adaptive response irrespective of tissues evaluated (Figure 8). These findings

lend further evidence that the role of CCR2 in the T cell response is more minute relative to its role in mediating the innate response.

CXCR6 is a chemokine receptor expressed primarily on Th1 cells including NK, CD4 and CD8 T cells [113]. Here we sought to determine how IL-15 affected CXCR6 expression on CD8 T cells. Although expression of CXCR6 was relatively abundant at day 8 post-infection, frequencies of expression were unchanged between wildtype and IL-15$^{-/-}$ animals (Figure 9). These trends were maintained at days 10 and 12 post-infection in all tissues evaluated. This data shows that IL-15 fails to play a role in the modulation of CXCR6 expression during the adaptive phase of influenza infection.

CXCR3 ligands are differentially expressed in IL-15$^{-/-}$ animals during early influenza infection.

After evaluating the expression of chemokine receptors important in the recruitment of CD8 T cells during the response to influenza infection, we sought to determine how the expression of their respective ligands compared. The chemokine receptor CXCR3 has been previously shown to bind CXCL9, CXCL10, and CXCL11 [75]. Although the three ligands function cooperatively, with one ligand compensating for the absence of another, CXCL10 has been the focus of the majority of previous studies on the CXCR3 ligands [100]. Here, we evaluated the expression of CXCL9 and CXCL10 in the BAL (Figure 10A) and serum (Figure 11A) of naïve wildtype and influenza-infected wildtype and IL-15$^{-/-}$ animals at days 3, 5, and 7 post-infection. In the BAL, CXCL9 was expressed at similar levels in both infected wildtype and IL-15$^{-/-}$ animals throughout the assayed time-points (Figure 10A), however CXCL10 was expressed at consistently high levels on days 3, 5, and 7 post infection

in the IL-15$^{-/-}$ animals (Figure 10A). Their wildtype counterparts expressed similar levels of CXCL10 compared to the IL-15$^{-/-}$ animals only during the later stages of infection. These data suggests that because IL-15$^{-/-}$ animals are preferentially expressing CXCL10, they may primarily be using this ligand to bind and recruit effector cells to shape the later adaptive response. However, since no defect was found in CXCL9 expression (or the untested CXCL11) further evaluation is necessary to confirm this hypothesis.

In the serum, expression of CXCL9 is constitutive at the early time-points (days 3 and 5) but is upregulated similarly in infected wildtype and IL-15$^{-/-}$ animals at day 7 (Figure 11A). CXCL10 expression is upregulated throughout the time-points assayed among infected wildtype and IL-15$^{-/-}$ animals with concentrations of CXCL10 remaining similar between both groups (Figure 11A). Levels of expression between infected wildtype and IL-15$^{-/-}$ animals remained similar throughout the course of infection indicating that IL-15 fails to play a role in the expression of CXCL9 and CXCL10 in the serum. The expression of these ligands was upregulated (especially CXCL10) as the adaptive response begins to form, at days 5 and 7 post-infection. Although we observed that CXCR3 ligand levels were upregulated during the course of infection, concentrations of CXCL9 and CXCL10 in the serum pale in comparison to those found in the BAL. So, while ligand expression can still be detected in the serum, ultimately the sites of infection (BAL) are where the majority of chemokine ligands responsible for the recruitment of Teffs are found.

CCR1, CCR3, and CCR5 ligands are preferentially expressed in the BAL of IL-15$^{-/-}$ animals during the early phase of influenza infection.

The ligands CCL2, CCL3, CCL4, and CCL5 can bind a number of chemokine receptors between them, but all share the ability to bind CCR5 [75]. Similar to CXCR3, CCR5 has been shown to play a role in the recruitment of effector CD8 T cells in respiratory virus response as well as normal homeostatic recruitment of effector T cells to the lung interstitium [102, 110]. In the BAL, we observed that CCL3, CCL4, and CCL5 expression was consistently high in infected IL-15$^{-/-}$ animals, especially at days 3 and 5 post-infection (Figure 10B). Expression of these ligands in infected wildtype animals was equivalent to IL-15$^{-/-}$ animals at days 5 and 7 post-infection. The expression of CCL2 in the BAL was unaffected by the absence of IL-15 (Figure 10B). The consistent high overall expression of these ligands in IL-15$^{-/-}$ animals may be a result of diminished frequencies of respondent cell types, leaving the ligands unconsumed.

In the serum, we observed that there is constitutive expression of CCL2, CCL4, and CCL5 following influenza infection, and that IL-15 deficiency does not affect this expression (Figure 11B). However, IL-15$^{-/-}$ animals preferentially express CCL3 at days 3 and 5 post-infection, mirroring the findings from the BAL (Figure 11B and 10B). Overall these findings demonstrate that differential chemokine ligand expression is more prevalent at the site of infection (BAL), which is necessary to attract different effector cells to the sites of viral replication.

2.4 Discussion

The migration of circulating effector cells to sites of infection is critical to the control and clearance of any infection. CD8 T cell are additionally important because this population of cells confers protection against a range of virus strains regardless of

hemagglutinin and neuraminidase expression patterns, or heterosubtypic immunity. For a period of time post influenza infection this population of CD8 T cells survives and is capable of eliminating different subtypes of influenza. However, if this population of CD8 T cells fails to reencounter viral antigens then they undergo contraction, dwindling further in cell number. Being able to augment this population's survival may be an important step in the development of a CD8 T cell-mediated anti-viral vaccine towards influenza able to protect against a number of unique viral strains. This strategy would not only be more cost effective in the long run than traditional antibody mediated trivalent vaccines, but also confer a broader range of protection as well. In this study we sought to further our understanding of the mechanisms involved behind IL-15 mediated Teff migration.

Previous work done by our lab has shown that IL-15c have the capacity to mediate the direct migration of influenza-specific CD8 Teffs [14]. While these findings support a model for direct chemotaxis to IL-15c, they did not eliminate the possibility that IL-15 is indirectly affecting CD8 Teff migration by modulating the expression of chemokines or chemokine receptors, or via a secondary chemotactic signal originating from an accessory cell. This study focuses primarily on the former of these two mechanisms, where we evaluate how chemokines and chemokine receptors are modulated by IL-15 *in vivo*.

IL-15 is produced by a number of different cell types including macrophages, dendritic cells, endothelial, and epithelial cells. To that end, we sought to determine potential sources of IL-15 as well as whether IL-15/IL-15Rα is being secreted as a soluble protein. The detection of IL-15 *in vivo* is difficult because of heavy post-transcriptional regulation of IL-15 mRNA. Generally, secreted IL-15 is found bound to it's Rα subunit as a complex. Our findings report that mouse lung epithelials (MLE-15s) express higher levels of IL-15

mRNA as measured by qRT-PCR (Figure 2A). However IL-15/IL-15Rα complexes failed to be detected at significant levels as measured by ELISA (Figure 2B). 1G11 (murine lung endothelial cells) failed to produce any IL-15 mRNA expression beyond basal levels, or secrete IL-15/IL-15Rα complexes in the supernatant of infected cells. These data indicates that although MLE-15s are one of the cellular sources responsible for the local production of IL-15 following influenza infection, more signals may be required to induce secretion of IL-15 out of the cells.

The signaling of chemokines through their receptors is critical in the migration of cells to the sites of infection. Chemokine signaling initiates a signaling cascade that results in the activation of integrins, and binding to ligands on the surface of endothelial cells, ultimately leading to lymphocyte transmigration to the sites to infection. In order to determine the mechanism of how the migration of influenza-specific CD8 T cells is mediated by IL-15 we first sought to determine if chemokine signaling was indeed playing a role in the migration of these cells to the BAL. A portion of the enriched CD8 T cells that were isolated from the spleens of infected congenically mismatched donors were incubated with Pertussis toxin, disabling chemokine signaling (Figure 3A). Blocking chemokine receptor signaling with Ptx treatment mitigated IL-15 induced migration *in vivo* (Figure 3C). These findings implicate chemokine receptor signaling as one of the mechanisms by which IL-15 is able to mediate Teff migration to the lung airways.

Our findings also show that in IL-15 deficient animals, expression of the chemokine receptors CXCR3, CCR5, and CX3CR1 differs from their wildtype counterparts. CXCR3, a chemokine receptor primarily implicated in the migration of effector CD8 T cells, is expressed at significantly lower levels in IL-15$^{-/-}$ animals (Figure 4). However, CCR5 and

CX3CR1 are preferentially expressed by IL-15$^{-/-}$ animals (Figures 5 and 6). Although the frequency of cells expressing CCR5 is significantly higher in the BAL of IL-15$^{-/-}$ animals, overall numbers of NP-tet+ CCR5+ cells remain lower than in wildtype animals. (Figure 7). The expression of the chemokine receptors CCR2 and CXCR6 remained consistent in wildtype and IL-15$^{-/-}$ animals, suggesting that these chemokine receptors may be reliant on other signaling molecules for their expression during influenza (Figures 8 and 9).

Chemokine ligand expression was measured in the BAL and serum of naïve and infected wildtype and IL-15$^{-/-}$ animals. We observe that in the serum CXCL9 and CXCL10, two of the CXCR3 ligands, are expressed similarly (Figure 11A). Both ligands are expressed at equal concentrations relative to their wildtype counterparts. These data indicates that IL-15 does not play a role in the modulation of CXCL9 and CXCL10 in the serum during flu infection. However in the BAL, we observe that CXCL9 and CXCL10 are differentially expressed relative to one another (Figure 10A). CXCL9 levels remain consistent in wildtype and IL-15$^{-/-}$ animals throughout the course of infection but in IL-15$^{-/-}$ animals, especially in the early time-points evaluated, CXCL10 is preferentially expressed. During the later phases expression of CXCL10 is equivalent among wildtype and IL-15$^{-/-}$ animals. These findings suggest that CXCL10 is recruiting cells to the lung airways earlier during infection than CXCL9, but as the infection progresses Teffs may use both ligands interchangeably as is evidenced by high concentrations of both later in infection. The roles of the CXCR3 ligands need to be further evaluated to gain a more clear understanding of their interrelationship in influenza and IL-15 mediated Teff migration.

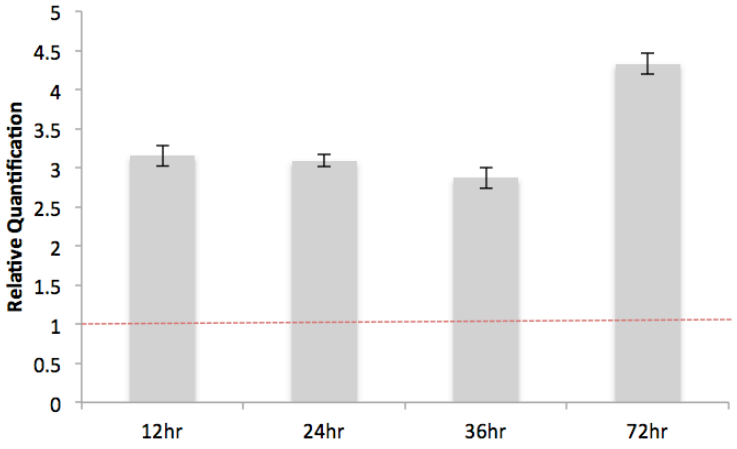
The chemokine ligands CCL2, CCL3, CCL4, and CCL5 all are capable of binding CCR5. Here we show that in the serum, CCL2, CCL4, and CCL5 were constitutively

expressed irrespective of the availability of IL-15 (Figure 11B). This indicates that IL-15 is dispensable for the expression of these ligands. Alternatively, IL-15 deficiency results in the preferential expression of CCL3, another of the CCR5 ligands (Figure 11B). Similarly in the BAL CCL3, CCL4, and CCL5 are all expressed at high concentrations throughout the course of infection in IL-15$^{-/-}$ animals, especially at days 3 and 5 post infection (Figure 11d-f). These high levels of infection correlated with expression levels of wildtype animals at days 5 and 7 post infection. Although CCL2 expression trended towards being higher in IL-15$^{-/-}$ animals in the BAL, levels remained relatively similar to infected wildtype animals (Figure 10B). The findings that CCL3 is preferentially expressed by IL-15$^{-/-}$ animals in both the serum and the BAL possibly outline a role for CCL3 in the recruitment of IL-15 dependent subsets of effectors. These results indicate a level of cooperative binding among the various CCR5 ligands to insure maximal likelihood of attracting target effectors to the site of infection.

Taken together, the data presented in this thesis show that chemokine/chemokine receptor signaling plays a role in IL-15 mediated CD8 Teff migration, and that in the absence of IL-15 the expression of chemokine receptors on respondent Teffs, as well as the expression of ligands in the BAL of influenza infected animals are also affected. This research is another step towards completely elucidating the mechanisms behind IL-15 mediated CD8 Teff migration to the lung airways, and gaining a greater understanding of the requirements for maximal recruitment of this population of cells. The research presented here also raises some outstanding questions of its own, such as which cell types are primarily responding to these differentially expressed chemokines, which cell types are responsible for

the production of these chemokines, and how is overall timing of chemokine and chemokine receptor expression impacting the immune response.

Figure 2 **A.**


Relative Quantification
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
12hr
24hr
36hr
72hr

B.

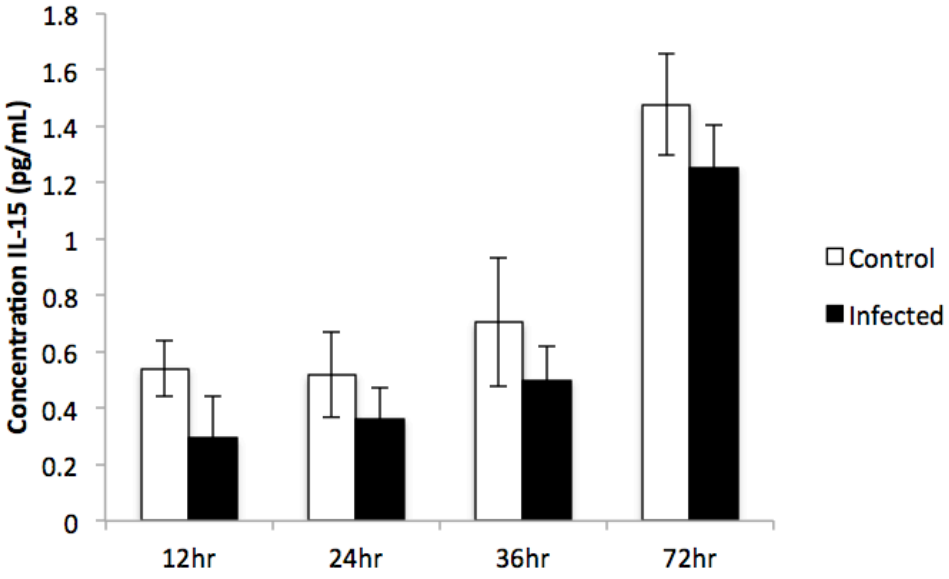

Concentration IL-15 (pg/mL)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
12hr
24hr
36hr
72hr
Control
Infected

Figure 2. Expression of IL-15 mRNA and soluble IL-15/IL-15Rα complexes by MLE-15 cells after influenza infection. (A) Gene expression levels of IL-15 were quantified in mock versus x31 infected MLE-15 cells 12, 24, 36, and 72 hours p.i. by quantitative RT-PCR. Values are expressed as a relative quantification with mock infected cells as the normalized baseline (dashed line). Values are represented as mean ± standard deviation compared with mock infected cells. (B) Secreted IL-15/IL-15Rα complexes were measured using the Ready-Set-Go eBioscience ELISA kit. Supernatants from mock and x31 infected MLE-15 cell cultures were collected and run in duplicate. Values are measure as a concentration in pg/mL based on a standard curve, with values derived from mean ± standard deviation comparing mock and infected supernatants. This is representative data from 2 experiments.

Figure 3

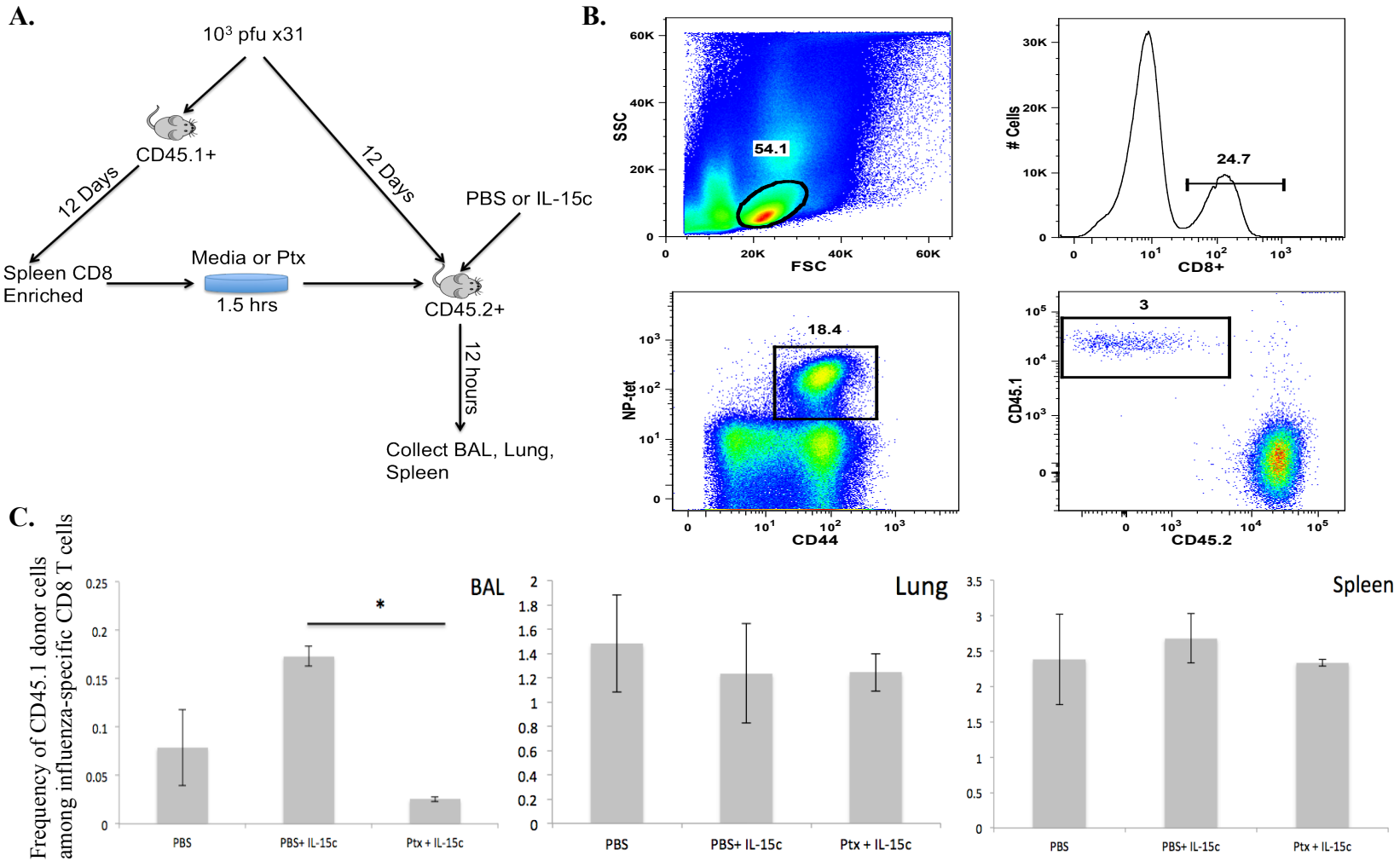

A.
10³ pfu x31
CD45.1+
12 Days
12 Days
PBS or IL-15c
Spleen CD8 Enriched
Media or Ptx
1.5 hrs
CD45.2+
12 hours
Collect BAL, Lung, Spleen
B.
SSC
FSC
54.1
Cells
CD8+
24.7
NP-tet
CD44
18.4
CD45.1
CD45.2
3
C.
Frequency of CD45.1 donor cells among influenza-specific CD8 T cells
BAL
Lung
Spleen
*
PBS
PBS+ IL-15c
Ptx + IL-15c

Figure 3. Ptx treatment of anti-influenza Teffs reduces their recovery from the lung airways. (A) Methods schematic for Ptx treatment of Teffs prior to IL-15 induced migration (B) Representative gating strategy for identification of CD45.1+ donor cells. (C) Lymphocytes recovered from the BAL, lung, and spleen 12 hours post adoptive transfer as represented in Figure 3A. Recipients also received either PBS intranasally or IL-15c 5 hours post-transfer. Values are reported as a mean ± standard deviation. p values < 0.05 are reported as significant via an unpaired student's T-test. Data are representative of 3 experiments.

Figure 4

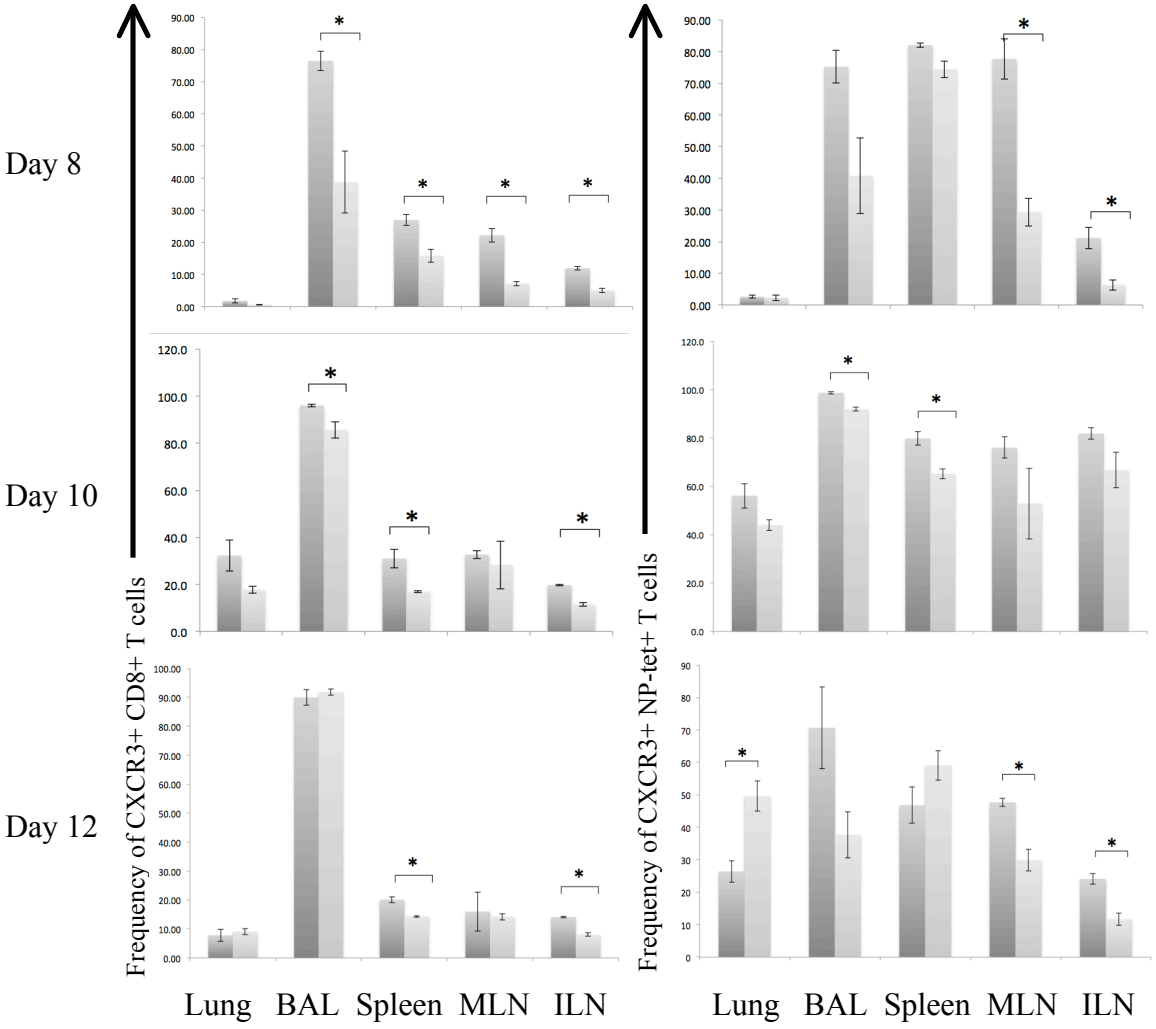

Day 8
Day 10
Day 12
Frequency of CXCR3+ CD8+ T cells
Frequency of CXCR3+ NP-tet+ T cells
Lung
BAL
Spleen
MLN
ILN
Lung
BAL
Spleen
MLN
ILN

Figure 4. CXCR3 expression is decreased on Teffs in IL-15 deficient animals. CXCR3 expression was measured among total CD8+ (left panels) and anti-NP-specific (right panels) Teffs via flow cytometry at days 8, 10, and 12 p.i. Fluorescence minus one expression (FMOs) was used to set the negative expression gates, and positive expression is reported as a frequency of CXCR3+ staining cell. p values < 0.05 are reported as significant via an unpaired student's T-test. Data are representative of 3 experiments.

Figure 5

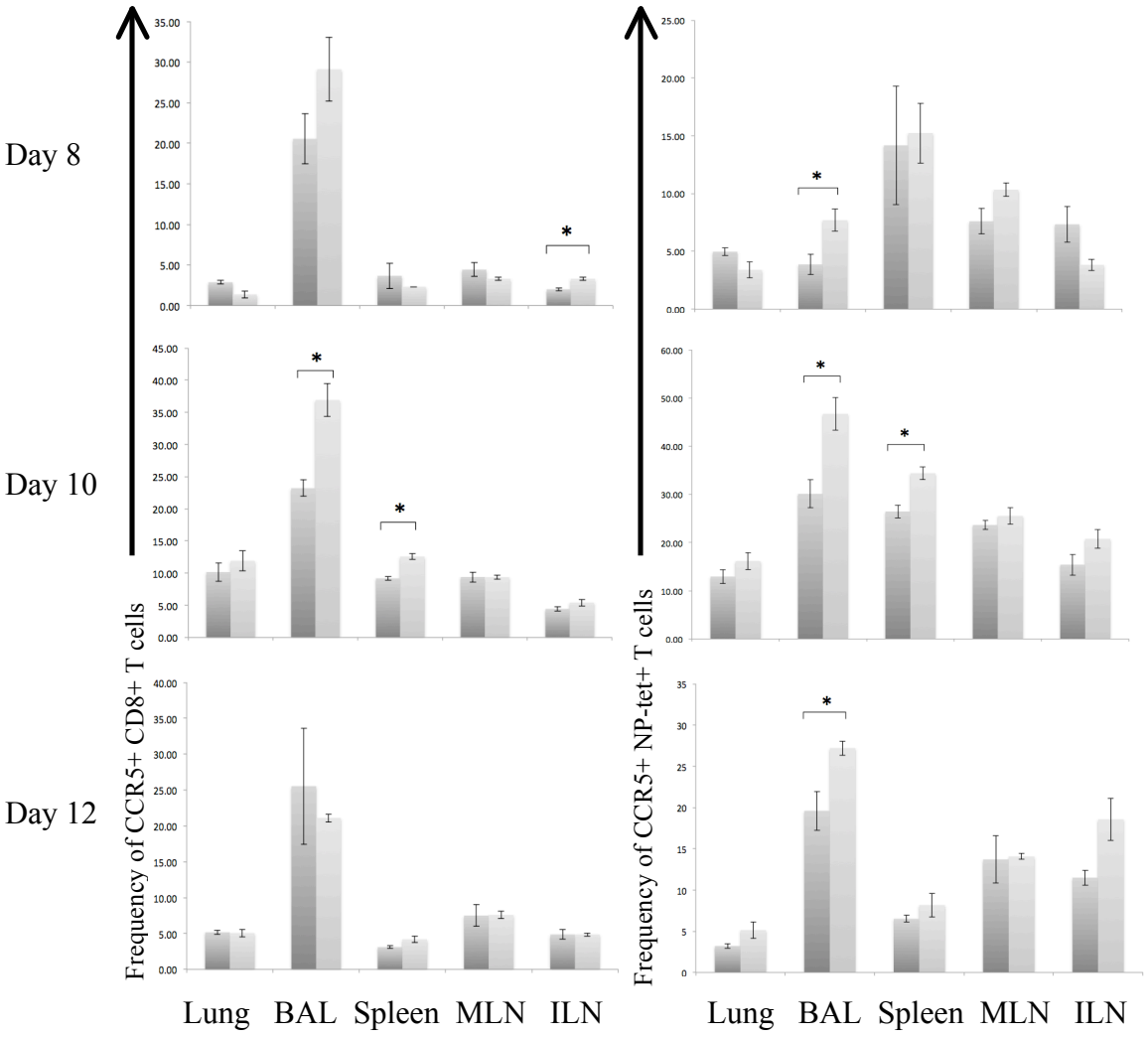

Day 8
Day 10
Day 12
Frequency of CCR5+ CD8+ T cells
Frequency of CCR5+ NP-tet+ T cells
Lung
BAL
Spleen
MLN
ILN
Lung
BAL
Spleen
MLN
ILN

Figure 5. CCR5 is expressed on more NP-specific CD8 Teffs in the lung airways of IL-15 deficient animals following influenza infection. CCR5 expression was measured among total CD8+ (left panels) and anti-NP-specific (right panels) Teffs via flow cytometry at days 8, 10, and 12 p.i. Fluorescence minus one expression (FMOs) was used to set the negative expression gates, and positive expression is reported as a frequency of CCR5+ staining cell. An unpaired student's T-test was used to determine significance where p values <0.05 are reported as significant. Data are representative of 3 experiments.

Figure 6

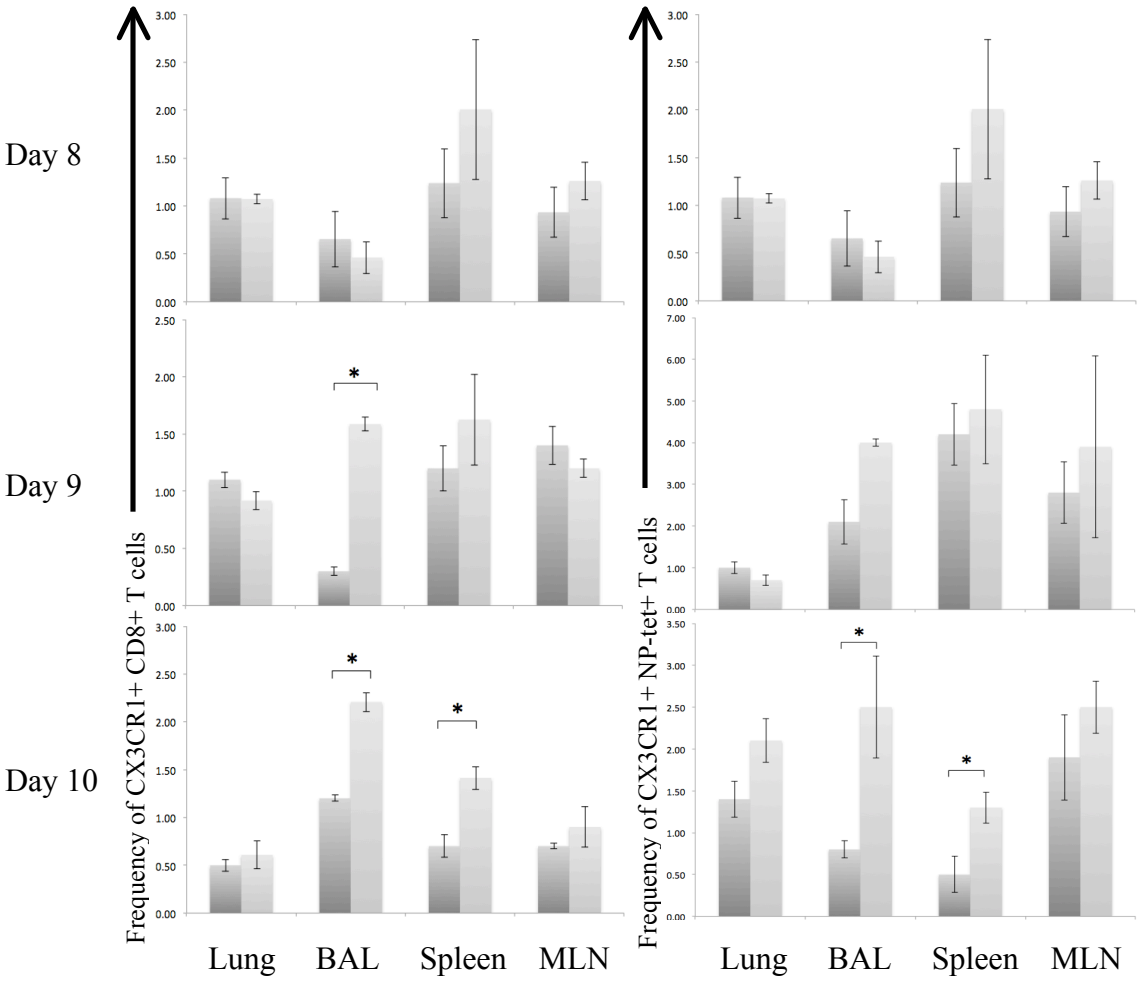

Day 8
Day 9
Day 10
Frequency of CX3CR1+ CD8+ T cells
Frequency of CX3CR1+ NP-tet+ T cells
Lung
BAL
Spleen
MLN
Lung
BAL
Spleen
MLN

Figure 6. CX3CR1 is preferentially expressed by IL-15$^{-/-}$ animals on influenza-specific CD8 T cells. CX3CR1 expression was measured among total CD8+ (left panels) and anti-NP-specific (right panels) Teffs via flow cytometry at days 8, 9, and 10 p.i. Fluorescence minus one expression (FMOs) was used to set the negative expression gates, and positive expression is reported as a frequency of CX3CR1+ staining cells. An unpaired student's T-test was used to determine significance where p values <0.05 are reported as significant. Data are representative of 2 experiments.

Figure 7

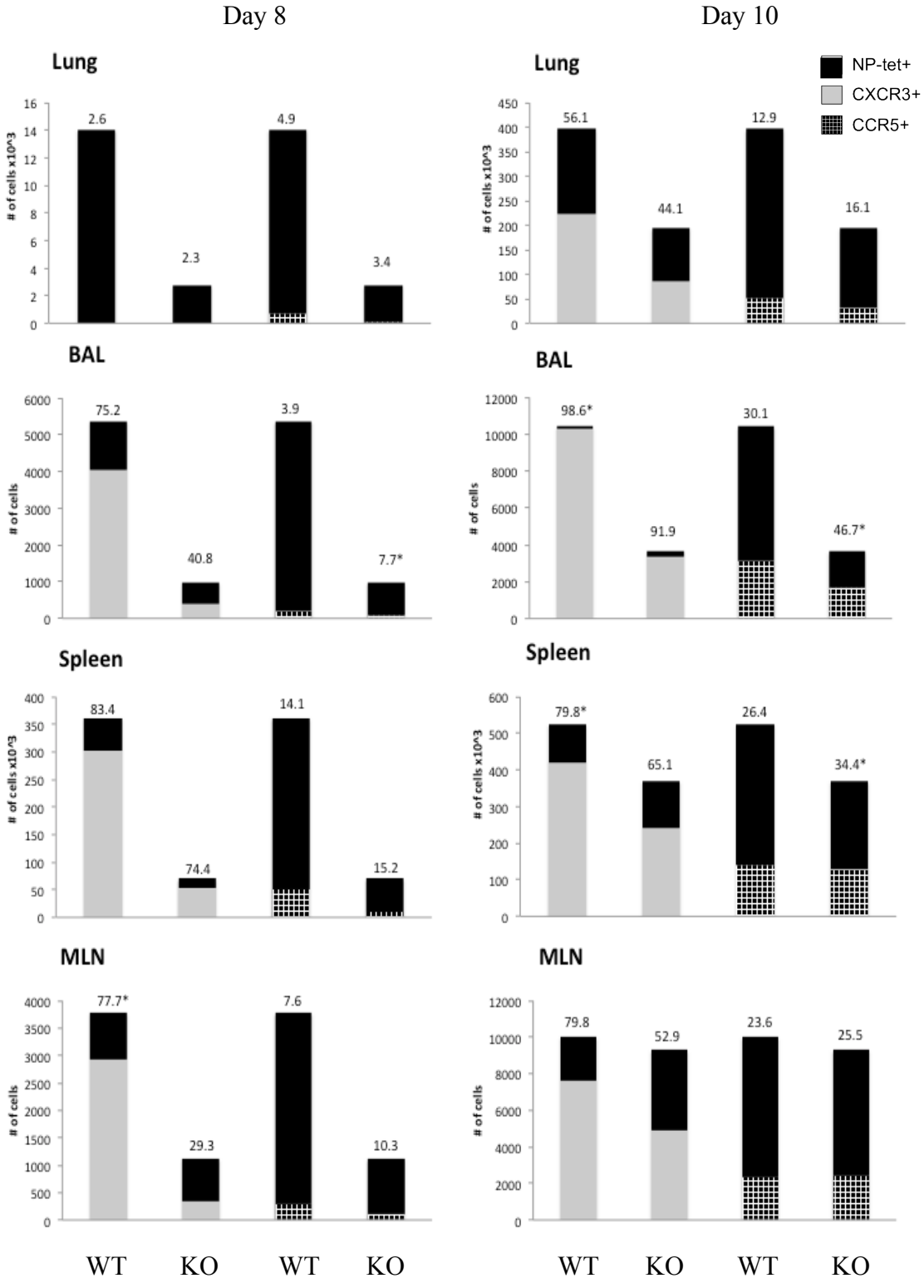

Day 8
Day 10
Lung
BAL
Spleen
MLN
NP-tet+
CXCR3+
CCR5+
# of cells x10^3
# of cells
2.6
2.3
4.9
3.4
56.1
44.1
12.9
16.1
75.2
40.8
3.9
7.7*
98.6*
91.9
30.1
46.7*
83.4
74.4
14.1
15.2
79.8*
65.1
26.4
34.4*
77.7*
29.3
7.6
10.3
79.8
52.9
23.6
25.5
WT
KO
WT
KO
WT
KO
WT
KO

Figure 7. IL-15$^{-/-}$ animals, despite their decreased CD8 T cell numbers, differentially express CCR5 and CXCR3 on influenza-specific T cells in the lung airways. Total number of NP-tet+ CD8 T cells were determined and compared between wildtype and IL-15$^{-/-}$ animals. Shaded in areas of the bar graph representing the frequency of CXCR3+ or CCR5+ NP-tet+ T cells as indicated. Numbers displayed atop the bar graphs represent the frequency of indicated shaded chemokine receptor as proportion of total NP-specific CD8 T cell. Asterisks (*) next to frequencies indicate significance between wildtype and IL-15$^{-/-}$ animals within a group. An unpaired student's T-test was used to determine significance where p values <0.05 are reported as significant.

Figure 8

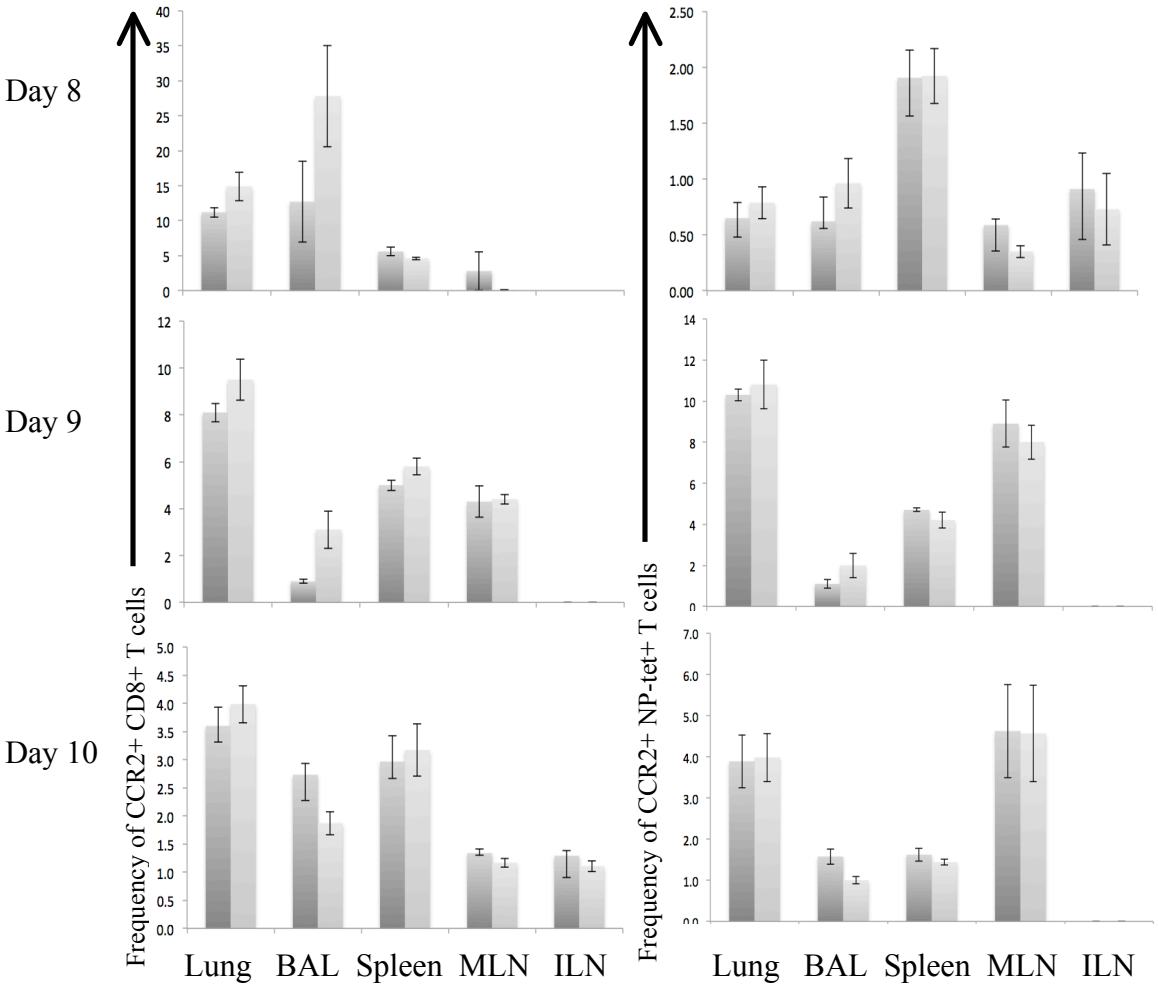

Day 8
Day 9
Day 10
Frequency of CCR2+ CD8+ T cells
Frequency of CCR2+ NP-tet+ T cells
Lung
BAL
Spleen
MLN
ILN
Lung
BAL
Spleen
MLN
ILN

Figure 8. CCR2 expression is unaltered between wildtype and IL-15$^{-/-}$ animals following influenza infection. CCR2 expression was measured among total CD8+ (left panels) and anti-NP-specific (right panels) Teffs via flow cytometry at days 8, 9, and 10 p.i. Fluorescence minus one expression (FMOs) was used to set the negative expression gates, and positive expression is reported as a frequency of CCR2+ staining cells. An unpaired student's T-test was used to determine significance where p values <0.05 are reported as significant. Data are representative of 2 experiments.

Figure 9

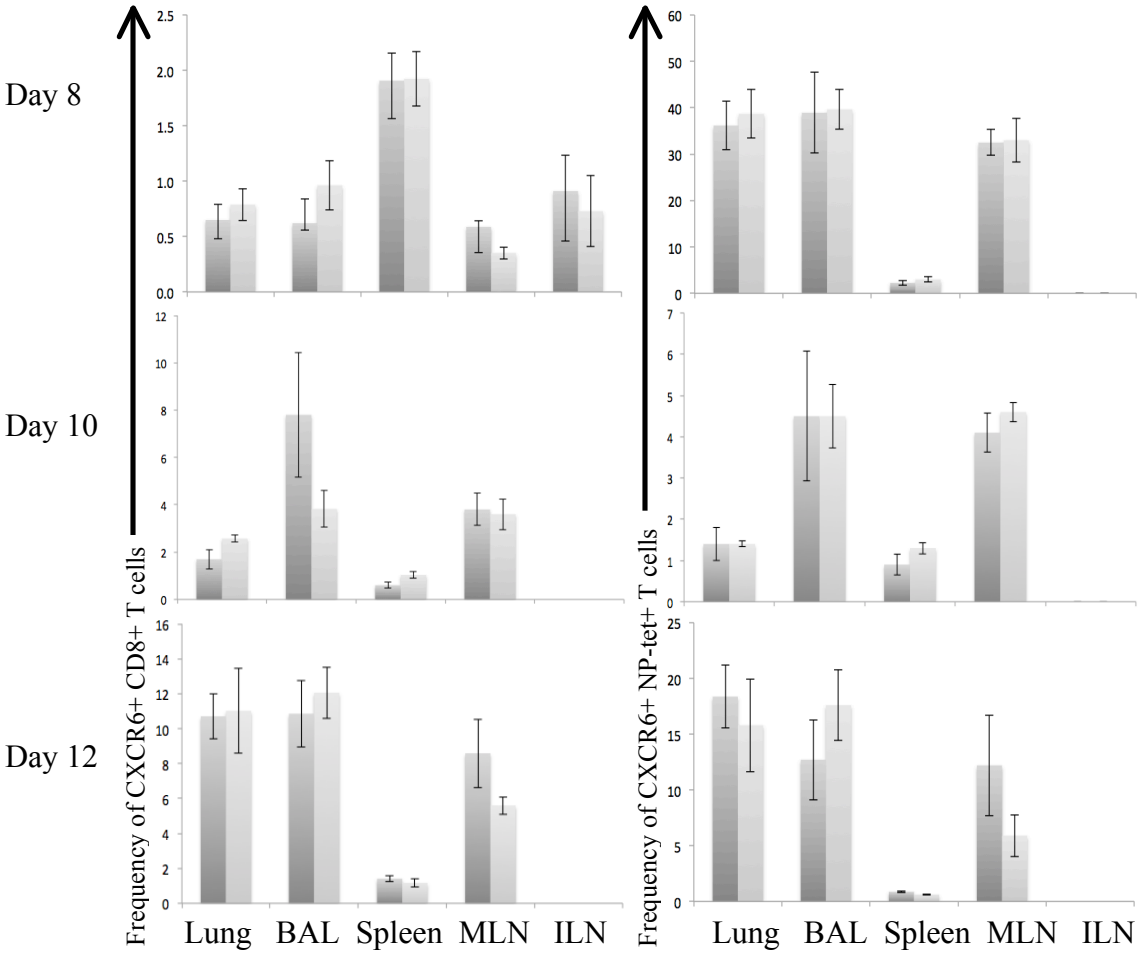

Day 8
Day 10
Day 12
Frequency of CXCR6+ CD8+ T cells
Frequency of CXCR6+ NP-tet+ T cells
Lung
BAL
Spleen
MLN
ILN
Lung
BAL
Spleen
MLN
ILN

Figure 9. CXCR6 is expressed homeostatically in both wildtype and IL-15$^{-/-}$ animals following influenza infection. CXCR6 expression was measured among total CD8+ (left panels) and anti-NP-specific (right panels) Teffs via flow cytometry at days 8, 10, and 12 p.i. Fluorescence minus one expression (FMOs) was used to set the negative expression gates, and positive expression is reported as a frequency of CXCR6+ staining cells. An unpaired student's T-test was used to determine significance where p values <0.05 are reported as significant. Data are representative of 3 experiments.

Figure 10

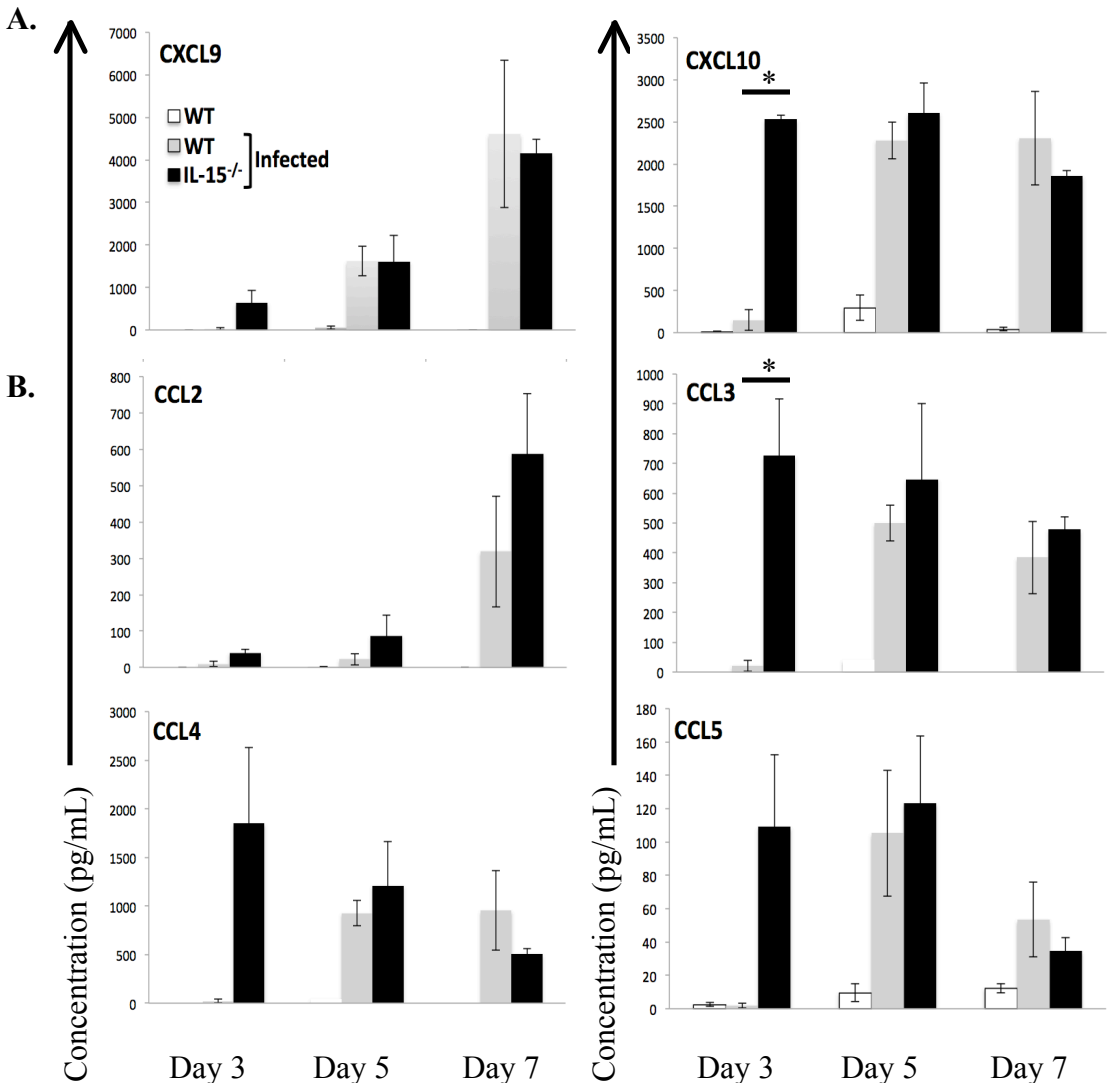

BAL
A.
B.
CXCL9
WT
WT
IL-15-/-
Infected
CXCL10
*
CCL2
CCL3
*
CCL4
CCL5
Concentration (pg/mL)
Concentration (pg/mL)
Day 3
Day 5
Day 7
Day 3
Day 5
Day 7

Figure 10. Chemokine expression in the lung airways of naïve and infected wildtype and IL-15$^{-/-}$ animals. (A-B) CXCL9, CXCL10, CCL2, CCL3, CCL4, and CCL5 expression evaluated in the BAL via multiplex. BAL was obtained on days 3, 5, and 7 p.i. by collecting three 1mL PBS washes of the lung airways. Samples were processed according to the manufacturers instructions and run in duplicate. Values are represented as a mean ± standard deviation being reported as a concentration in pg/mL. Data are representative of 3 experiments

Figure 11

Serum

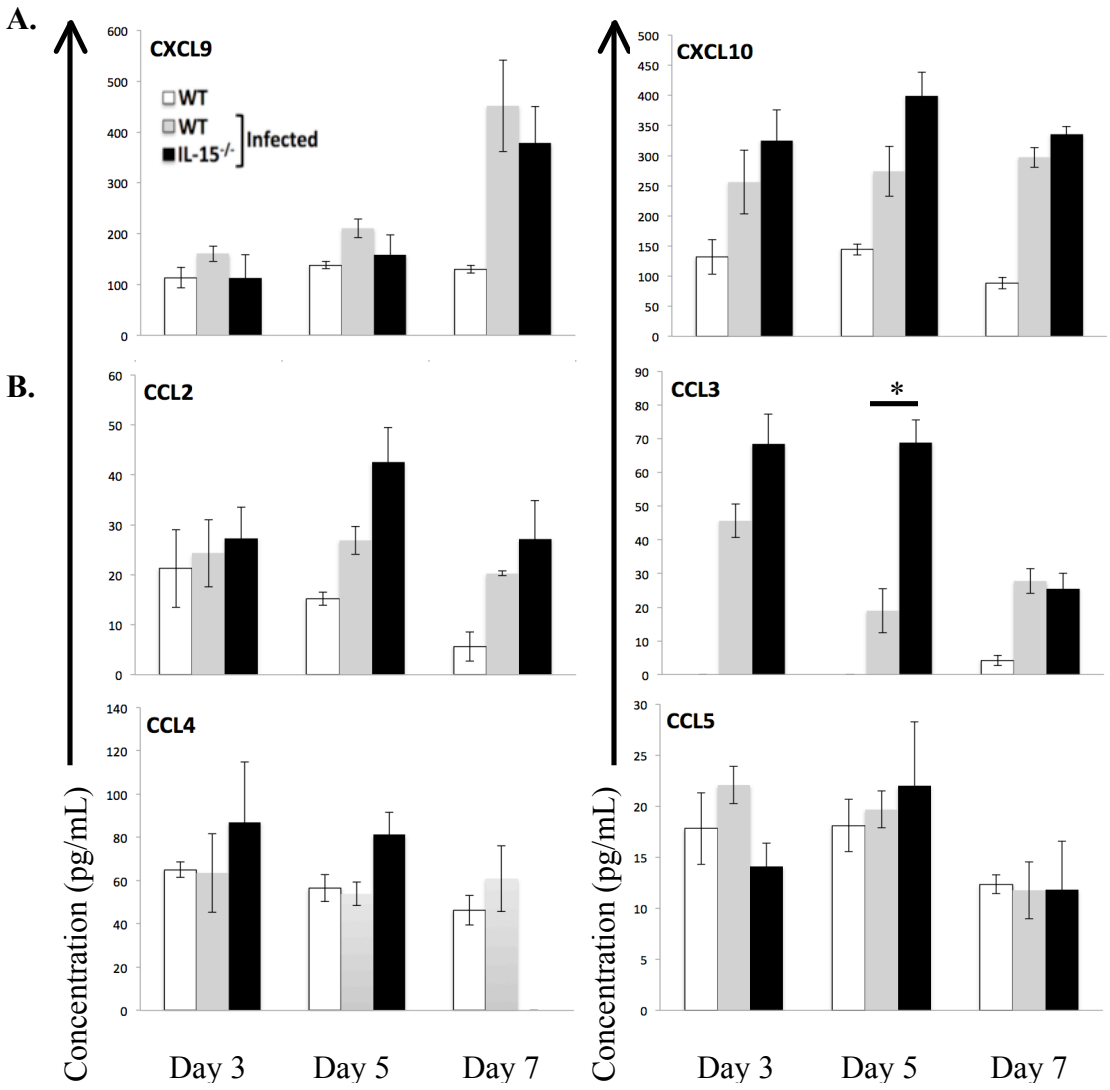


Figure 11. Chemokine expression in the serum of naïve and infected wildtype and IL-15$^{-/-}$ animals. (A-B) CXCL9, CXCL10, CCL2, CCL3, CCL4, and CCL5 expression evaluated in the serum on days 3, 5, and 7 p.i. Serum was extracted from the liquid phase and run in duplicate on the multiplex. Values are represented as a mean ± standard deviation being reported as a concentration in pg/mL. Data are representative of 3 experiments.

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