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# Chemokine receptor CXCR3 affects the balance between effector and memory CD8 T-cell generation

by

Joyce K. Hu

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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This dissertation is dedicated to my parents,

Christina and Michael Hu.

Their love and support has given me the courage to pursue new things and their values in dedication and patience will continue to influence me throughout my life.

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**Contributions to thesis work**

Chapter 2 is a reprint of the material as it appears in *PNAS*, Hu JK, Kagari T, Clingan JM, Matloubian M (2011) 108: E118-27. Mehrdad Matloubian and I designed the experiments. I performed experiments and analyzed data for every figure. Mehrdad processed livers and lungs for Fig.4A and C. Jon isolated RNA, made cDNA, performed the Q-PCR, and analyzed data for Fig.1C. He also sorted marginal zone macrophages and marginal metallophilic macrophages, isolated RNA, made cDNA, performed the Q-PCR, and analyzed data for Fig.5B. Takashi sorted KLRG1$^{lo}$ and KLRG1$^{hi}$ effector and memory cell subsets, isolated RNA, and made cDNA for Fig.1D and Fig.S5A. Mehrdad and I wrote the paper.

**Chemokine receptor CXCR3 affects the balance between effector and memory CD8 T-cell generation**

by

Joyce K. Hu

During infection, pathogen-specific T cells must efficiently recognize their cognate antigen, expand into effector cells, and form memory cells that provide protection during subsequent encounters with the same pathogen. Chemokines and chemokine receptors play essential roles in these processes. They mediate T cell surveillance of antigens in secondary lymphoid organs (SLOs) and facilitate T cell contacts with other immune cells that result in activation, expansion into effector cells, and formation of functional memory cells. Within the spleen, activated CD8 T cells move from distinct compartments of the white pulp (WP) to the marginal zone (MZ) and red pulp (RP), where they exit the spleen and migrate to inflamed tissues. The molecular cues that guide activated CD8 T cells to splenic compartments and the effect of these movements on T cell differentiation are not completely understood. The chemokine receptor CXCR3 is expressed on activated CD8 T cells and guides these cells towards the interferon-inducible chemokines CXCL9, CXCL10, and CXCL11. We used wild-type (WT) and CXCR3 knock-out (KO) T cell receptor (TCR) transgenic P14 CD8 T cells to determine if CXCR3 directs activated CD8 T cells to specific splenic compartments and if CXCR3-directed movements influence CD8 T cell differentiation. Using lymphocytic choriomeningitis virus (LCMV) infection, we

found that CXCR3 directed activated CD8 T cells towards CXCR3 ligands in the MZ, where LCMV antigens were abundant. In contrast, CXCR3 KO CD8 T cells were concentrated in the T zone away from antigen in the MZ. Because antigen and inflammation promote the generation of short-lived effector cells, we examined whether CXCR3-mediated positioning affected CD8 T cell differentiation. CD8 T cell clustering in the MZ was associated with increased generation of short-lived effector cells and decreased formation of memory precursor cells. This led to the generation of fewer memory cells that were phenotypically and qualitatively different. These data demonstrate that CXCR3 increases CD8 T cell access to antigen and potentially inflammation in the MZ that affects the balance between effector and memory cell generation.

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# Chapter 1

# Introduction

## Coordinated immune cell movements are essential for immune responses

*Secondary lymphoid organs and antigen entry*

During infection, efficient recognition of cognate antigens by pathogen-specific T and B cells is essential to mount an effective immune response. However, the precursor frequencies of T and B cells are relatively low compared to their overall numbers in the body. For example, during infection with lymphocytic choriomeningitis virus (LCMV), precursor frequencies of the immunodominant gp33-41 epitope is 1 in $2x10^5$ CD8 T cells or 100-200 in 2-$4x10^7$ total estimated naïve CD8 T cells in an entire mouse [1]. Due to these low precursor frequencies, antigen surveillance in highly organized immune centers is essential. Within these organized centers, called secondary lymphoid organs (SLOs), naïve T and B cells survey for cognate antigens and interact with other immune cells to elicit an immune response.

The architecture of SLOs (Figure 1) facilitates antigen surveillance and immune cell interactions. SLOs include the spleen, lymph nodes (LNs), and mucosa-associated lymphoid tissue (MALT), each of which acquires antigens that have entered via different routes. The splenic red pulp filters blood for antigens and microorganisms [2], lymph nodes filter the lymph for antigens arriving from the skin or mucosa [3], and MALT acquire antigens from mucosal tissues. The spleen is composed of the red pulp (RP) that filters blood and the white pulp (WP) that enables T and B cell responses. Structured similarly, the splenic WP and LN contain a central T cell compartment called the periarteriolar lymphoid sheath (PALS) in the WP or the T zone in LNs. B cell follicles border

the central T cell compartment and both T and B cell compartments are encapsulated within the marginal zone (MZ) of the spleen or the subcapsular sinus (SCS) of the LN.

During infection, antigens enter SLOs in free form or in association with immune cells [3]. In the spleen, antigens enter from the blood into arterioles that terminate in the marginal sinus or marginal zone (MZ) [2]. From there, low molecular weight antigens can enter the white pulp via a conduit system [4] while larger antigens can flow from the MZ into the cords of the splenic RP or directly into venous sinuses back to the blood. In the lymph node, antigens enter through the afferent lympatics into the SCS. While small molecules may enter the lymph node parenchyma through a reticular conduit [4,5], other molecules can move into medullary sinuses where they exit through efferent lymphatics [4].

Alternatively, immune cells in different SLO compartments can capture antigens. On either side of the marginal sinus, marginal zone macrophages (MZM) nearest to the RP and marginal metallophilic macrophages (MMM) nearest to the WP internalize and degrade pathogens [6,7]. Marginal zone B cells capture antigen and become IgM-secreting plasma cells [8] or they migrate to the WP where they shuttle antigen to B cell follicles [9] or participate in B cell [10] and T cell [11] responses. Marginal zone dendritic cells, when activated, migrate into the white pulp and participate in adaptive immune responses [7,12,13]. Meanwhile, in the WP, dendritic cells can take up antigens that have moved through the splenic conduit system [3,7]. In the RP, macrophages encounter pathogens from blood such as bacteria and secrete molecules that

limit their growth [14]. Similarly to macrophages in the marginal zone, macrophages in the SCS of LNs also take up and degrade pathogens [3]. Additionally, subcapsular sinus macrophages can transport antigen in the form of immune complexes to follicular B cells, which can then transport these complexes to follicular dendritic cells or the T zone to initiate a response [15]. In peripheral tissue, antigens can also be taken up and processed by dendritic cells, which mature and migrate to draining lymph nodes for presentation [16].

*Role of chemokines in antigen surveillance and immune cell activation, function, and differentiation*

Chemokines and chemokine receptors are critical for antigen surveillance, immune cell compartmentalization in SLOs, and immune cell movements during infection. Chemokines are small protein chemoattractants classified by structure according to the number and spacing of conserved cysteine residues [17]. They bind to seven transmembrane chemokine receptors that signal through G proteins to direct cell movement. Chemokines can further be grouped into homeostatic and inflammatory chemokines, although some chemokines have characteristics of both groups [18,19]. Homeostatic chemokines are constitutively expressed during homeostatic, or non-inflammatory, conditions and are important for lymphocyte recirculation and compartmentalization in SLOs. In contrast, inflammatory chemokines are induced by inflammatory stimuli and are important for the immune response during infection and disease. Thus, regulation of homeostatic and inflammatory

chemokines is crucial for the switch between immune cell vigilance during normal states to immune cell responses during disease states.

Lymphocyte entry into SLOs, compartmentalization within SLOs, and exit from SLOs requires chemokine receptors and the sphingosine 1-phosphate receptor-1 ($S1P_1$). $S1P_1$ is a 7-transmembrane receptor that binds the lysophospholipid sphingosine 1-phosphate (S1P) and mediates lymphocyte exit from the thymus and SLOs [20,3,21]. T and B cells enter the spleen through arterioles into the marginal sinus, marginal zone, or red pulp [3,2]. From there, they move into the white pulp via bridging channels [22] in a pertussis toxin sensitive, or $G\alpha_i$ dependent [23], and integrin-dependent manner [24]. In lymph nodes, T and B cells enter through high endothelial venules (HEV) in a multi-step process involving selectin-mediated rolling, chemokine-mediated integrin activation, integrin-mediated adhesion, and transmigration across the HEV [3]. After entry, chemokines attract T and B cells to distinct compartments of the splenic WP or LN. CCR7-expressing naïve T cells are attracted to the homeostatic chemokines CCL19 [25] and CCL21 [26,27], which are secreted by stromal cells in the splenic PALS and LN T zone [28]. CXCR5-expressing naïve B cells are attracted to the homeostatic chemokine CXCL13 expressed by follicular stromal cells in B cell follicles. After surveying for cognate peptide-MHC and antigen, T and B cells use the $S1P_1$ receptor to exit LNs [20] towards a gradient of increased S1P within the efferent lymphatics [29].

Chemokine receptor modulation is important for antigen transport and cell-cell interactions. Dendritic cells modulate chemokine receptor expression to

move from peripheral tissues to draining lymph nodes to present antigen. Immature dendritic cells (DCs) express inflammatory chemokine receptors such as CCR1, CCR5, CCR6, and CXCR1 that allow these cells to migrate to peripheral tissues to survey for pathogenic antigens [30]. In particular, skin-resident dendritic cells express CCR1, CCR5, and CXCR1, which allow them to survey the skin. In response to activation signals, DCs produce inflammatory chemokines including CXCL8, CXCL10, CCL3, and CCL4 which may enhance neutrophil and monocyte recruitment to the affected tissue [31]. As dendritic cells mature, they downregulate inflammatory chemokine receptors and upregulate the homeostatic chemokine receptor CCR7 that guides them towards draining lymph nodes [32-34]. While migrating to lymph nodes or while in lymph nodes, DCs secrete CCL18, which preferentially attracts naïve T cells [35], and CCL17 [36] and CCL22 [37] which attracts activated T cells. T and B cells also modulate chemokine receptor expression during the immune response. Activated CD4 T helper cells upregulate CXCR5 and activated B cells upregulate CCR7, which allows these cells to move towards the T-B boundary where they can give and receive help [38,39]. Another subset of CD4 T helper cells, called follicular B helper T cells, downregulate CCR7 and upregulate CXCR5 [40], allowing them to move into B cell follicles to aid the germinal center response [41].

In addition to directing immune cells within the lymph node, chemokines also direct immune cells to peripheral tissues where they perform effector functions. Activated CD4 and CD8 T cells upregulate expression of

inflammatory chemokine receptors CCR5, CXCR6, and CXCR3. CCR5-mediated migration of effector and memory cells to peripheral tissues is important to combat particular infections. In one example, CCR5 expression on activated CD4 and CD8 T cells increased infiltration into the brain during West Nile virus infection and correlated with increased survival [42]. In another example, CCR5 mediated early memory CD8 T cell infiltration into the lung during secondary infection with Sendai virus, which was associated with decreased viral titers in the lung [43]. Similarly, CXCR6 may mediate T cell infiltration into inflamed peripheral sites [44-46]. Correlative studies showed that CXCR6-expressing T cells infiltrated rheumatoid joints, inflamed livers, and spinal cords of mice with experimental autoimmune encephalomyelitis (EAE). A study using CXCR6 knockout mice demonstrated that CXCR6 partially mediated CD8 T cell infiltration into inflamed livers that expressed the CXCR6 ligand CXCL16 [47]. Likewise, CXCR3 may direct T cells into inflamed tissues in models of infection, graft-versus-host disease, and autoimmune disease. CXCR3 is expressed on effector and memory CD4 and CD8 T cells and attracts these cells to inflamed sites expressing the interferon-inducible CXCR3 ligands CXCL9, CXCL10, and CXCL11 [48-50]. The outcomes of CXCR3-mediated infiltration into inflamed tissues vary depending on the infection. Intracranial infection of mice with lymphocytic choriomeningitis virus (LCMV) leads to death at seven to ten days post-infection. In this infection model, CXCR3 expression increased T cell accumulation in the brain parenchyma and accelerated mortality in mice [51,52], possibly due to heightened immunopathology in the central

nervous system (CNS). On the other hand, CXCR3 played a protective role in intracerebral infection with dengue virus. CXCR3-mediated effector CD8 T cell recruitment to sites of dengue virus infection correlated with lower viral loads and a lower frequency of paralysis and death [53]. Similarly, during subcutaneous infections of *Leishmania major*, CXCR3 expression increased CD4 and CD8 T cell infiltration into lesions and correlated with decreased parasite loads at eight weeks post-infection [54]. Interestingly, recruitment of IFN-γ-expressing T cells to inflamed tissues may increase CXCR3 ligand expression, resulting in further recruitment of T cells to that tissue [55-58]. This can be seen during HSV-2 infection, where effector CD8 T cell migration to the vaginal site of infection depended on CXCR3 ligand induction by IFN-γ-expressing CD4 T cells at the site of infection [59]. The amplification of signals for T cell recruitment could lead to aggravation of a disease or to protection during disease depending on the T cells recruited. In graft-versus-host disease, CXCR3 recruited effector T cells into CXCR3 ligand-expressing allografts, leading to increased allograft rejection [60,61]. However, in experimental autoimmune encephalomyelitis (EAE), CXCR3 expression reduced EAE severity during its chronic phase possibly by recruitment of $Foxp3^+$ regulatory T cells ($T_{reg}$) to the central nervous system (CNS) [62]. These studies demonstrate the variable outcomes of CXCR3-mediated T cell recruitment to inflamed peripheral tissues. Although most studies focused on T cell recruitment to peripheral tissues, one study suggested that CXCR3 may guide T cells within lymph nodes. In tonsils and lymph nodes of HIV-infected patients, CXCL9 expression around germinal centers was

associated with the localization of CXCR3-expressing CD4 T cells around and within these germinal centers [63]. Overall, these studies show that inflammatory chemokine receptors CCR5, CXCR6, and CXCR3 are important for the trafficking of effector cells to sites where they perform effector functions.

Importantly, chemokines also influence T cell differentiation. CD8 T cell differentiation into functional memory cells requires CD4 T cell help [64-67]. CD4 T cells can help CD8 T cells by licensing DCs to give CD8 T cells the proper signals to form memory cells [67]. In inflamed lymph nodes, CCR5-expressing CD8 T cells were attracted to the CCR5 ligands CCL3 and CCL4 that were expressed by licensed DCs [68]. Thus, CCR5 promoted contacts between CD8 T cells and licensed DCs, which promoted the generation of functional memory cells. Moreover, chemokines influence CD4 T cell differentiation into T helper cell subsets. CD4 T cell differentiation into helper cell subsets is influenced by the cytokine microenvironment during activation [69]. For example, IL-12 promotes differentiation into T helper type 1 (Th1) cells [70], IL-4 and IL-13 promote differentiation into T helper type 2 (Th2) cells [71-74], and IL-6 and IL-21 promote differentiation into follicular T helper ($T_{FH)}$ cells [75-77]. Chemokines can promote the expression of cytokines that influence CD4 T cell differentiation [78]. For example, expression of CCL3 and CCL4 after *Toxoplasma gondii* infection induced IL-12 expression in DCs in a CCR5-dependent manner, thus creating a microenvironment favoring the differentiation of Th1 cells [79]. In contrast, CCL2 inhibited IL-12 expression in APCs and enhanced IL-4 expression in activated CD4 T cells, thereby promoting the differentiation into Th2 cells [78].

In conclusion, chemokines are important for antigen surveillance and T cell activation, differentiation, and function.

## CD8 T cell differentiation into effector and memory cells

### *The formation of effector and memory CD8 T cells*

The formation of effector and memory CD8 T cells is essential for immune responses to repeated encounters with intracellular pathogens. During an acute infection, pathogen-specific CD8 T cells are activated and expand to form a heterogeneous population of effector CD8 T cells. These effector cells secrete inflammatory cytokines and kill infected cells, leading to the clearance of the pathogen by the peak of CD8 T cell expansion. After the peak of expansion, a majority of effector CD8 T cells die during contraction. However, a small percentage of these effector cells survive contraction and form long-lived, self-renewing memory cells that respond efficiently to reencounters with the same or a similar pathogen. During chronic infections, CD8 T cells display similar kinetics of expansion and contraction. However, due to repeated stimulation by antigen, CD8 T cells become exhausted and form non-classical memory cells that depend on antigen stimulation to survive [80].

### *Effector and memory cell heterogeneity*

An important factor to consider when forming models on CD8 T cell differentiation is the heterogeneity of effector and memory cells. Models must sufficiently explain how these heterogeneous pools are achieved. Effector cell

populations are phenotypically and functionally heterogeneous in their proliferative capacity and their expression of various receptors and cytokines [81]. Memory cells are also heterogeneous and have been grouped into subsets that differ in their potential to migrate to different tissues and in their potential to secrete cytokines and expand during secondary infection [82-85]. Two broad subsets of memory cells have been characterized and are called effector memory ($T_{EM}$) and central memory cells ($T_{CM}$) [86]. $T_{EM}$ express low levels of CD62L (L-selectin) and the chemokine receptor CCR7 whereas $T_{CM}$ express high levels of both receptors. CD62L is a cell adhesion molecule that mediates lymphocyte rolling on high endothelial venules (HEV), which is essential for entry into peripheral lymph nodes. Subsequent binding of CCR7 on lymphocytes to CCL21 on HEV initiates integrin binding, firm arrest, and transmigration across the HEV into the lymph node [87]. Due to the roles of CD62L and CCR7 in entry into peripheral lymph nodes, it was proposed that $CD62L^{+}CCR7^{+}$ $T_{CM}$ were enriched in peripheral lymph nodes while $CD62L^{-}CCR7^{-}$ $T_{EM}$ were enriched in peripheral tissues. In response to polyclonal stimulation of human blood lymphocytes, $T_{EM}$ expressed more effector molecules including perforin and interferon-$\gamma$ (IFN-$\gamma$) whereas $T_{CM}$ expressed slightly more interleukin-2 (IL-2) [82]. Due to the distinct homing capabilities and effector functions of these memory cell subsets, a hypothesis was formed about the role of $T_{EM}$ and $T_{CM}$. During recall responses, $T_{EM}$ would be located in peripheral tissues to provide the first line of defense while $T_{CM}$ would be located in lymph nodes where they would expand to form a second pool of effector cells.

However, subsequent examination of memory subsets showed that the characterization of these subsets proved to be more complex. Differences in immediate effector function in $T_{EM}$ and $T_{CM}$ aligned more with the location of the memory cells than with the expression of markers identifying $T_{EM}$ and $T_{CM}$. Specifically, it was shown that both $CD62L^{lo}$ $T_{EM}$ and $CD62L^{hi}$ $T_{CM}$ acquired traits of $T_{EM}$ when transferred from the spleen into the liver and lung [88]. Moreover, after stimulation with cognate peptide, $T_{EM}$ and $T_{CM}$ from the spleen both rapidly expressed similar amounts of effector cytokines such as IFN-$\gamma$ and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) [83,89,90]. However, splenic $T_{CM}$ still expressed more IL-2 than splenic $T_{EM}$ and had a greater proliferative capacity as originally hypothesized [83]. Finally, it was shown that T cells that were engineered to constitutively express CD62L still formed similar memory cell subsets compared to wild-type cells as analyzed by expression of memory markers and by homeostatic turnover [91]. These data suggest that expression of CD62L does not determine characteristics of $T_{EM}$ and $T_{CM}$. Instead, the incorporation of signals through TCR stimulation, which affects CD62L expression [92], and other factors such as the tissue environment may influence characteristics of $T_{EM}$ and $T_{CM}$. Understanding the heterogeneity and qualities of effector and memory cell subsets is important in the generation of models of T cell differentiation.

*Signals that influence CD8 T cell differentiation*

Three important signals of T cell activation are antigenic stimulation, costimulation, and inflammation. The strength of antigenic and inflammatory

stimulation contributes to differences in the CD8 T cell response. By separating antigenic and inflammatory signals during infection, it has been shown that these signals play distinct roles in CD8 T cell differentiation. One study used a recombinant vaccinia virus that was engineered to alter the amounts of ovalbumin-specific peptide presentation with the same dose of virus [93]. Using this system, it was shown that increasing antigenic stimulation in the presence of the same amount of inflammation increased the magnitude of the CD8 T cell response [93,94]. However, it did not noticeably alter the differentiation of CD8 T cells into terminally differentiated effectors versus memory precursor cells [94]. On the other hand, increasing the amount of inflammation in the presence of the same amount of antigen promoted CD8 T cell differentiation into terminally differentiated effectors [94]. To a similar effect, decreasing amounts of inflammation accelerated the generation of functional memory cells [95]. To examine the impact of varying amounts of antigen and inflammation on T cell differentiation, one may also increase or decrease competition for these signals by varying T cell precursor frequencies. Increasing the number of antigen-specific precursor T cells decreased the amount of available signals per T cell, which resulted in the generation of more memory precursor-like cells [96]. Moreover, increasing the number of antigen-specific precursor T cells promoted the generation of $T_{CM}$ over $T_{EM}$ cells [97]. Overall, decreasing the strength of antigen and inflammation increases the generation of memory precursors over terminal effectors and increases the generation of $T_{CM}$ over $T_{EM}$.

The duration of antigenic and inflammatory stimulation also influences CD8 T cell differentiation. One day of *in vitro* stimulation with antigen is sufficient to elicit CD8 T cell division at least seven times over the course of several days into functional effector cells [98-101]. However, using antibiotics to curb antigenic and inflammatory stimulation *in vivo* at one day post-infection with *Listeria monocytogenes* led to the formation of fewer memory cells [98]. This may be due to an insufficient amount of other signals necessary for memory cell formation, including CD4 T cell help [64]. Although these studies showed that CD8 effector T cells can form after one day of antigenic and inflammatory stimulation, they do not address whether prolonged antigenic and inflammatory stimulation later during infection influences differentiation into terminal effectors, memory cells, and memory cell subsets. One study demonstrated that curbing antigen and inflammation during later infection decreased the generation of terminally differentiated effectors and increased the generation of memory precursors and central memory cells [102].

In addition, the transcription factors T-bet, Blimp-1, and Eomesodermin are important for effector and memory CD8 T cell programming. Antigen and inflammatory factors induce T-bet, which is required for the generation of terminally differentiated effector cells as identified by the marker killer cell lectin-like receptor subfamily G member 1 (KLRG1) [94]. Increased expression of T-bet promotes the generation of KLRG1$^{hi}$ terminal effectors. Yet, some expression of T-bet is important for the formation of memory cells as it has been shown to regulate the expression memory precursor cell-specific genes [103,94].

Another transcription factor, Blimp-1, is a transcriptional repressor previously shown to be required for the differentiation of B cells into plasma cells [104,105]. In T cells, expression of Blimp-1 promoted CD8 T cell differentiation into KLRG1$^{hi}$ terminal effectors while deletion of Blimp-1 led to the formation of more memory cells and central memory cells [106,107]. Similarly, a paralogue of T-bet, Eomesodermin, plays a role in effector CD8 T cell differentiation and function [108]. T-bet and eomesodermin together are important for the expression of the effector cytokine IFN-$\gamma$ and the $\beta$ chain of the IL-15 receptor and IL-2 receptor, CD122, which is necessary for memory CD8 T cell turnover [109]. Therefore, T-bet, Blimp-1, and Eosmesodermin are important for the generation of effector cells while also affecting memory cell generation and maintenance.

As well, the cytokines IL-2, IL-7, and IL-15 play roles in CD8 T cell proliferation and survival and memory cell turnover [110]. Although IL-2 is required for *in vitro* expansion of T cells, CD8 T cells expand well into effector cells *in vivo* in IL-2- and IL-2 receptor-deficient mice [110,111]. Although not required for effector cell expansion, IL-2 expression during priming was important to generate memory cells that expanded fully during secondary infection [112]. Another cytokine, IL-7, has been shown to play roles in the survival of naïve CD8 T cells and the maintenance of memory CD8 T cells [113]. The IL-7 receptor (IL-7R) is expressed on naïve T cells, downregulated on activated T cells, and then upregulated again on CD8 T cells that have a greater potential to form memory cells [114]. However, overexpression of the IL-7R on effector CD8 T cells did not promote effector cell survival and the formation of memory cells [115], which

suggested that IL-7 did not direct memory CD8 T cell differentiation. Interestingly, TGF-β accelerated the apoptosis of KLRG1$^{hi}$ terminally differentiated cells that did not express the IL-7 receptor [116]. This implied that KLRG1$^{hi}$ effector cells that expressed the IL-7R are more resistant to TGF-β-mediated apoptosis. However, it has not been formally shown that IL-7R expression mediates the survival of effector cells. Another important cytokine that maintains memory cell homeostasis is IL-15 [117]. IL-15 is important for effector cell survival during contraction [118,116] as well as memory CD8 T cell turnover and homeostatic proliferation [119-121]. Thus, IL-2, IL-7, and IL-15 play important and distinct roles in the T cell response to and primary and successive encounters of a pathogen.

*Models for the generation of effector and memory CD8 T cells*

Signals that direct CD8 T cell differentiation into effector cells versus memory cells have been vigorously examined. However, questions still remain as to when CD8 T cells commit to one lineage or the other, and whether this decision is fixed or plastic. To better understand the models that have been proposed to depict CD8 T cell differentiation, it is important to define and characterize differences between short-lived effector cells and memory cells. While short-lived effector cells are terminally differentiated and eventually die, memory cells are long-lived, undergo homeostatic proliferation, and respond more efficiently than naïve cells to reencounters of the same pathogen.

Several models have been proposed to characterize CD8 T cell differentiation into effector and memory cells. Three models are prominent today and have evolved primarily from advances in the last decade. Previously, it was not known if effector and memory cells were derived from separate naïve T cell precursors [122]. Elegant studies following the differentiation of a single naïve CD8 T cell, either through single-cell transfer [123] or genetic tagging [124], demonstrated that a heterogeneous population of effector cells could differentiate from a single cell. The following models were generated to depict the differentiation of a naïve T cell into a heterogeneous population of effector and memory cells: 1) Fixed lineage model 2) Decreasing potential model and 3) Fate commitment with progressive differentiation model [103] (Figure 2).

The fixed lineage model states that naïve CD8 T cells differentiate directly into effector cells or directly into memory cells. In this model, naïve T cells bifurcate into two distinct and fixed cell lineages shortly after activation [125]. In support of this model, it was found that lymphocytes can asymmetrically divide into two daughter cells that differentially displayed phenotypic and functional qualities of an effector cell and a memory cell [126]. This data could support a model where naïve T cells differentiate into either effector cells or memory cells, which do not enter an effector cell stage. However, this study did not examine successive rounds of proliferation typical of *in vivo* infections to determine if these lineages were indeed fixed.

An alternative model is the decreasing potential model, which states that the potential of a CD8 T cell to form into a memory cell decreases with

increasing antigenic stimulation [122]. In this model, all activated CD8 T cells differentiate into effector cells. However, cells that receive less stimulation have a greater potential to recover and differentiate into memory cells while cells that receive more stimulation become terminally differentiated and die. In support of this model, lineage tracing demonstrated that effector cells expressing IFNγ formed memory cells after infection that expanded well during secondary infection [127]. This demonstrated that memory cells entered an effector cell stage before differentiating into memory cells. Additionally, several studies support the idea that increased stimulation increases the likelihood of cell death. For example, it was shown that inhibiting mTOR, which is activated downstream of TCR and cytokine signaling, improved the conversion of effector cells to memory cells [128,129]. This suggested that dampening TCR and cytokine signaling increased a cell's potential to form into a memory cell. Furthermore, increasing competition for stimulation promotes the generation of memory cells. During an immune response, antigen-specific T cells enter inflamed lymph nodes at different times during infection. Naïve T cells that enter a lymph node after the initiation of a T cell response compete with already expanding T cells for stimulation by DCs [130]. As a result, these "late-comers" experience less stimulation and form more central memory T cells that expand better during a recall response [130]. In similar studies, others have transferred different T cell precursor frequencies to demonstrate that increased competition for stimulation resulted in the formation of more central memory cells [131,96,97].

A decade later, the fate commitment and progressive differentiation model [103] was formed as an extension of the decreasing potential model with changes that accommodated new findings in T-cell differentiation. It states that precursors of terminal effector cells and memory cells can be identified during infection but that increasing stimulation can drive differentiation of memory precursors into terminally differentiated cells. In support of this model, terminally differentiated effector cells and memory precursor cells could be identified by expression of KLRG1 and IL-7 receptor. Furthermore, increased stimulation led to the increased formation of terminal effectors [94]. The fate commitment and progressive differentiation model further divides memory cells into effector memory and central memory subsets [82] and states that effector memory cells are transitional memory cells that eventually convert to central memory cells over time. The idea of effector memory cells as a transitional memory cell subset is based on the finding that effector memory cells that were transferred into new recipients converted to central memory cells over time [83]. However, it was later shown that low precursor frequencies of T cells could lead to the generation of a stable population of effector memory cells that did not convert into central memory cells [97]. Therefore, this model may need a further adjustment to include a stable lineage of effector memory cells.

Overall, these models are representative of our current knowledge of T cell differentiation and will likely evolve with an improved understanding of T cell differentiation over time.

**Lymphocytic choriomeningitis virus (LCMV) infection**

Lymphocytic choriomeningitis virus is a non-cytolytic arenavirus, which is an enveloped virus with a nucleocapsid containing two single-stranded RNA. The two single-stranded RNA of LCMV are ambisense and the large (L) strand encodes the polymerase and z protein while the small (S) strand encodes the nucleocapsid protein and two surface glycoproteins. There are several genetic variants of LCMV that differ in their virulence [132]. Two commonly used strains are the acute Armstrong strain, which is cleared within eight to ten days post-infection, and the chronic Clone-13 strain, which persists for several months [133] (Figure 3). Differences in these two strains include five point mutations, two of which result in coding changes: the first mutation changes a lysine to a glutamine in the polymerase and the second mutation changes a phenylalanine to a leucine in the glycoprotein [134-136]. The polymerase mutation in Clone-13 increases viral replication in macrophages and the mutation in the glycoprotein increases the number of macrophages infected [137]. Furthermore, the mutation in the glycoprotein in Clone-13 results in higher binding affinity to $\alpha$-dystroglycan, the receptor for the virus [138], which is preferentially expressed on DCs. This leads to improved virus binding and infection of DCs, suppression of the effector CD8 T cell response, and viral persistence [139,140]. The acute and chronic strains of LCMV also vary in the infection of different areas of the spleen [141]. At one day post-infection, Armstrong and Clone-13 infection is focused in marginal zones of the spleen. However, at three days post-infection, Armstrong infection is contained in marginal zone areas whereas Clone-13 spreads to the T

zones where the virus infects fibroblastic reticular cells (FRCs). This infection of FRCs contributes to disruption of the splenic architecture and FRC conduit network [142]. By 8 days post-infection, Armstrong infection is cleared in the spleen whereas Clone-13 infection persists in the white pulp and red pulp [141,142]. Additionally, lymphocytes play different roles in acute versus chronic infections with LCMV. CD8 T cells are critical for the clearance of both strains [143]. Although CD4 T cells are not required to generate the CTL response or to clear acute infection, CD4 T cells are critical for the eventual clearance of the chronic strain [143]. Similarly, B cells are not required for viral clearance during acute infection but are important for limiting virus dissemination and resolving infection with the chronic strain [144].

**Thesis work**

The aim of this thesis work was to understand how inflammatory chemokine receptor expression influences CD8 T cell differentiation into effector and memory cells. T cell differentiation is influenced by access to antigenic and inflammatory stimuli as well as access to survival factors. Importantly, chemokine receptor expression can influence access to stimuli by promoting movement to different microenvironments. Previously, it had been shown that activated CD8 T cells moved to different compartments of the spleen during the course of the infection [145]. Activated CD8 T cells formed clusters near B cell follicles and in the marginal zone before moving through bridging channels into the red pulp, where they exited from the spleen [145]. Yet, the molecular signals

involved in CD8 T cell movement to different areas of the spleen and the resulting effect on CD8 T cell differentiation was not explored.

Activated CD8 T cells upregulate expression of inflammatory chemokine receptor CXCR3, which directs them to areas that express the interferon-inducible ligands CXCL9, CXCL10, and CXCL11. A previous study demonstrated that CXCL9 was expressed in particular compartments of a chronically inflamed lymph node [63]. This suggested a potential role for CXCR3 in directing activated T cells to different microenvironments of the SLO during T cell expansion and differentiation.

Using acute LCMV ($LCMV_{Arm}$) as an infection model, we found that CXCL9 was expressed around follicles and in marginal zones of inflamed spleens at 3-5 dpi. Activated CD8 T cells upregulated CXCR3 by 3 dpi and formed clusters around B cell follicles and in marginal zones at 3-5 dpi. By cotransferring wild-type (WT) and CXCR3 knock-out (KO) LCMV-specific, TCR-transgenic P14 CD8 T cells into mice and infecting them, we found that CD8 T cells were directed to these areas in a CXCR3-dependent manner. Moreover, immunofluorescent staining revealed that LCMV antigen was abundant in the marginal zone during this time. Cells in the marginal zone include marginal zone macrophages and marginal zone metallophilic macrophages. These cells contained LCMV RNA and were likely infected with virus. Therefore, activated CXCR3-expressing CD8 T cells that moved to the marginal zone also gained access to infected cells with the potential to present viral peptide:MHC and

secrete inflammatory cytokines. In contrast, activated CXCR3 KO CD8 T cells were concentrated in the T zone away from the antigen-abundant marginal zone.

CXCR3-dependent access to antigenic and inflammatory stimuli was associated with the formation of more short-lived effector cells. However, both WT and CXCR3 KO cells proliferated similarly at 2.5 dpi and expressed similar levels of the effector molecules IFN-$\gamma$, TNF-$\alpha$, and Granzyme B at 8 dpi. This showed that CXCR3 was not required for expansion into functional effector cells. When we followed WT and CXCR3 KO cells into memory phases, we found that CXCR3 KO CD8 T cells generated 2-4-fold more memory cells. Moreover, these CXCR3 KO memory CD8 T cells were phenotypically different and contained greater proportions of central memory cells. Furthermore, CXCR3 KO memory CD8 T cells proliferated more than WT memory CD8 T cells during secondary infection. Although not required for the formation of effector or memory cells, the inflammatory chemokine receptor CXCR3 influenced CD8 T cell differentiation into effector cells at the expense of memory cells and affected the phenotype and quality of memory cells that were formed.

## References


1. Blattman JN (2002) Estimating the Precursor Frequency of Naive Antigen-specific CD8 T Cells. *Journal of Experimental Medicine* 195: 657-664.
2. Cesta MF (2006) Normal structure, function, and histology of the spleen. *Toxicologic pathology* 34: 455-65.
3. Cyster JG (2005) Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annual review of immunology* 23: 127-59.
4. Nolte M a, Beliën J a M, Schadee-Eestermans I, Jansen W, Unger WWJ, et al. (2003) A conduit system distributes chemokines and small blood-borne molecules through the splenic white pulp. *The Journal of experimental medicine* 198: 505-12.
5. Sainte-Marie G, Peng FS (1986) Diffusion of a lymph-carried antigen in the fiber network of the lymph node of the rat. *Cell and tissue research* 245: 481-6.
6. Jones C, Virji M, Crocker PR (2003) Recognition of sialylated meningococcal lipopolysaccharide by siglecs expressed on myeloid cells leads to enhanced bacterial uptake. *Molecular Microbiology* 49: 1213-1225.
7. Mebius RE, Kraal G (2005) Structure and function of the spleen. *Nature reviews. Immunology* 5: 606-16.
8. Lopes-Carvalho T, Kearney JF (2004) Development and selection of marginal zone B cells. *Immunological reviews* 197: 192-205.
9. Cinamon G, Zachariah M a, Lam OM, Foss FW, Cyster JG (2008) Follicular shuttling of marginal zone B cells facilitates antigen transport. *Nature immunology* 9: 54-62.
10. Song H, Cerny J (2003) Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen. *The Journal of experimental medicine* 198: 1923-35.
11. Attanavanich K, Kearney JF (2004) Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells. *Journal of immunology (Baltimore, Md. : 1950)* 172: 803-11.
12. Yu P, Wang Y, Chin RK, Martinez-Pomares L, Gordon S, et al. (2002) B cells control the migration of a subset of dendritic cells into B cell follicles via CXC chemokine ligand 13 in a lymphotoxin-dependent fashion. *Journal of immunology (Baltimore, Md. : 1950)* 168: 5117-23.
13. Martínez-Pomares L, Kosco-Vilbois M, Darley E, Tree P, Herren S, et al. (1996) Fc chimeric protein containing the cysteine-rich domain of the murine mannose receptor binds to macrophages from splenic marginal zone and lymph node subcapsular sinus and to germinal centers. *The Journal of experimental medicine* 184: 1927-37.
14. Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, et al. (2004) Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* 432: 917–921.

15. Phan TG, Grigorova I, Okada T, Cyster JG (2007) Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nature immunology* 8: 992-1000.
16. Cyster JG (1999) Chemokines and the Homing of Dendritic Cells to the T Cell Areas of Lymphoid Organs. *Journal of Experimental Medicine* 189: 447-450.
17. Murphy PM, Baggiolini M, Charo IF, Hébert C a, Horuk R, et al. (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacological reviews* 52: 145-76.
18. Rossi D, Zlotnik a (2000) The biology of chemokines and their receptors. *Annual review of immunology* 18: 217-42.
19. Moser B, Loetscher P (2001) Lymphocyte traffic control by chemokines. *Nature immunology* 2: 123-8.
20. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, et al. (2004) Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* 427: 355-60.
21. Schwab SR, Cyster JG (2007) Finding a way out: lymphocyte egress from lymphoid organs. *Nature immunology* 8: 1295-301.
22. Bajénoff M, Glaichenhaus N, Germain RN (2008) Fibroblastic reticular cells guide T lymphocyte entry into and migration within the splenic T cell zone. *Journal of immunology (Baltimore, Md. : 1950)* 181: 3947-54.
23. Cyster JG, Goodnow CC (1995) Pertussis toxin inhibits migration of B and T lymphocytes into splenic white pulp cords. *The Journal of experimental medicine* 182: 581.
24. Lo CG, Lu TT, Cyster JG (2003) Integrin-dependence of Lymphocyte Entry into the Splenic White Pulp. *Journal of Experimental Medicine* 197: 353-361.
25. Yoshida R, Imai T, Hieshima K, Kusuda J, Baba M, et al. (1997) Molecular cloning of a novel human CC chemokine EBI1-ligand chemokine that is a specific functional ligand for EBI1, CCR7. *The Journal of biological chemistry* 272: 13803-9.
26. Hedrick J a, Zlotnik a (1997) Identification and characterization of a novel beta chemokine containing six conserved cysteines. *Journal of immunology (Baltimore, Md. : 1950)* 159: 1589-93.
27. Gunn MD, Tangemann K, Tam C, Cyster JG, Rosen SD, et al. (1998) A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America* 95: 258-63.
28. Luther S a, Tang HL, Hyman PL, Farr a G, Cyster JG (2000) Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. *Proceedings of the National Academy of Sciences of the United States of America* 97: 12694-9.
29. Schwab SR, Pereira JP, Matloubian M, Xu Y, Huang Y, et al. (2005) Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science (New York, N.Y.)* 309: 1735-9.

30. Sallusto F, Palermo B, Lenig D, Miettinen M, Matikainen S, et al. (1999) Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *European journal of immunology* 29: 1617-25.
31. Sallusto F, Schaerli P, Loetscher P, Schaniel C, Lenig D, et al. (1998) Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *European journal of immunology* 28: 2760-9.
32. Sozzani S, Allavena P, D'Amico G, Luini W, Bianchi G, et al. (1998) Cutting edge: differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. *The Journal of Immunology* 161: 1083.
33. Yanagihara S, Komura E, Nagafune J, Watarai H, Yamaguchi Y (1998) EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation. *Journal of immunology (Baltimore, Md. : 1950)* 161: 3096-102.
34. Saeki H, Moore AM, Brown MJ, Hwang ST (1999) Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *The Journal of Immunology* 162: 2472.
35. Adema GJ, Hartgers F, Verstraten R, Vries E de, Marland G, et al. (1997) A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. *Nature* 387: 713-7.
36. Lieberam I, Förster I (1999) The murine beta-chemokine TARC is expressed by subsets of dendritic cells and attracts primed CD4+ T cells. *European journal of immunology* 29: 2684-94.
37. Tang HL (1999) Chemokine Up-Regulation and Activated T Cell Attraction by Maturing Dendritic Cells. *Science* 284: 819-822.
38. Randolph D a (1999) The Role of CCR7 in TH1 and TH2 Cell Localization and Delivery of B Cell Help in Vivo. *Science* 286: 2159-2162.
39. Ansel KM (1999) In Vivo-activated CD4 T Cells Upregulate CXC Chemokine Receptor 5 and Reprogram Their Response to Lymphoid Chemokines. *Journal of Experimental Medicine* 190: 1123-1134.
40. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, et al. (2000) Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *The Journal of experimental medicine* 192: 1545-52.
41. Reinhardt RL, Liang H-E, Locksley RM (2009) Cytokine-secreting follicular T cells shape the antibody repertoire. *Nature immunology* 10: 385-93.
42. Glass WG, Lim JK, Cholera R, Pletnev AG, Gao J-L, et al. (2005) Chemokine receptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection. *The Journal of experimental medicine* 202: 1087-98.
43. Kohlmeier JE, Miller SC, Smith J, Lu B, Gerard C, et al. (2008) The chemokine receptor CCR5 plays a key role in the early memory CD8+ T cell response to respiratory virus infections. *Immunity* 29: 101-13.
44. Kim CH, Kunkel EJ, Boisvert J, Johnston B, Campbell JJ, et al. (2001) Bonzo/CXCR6 expression defines type 1-polarized T-cell subsets with

extralymphoid tissue homing potential. *The Journal of clinical investigation* 107: 595-601.
45. Nanki T, Shimaoka T, Hayashida K, Taniguchi K, Yonehara S, et al. (2005) Pathogenic role of the CXCL16-CXCR6 pathway in rheumatoid arthritis. *Arthritis and rheumatism* 52: 3004-14.
46. Kim JV, Jiang N, Tadokoro CE, Liu L, Ransohoff RM, et al. (2010) Two-photon laser scanning microscopy imaging of intact spinal cord and cerebral cortex reveals requirement for CXCR6 and neuroinflammation in immune cell infiltration of cortical injury sites. *Journal of immunological methods* 352: 89-100.
47. Sato T, Thorlacius H, Johnston B, Staton TL, Xiang W, et al. (2005) Role for CXCR6 in recruitment of activated CD8+ lymphocytes to inflamed liver. *Journal of immunology (Baltimore, Md. : 1950)* 174: 277-83.
48. Farber J (1997) Mig and IP-10: CXC chemokines that target lymphocytes. *J. Leukoc. Biol.* 61: 246-257.
49. Luster AD (1987) Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). *Journal of Experimental Medicine* 166: 1084-1097.
50. Cole KE (1998) Interferon-inducible T Cell Alpha Chemoattractant (I-TAC): A Novel Non-ELR CXC Chemokine with Potent Activity on Activated T Cells through Selective High Affinity Binding to CXCR3. *Journal of Experimental Medicine* 187: 2009-2021.
51. Allan JE, Dixon JE, Doherty PC (1987) Nature of the inflammatory process in the central nervous system of mice infected with lymphocytic choriomeningitis virus. *Current topics in microbiology and immunology* 134: 131-43.
52. Christensen JE, Nansen A, Moos T, Lu B, Gerard C, et al. (2004) Efficient T-cell surveillance of the CNS requires expression of the CXC chemokine receptor 3. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 24: 4849-58.
53. Hsieh M-F, Lai S-L, Chen J-P, Sung J-M, Lin Y-L, et al. (2006) Both CXCR3 and CXCL10/IFN-Inducible Protein 10 Are Required for Resistance to Primary Infection by Dengue Virus. *J. Immunol.* 177: 1855-1863.
54. Rosas LE, Barbi J, Lu B, Fujiwara Y, Gerard C, et al. (2005) CXCR3-/- mice mount an efficient Th1 response but fail to control Leishmania major infection. *European journal of immunology* 35: 515-23.
55. Lacotte S, Brun S, Muller S, Dumortier H (2009) CXCR3, inflammation, and autoimmune diseases. *Annals of the New York Academy of Sciences* 1173: 310-7.
56. Reinhart T a (2002) Increased expression of the inflammatory chemokine CXC chemokine ligand 9/monokine induced by interferon-gamma in lymphoid tissues of rhesus macaques during simian immunodeficiency virus infection and acquired immunodeficiency syndrome. *Blood* 99: 3119-3128.
57. Chuang Y-H, Lian Z-X, Cheng C-M, Lan RY, Yang G-X, et al. (2005) Increased levels of chemokine receptor CXCR3 and chemokines IP-10 and

MIG in patients with primary biliary cirrhosis and their first degree relatives. *Journal of autoimmunity* 25: 126-32.
58. Groom JR, Luster AD (2011) CXCR3 in T cell function. *Experimental cell research* 317: 620-31.
59. Nakanishi Y, Lu B, Gerard C, Iwasaki A (2009) CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. *Nature* 462: 510-3.
60. Hancock WW, Lu B, Gao W, Csizmadia V, Faia K, et al. (2000) Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *The Journal of experimental medicine* 192: 1515-20.
61. Uppaluri R, Sheehan KCF, Wang L, Bui JD, Brotman JJ, et al. (2008) Prolongation of cardiac and islet allograft survival by a blocking hamster anti-mouse CXCR3 monoclonal antibody. *Transplantation* 86: 137-47.
62. Muller M, Carter SL, Hofer MJ, Manders P, Getts DR, et al. (2007) CXCR3 signaling reduces the severity of experimental autoimmune encephalomyelitis by controlling the parenchymal distribution of effector and regulatory T cells in the central nervous system. *The Journal of Immunology* 179: 2774.
63. Rabin RL, Alston MA, Sircus JC, Knollmann-Ritschel B, Moratz C, et al. (2003) CXCR3 Is Induced Early on the Pathway of CD4+ T Cell Differentiation and Bridges Central and Peripheral Functions. *J. Immunol.* 171: 2812-2824.
64. Sun JC, Bevan MJ (2003) Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science (New York, N.Y.)* 300: 339-42.
65. Shedlock DJ, Shen H (2003) Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science (New York, N.Y.)* 300: 337-9.
66. Janssen EM, Lemmens EE, Wolfe T, Christen U, Herrath MG von, et al. (2003) CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 421: 852-6.
67. Bevan MJ (2004) Helping the CD8(+) T-cell response. *Nature reviews. Immunology* 4: 595-602.
68. Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, et al. (2006) Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature* 440: 890-5.
69. O'Garra a (1998) Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 8: 275-83.
70. Hsieh C, Macatonia S, Tripp C, Wolf S, O'Garra A, et al. (1993) Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science* 260: 547-549.
71. Gros G Le, Ben-Sasson SZ, Seder R, Finkelman F, Paul W (1990) Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *The Journal of experimental medicine* 172: 921.
72. Swain SL, Weinberg a D, English M, Huston G (1990) IL-4 directs the development of Th2-like helper effectors. *Journal of immunology (Baltimore, Md. : 1950)* 145: 3796-806.

73. Kaplan MH, Schindler U, Smiley ST, Grusby MJ (1996) Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 4: 313-9.
74. McKenzie GJ, Emson CL, Bell SE, Anderson S, Fallon P, et al. (1998) Impaired development of Th2 cells in IL-13-deficient mice. *Immunity* 9: 423-32.
75. Diehl S, Rincón M (2002) The two faces of IL-6 on Th1/Th2 differentiation. *Molecular immunology* 39: 531-6.
76. Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, et al. (2008) Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29: 138-49.
77. Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, et al. (2008) A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* 29: 127-37.
78. Luther S a, Cyster JG (2001) Chemokines as regulators of T cell differentiation. *Nature immunology* 2: 102-7.
79. Aliberti J, Reis e Sousa C, Schito M, Hieny S, Wells T, et al. (2000) CCR5 provides a signal for microbial induced production of IL-12 by CD8 alpha+ dendritic cells. *Nature immunology* 1: 83-7.
80. Wherry EJ, Barber DL, Kaech SM, Blattman JN, Ahmed R (2004) Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proceedings of the National Academy of Sciences of the United States of America* 101: 16004-9.
81. Peixoto A, Evaristo C, Munitic I, Monteiro M, Charbit A, et al. (2007) CD8 single-cell gene coexpression reveals three different effector types present at distinct phases of the immune response. *The Journal of experimental medicine* 204: 1193-205.
82. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708-12.
83. Wherry EJ, Teichgräber V, Becker TC, Masopust D, Kaech SM, et al. (2003) Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nature immunology* 4: 225-34.
84. Masopust D, Vezys V, Marzo AL, Lefrançois L (2001) Preferential localization of effector memory cells in nonlymphoid tissue. *Science (New York, N.Y.)* 291: 2413-7.
85. Hikono H, Kohlmeier JE, Takamura S, Wittmer ST, Roberts AD, et al. (2007) Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. *The Journal of experimental medicine* 204: 1625-36.
86. Sallusto F, Geginat J, Lanzavecchia A (2004) Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annual review of immunology* 22: 745-63.
87. Rosen SD (2004) Ligands for L-selectin: homing, inflammation, and beyond. *Annual review of immunology* 22: 129-56.

88. Marzo AL, Yagita H, Lefrançois L (2007) Cutting edge: migration to nonlymphoid tissues results in functional conversion of central to effector memory CD8 T cells. *Journal of immunology (Baltimore, Md. : 1950)* 179: 36-40.
89. Unsoeld H, Krautwald S, Voehringer D, Kunzendorf U, Pircher H (2002) Cutting edge: CCR7+ and CCR7- memory T cells do not differ in immediate effector cell function. *Journal of immunology (Baltimore, Md. : 1950)* 169: 638-41.
90. Ravkov EV, Myrick CM, Altman JD (2003) Immediate early effector functions of virus-specific CD8+CCR7+ memory cells in humans defined by HLA and CC chemokine ligand 19 tetramers. *Journal of immunology (Baltimore, Md. : 1950)* 170: 2461-8.
91. Wirth TC, Badovinac VP, Zhao L, Dailey MO, Harty JT (2009) Differentiation of central memory CD8 T cells is independent of CD62L-mediated trafficking to lymph nodes. *Journal of immunology (Baltimore, Md. : 1950)* 182: 6195-206.
92. Chao CC, Jensen R, Dailey MO (1997) Mechanisms of L-selectin regulation by activated T cells. *The Journal of Immunology* 159: 1686.
93. Wherry EJ, Puorro K a, Porgador a, Eisenlohr LC (1999) The induction of virus-specific CTL as a function of increasing epitope expression: responses rise steadily until excessively high levels of epitope are attained. *Journal of immunology (Baltimore, Md. : 1950)* 163: 3735-45.
94. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, et al. (2007) Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27: 281-95.
95. Badovinac VPVP, Harty JTJT (2007) Manipulating the rate of memory CD8 T cell generation after acute infection. *The Journal of Immunology* 179: 53.
96. Badovinac VP, Haring JS, Harty JT (2007) Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity* 26: 827-41.
97. Marzo AL, Klonowski KD, Bon A Le, Borrow P, Tough DF, et al. (2005) Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nature immunology* 6: 793-9.
98. Williams MA, Bevan MJ (2004) Shortening the Infectious Period Does Not Alter Expansion of CD8 T Cells but Diminishes Their Capacity to Differentiate into Memory Cells. *J. Immunol.* 173: 6694-6702.
99. Stipdonk MJ van, Lemmens EE, Schoenberger SP (2001) Naïve CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nature immunology* 2: 423-9.
100. Kaech SM, Ahmed R (2001) Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. *Nature immunology* 2: 415-22.
101. Wong P, Pamer EG (2001) Cutting Edge: Antigen-Independent CD8 T Cell Proliferation. *J. Immunol.* 166: 5864-5868.

102. Sarkar S, Kalia V, Haining WN, Konieczny BT, Subramaniam S, et al. (2008) Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *The Journal of experimental medicine* 205: 625-40.
103. Kaech SM, Wherry EJ (2007) Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity* 27: 393-405.
104. Turner C a, Mack DH, Davis MM (1994) Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell* 77: 297-306.
105. Shapiro-Shelef M, Lin K-I, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, et al. (2003) Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* 19: 607-20.
106. Rutishauser RL, Martins G a, Kalachikov S, Chandele A, Parish I a, et al. (2009) Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 31: 296-308.
107. Kallies A, Xin A, Belz GT, Nutt SL (2009) Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses. *Immunity* 31: 283-95.
108. Pearce EL, Mullen AC, Martins G a, Krawczyk CM, Hutchins AS, et al. (2003) Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science (New York, N.Y.)* 302: 1041-3.
109. Intlekofer AM, Takemoto N, Wherry EJ, Longworth S a, Northrup JT, et al. (2005) Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nature immunology* 6: 1236-44.
110. Schluns KS, Lefrançois L (2003) Cytokine control of memory T-cell development and survival. *Nature reviews. Immunology* 3: 269-79.
111. Kundig T, Schorle H, Bachmann M, Hengartner H, Zinkernagel R, et al. (1993) Immune responses in interleukin-2-deficient mice. *Science* 262: 1059-1061.
112. Williams M a, Tyznik AJ, Bevan MJ (2006) Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* 441: 890-3.
113. Schluns KS, Kieper WC, Jameson SC, Lefrançois L (2000) Interleukin-7 mediates the homeostasis of naïve and memory CD8 T cells in vivo. *Nature immunology* 1: 426-32.
114. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, et al. (2003) Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nature immunology* 4: 1191-8.
115. Hand TW, Morre M, Kaech SM (2007) Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. *Proceedings of the National Academy of Sciences of the United States of America* 104: 11730-5.

116. Effector S-lived, Cells CDT, Sanjabi S, Mosaheb MM, Flavell RA (2009) Opposing effects of TGF-beta and IL-15 cytokines control the number of short-lived effector CD8+ T cells. *Immunity* 31: 131-44.
117. Surh CD, Sprent J (2008) Homeostasis of naive and memory T cells. *Immunity* 29: 848-62.
118. Yajima T, Yoshihara K, Nakazato K, Kumabe S, Koyasu S, et al. (2006) IL-15 Regulates CD8+ T Cell Contraction during Primary Infection. *J. Immunol.* 176: 507-515.
119. Schluns KS, Williams K, Ma A, Zheng XX, Lefrancois L (2002) Cutting Edge: Requirement for IL-15 in the Generation of Primary and Memory Antigen-Specific CD8 T Cells. *J. Immunol.* 168: 4827-4831.
120. Becker TC, Wherry EJ, Boone D, Murali-Krishna K, Antia R, et al. (2002) Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *The Journal of experimental medicine* 195: 1541-8.
121. Goldrath AW, Sivakumar PV, Glaccum M, Kennedy MK, Bevan MJ, et al. (2002) Cytokine Requirements for Acute and Basal Homeostatic Proliferation of Naive and Memory CD8+ T Cells. *Journal of Experimental Medicine* 195: 1515-1522.
122. Ahmed R, Gray D (1996) Immunological Memory and Protective Immunity: Understanding Their Relation. *Science* 272: 54-60.
123. Stemberger C, Huster KM, Koffler M, Anderl F, Schiemann M, et al. (2007) A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets. *Immunity* 27: 985-97.
124. Gerlach C, Heijst JWJ van, Swart E, Sie D, Armstrong N, et al. (2010) One naive T cell, multiple fates in CD8+ T cell differentiation. *The Journal of experimental medicine* 207: 1235-46.
125. Farber DL (1998) Cutting Edge Commentary: Differential TCR Signaling and the Generation of Memory T Cells. *J. Immunol.* 160: 535-539.
126. Chang JT, Palanivel VR, Kinjyo I, Schambach F, Intlekofer AM, et al. (2007) Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science (New York, N.Y.)* 315: 1687-91.
127. Bannard O, Kraman M, Fearon DT (2009) Secondary replicative function of CD8+ T cells that had developed an effector phenotype. *Science (New York, N.Y.)* 323: 505-9.
128. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, et al. (2009) mTOR regulates memory CD8 T-cell differentiation. *Nature* 460: 108-12.
129. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, et al. (2009) Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* 460: 103-7.
130. Catron DM, Rusch LK, Hataye J, Itano A a, Jenkins MK (2006) CD4+ T cells that enter the draining lymph nodes after antigen injection participate in the primary response and become central-memory cells. *The Journal of experimental medicine* 203: 1045-54.
131. Obar JJ, Khanna KM, Lefrançois L (2008) Endogenous naive CD8+ T cell precursor frequency regulates primary and memory responses to infection. *Immunity* 28: 859-69.

132. Dutko FJ, Oldstone MB (1983) Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. *The Journal of general virology* 64 (Pt 8): 1689-98.
133. Ahmed R, Oldstone MB (1988) Organ-specific selection of viral variants during chronic infection. *The Journal of experimental medicine* 167: 1719-24.
134. Salvato M, Shimomaye E, Southern P, Oldstone MB (1988) Virus-lymphocyte interactions. IV. Molecular characterization of LCMV Armstrong (CTL+) small genomic segment and that of its variant, Clone 13 (CTL-). *Virology* 164: 517-22.
135. Salvato M, Borrow P, Shimomaye E, Oldstone MB (1991) Molecular basis of viral persistence: a single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with suppression of the antiviral cytotoxic T-lymphocyte response and establishment of persistence. *Journal of virology* 65: 1863-9.
136. Matloubian M, Somasundaram T, Kolhekar SR, Selvakumar R, Ahmed R (1990) Genetic basis of viral persistence: single amino acid change in the viral glycoprotein affects ability of lymphocytic choriomeningitis virus to persist in adult mice. *The Journal of experimental medicine* 172: 1043.
137. Matloubian M, Kolhekar SR, Somasundaram T, Ahmed R (1993) Molecular determinants of macrophage tropism and viral persistence: importance of single amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus. *Journal of virology* 67: 7340-9.
138. Cao W, Henry M, Borrow P, Yamada H, Elder J, et al. (1998) Identification of -Dystroglycan as a Receptor for Lymphocytic Choriomeningitis Virus and Lassa Fever Virus. *Science* 282: 2079-2081.
139. Sevilla N, Kunz S, Holz a, Lewicki H, Homann D, et al. (2000) Immunosuppression and resultant viral persistence by specific viral targeting of dendritic cells. *The Journal of experimental medicine* 192: 1249-60.
140. Smelt SC, Borrow P, Kunz S, Cao W, Tishon A, et al. (2001) Differences in Affinity of Binding of Lymphocytic Choriomeningitis Virus Strains to the Cellular Receptor alpha-Dystroglycan Correlate with Viral Tropism and Disease Kinetics. *Journal of Virology* 75: 448.
141. Li Q, Skinner PJ, Ha SJ, Duan L, Mattila TL, et al. (2009) Visualizing antigen-specific and infected cells in situ predicts oucomes in early viral infection. *Science* 323: 1726.
142. Mueller SN, Matloubian M, Clemens DM, Sharpe AH, Freeman GJ, et al. (2007) Viral targeting of fibroblastic reticular cells contributes to immunosuppression and persistence during chronic infection. *Proceedings of the National Academy of Sciences of the United States of America* 104: 15430-5.
143. Matloubian M, Concepcion RJ, Ahmed R (1994) CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *Journal of virology* 68: 8056-63.

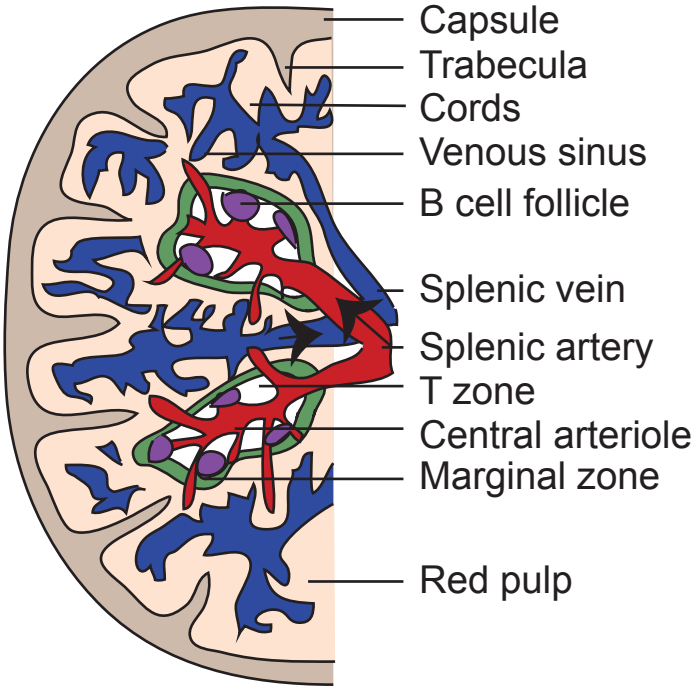
144. Whitmire JK, Asano MS, Kaech SM, Sarkar S, Hannum LG, et al. (2009) Requirement of B cells for generating CD4+ T cell memory. *Journal of immunology (Baltimore, Md. : 1950)* 182: 1868-76.
145. Khanna KM, McNamara JT, Lefrançois L (2007) In situ imaging of the endogenous CD8 T cell response to infection. *Science (New York, N.Y.)* 318: 116-20.
146. Wherry EJJ, Ahmed R (2004) Memory CD8 T-Cell Differentiation during Viral Infection. *Journal of virology* 78: 5535.

**Figure 1**

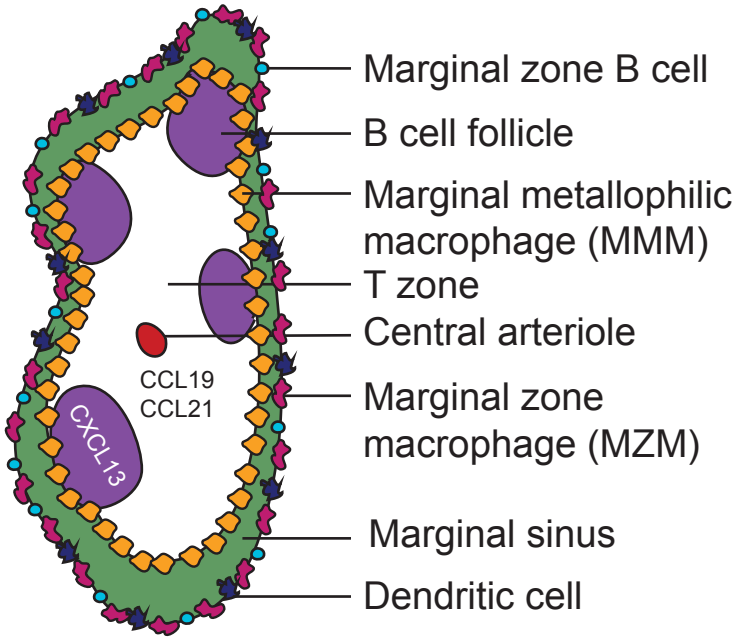
**Spleen and lymph node architecture**

Architecture of spleen (A), splenic white pulp (B), and lymph node (C), adapted from reviews [3,7]. Arrows show the flow of blood through arteries (red) into the spleen and through veins out of the spleen (blue) (A) or the flow of lymph through afferent lymphatics into the lymph node and through efferent lymphatics out of the lymph node (C).

**A** Spleen




**B** White pulp



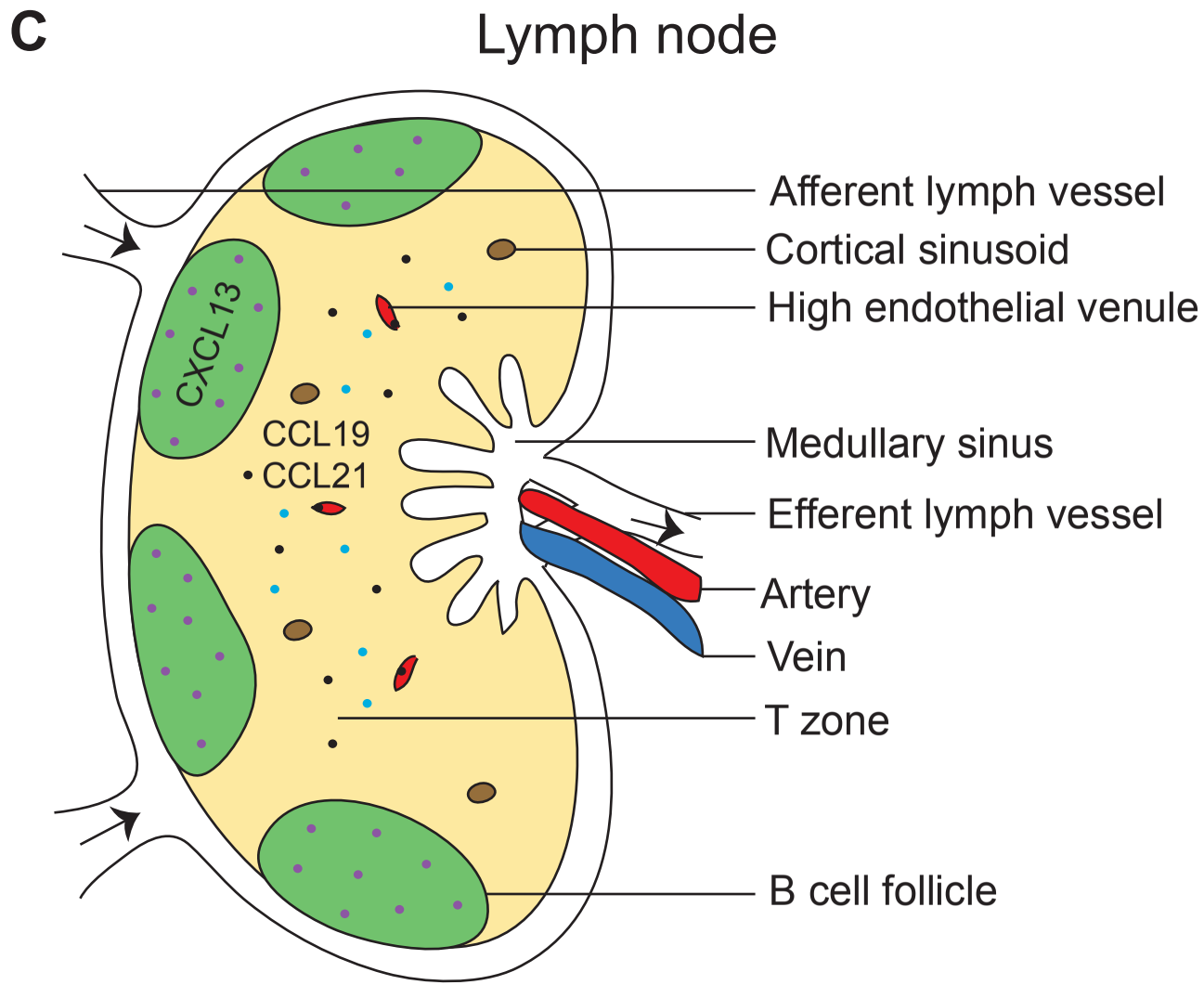

C
Lymph node
Afferent lymph vessel
Cortical sinusoid
High endothelial venule
Medullary sinus
Efferent lymph vessel
Artery
Vein
T zone
B cell follicle
CXCL13
CCL19
CCL21

**Figure 2**

**T cell differentiation models**

Fixed lineage model (A), decreasing potential model (B), and fate commitment with progressive differentiation model, adapted from reviews [103,122].

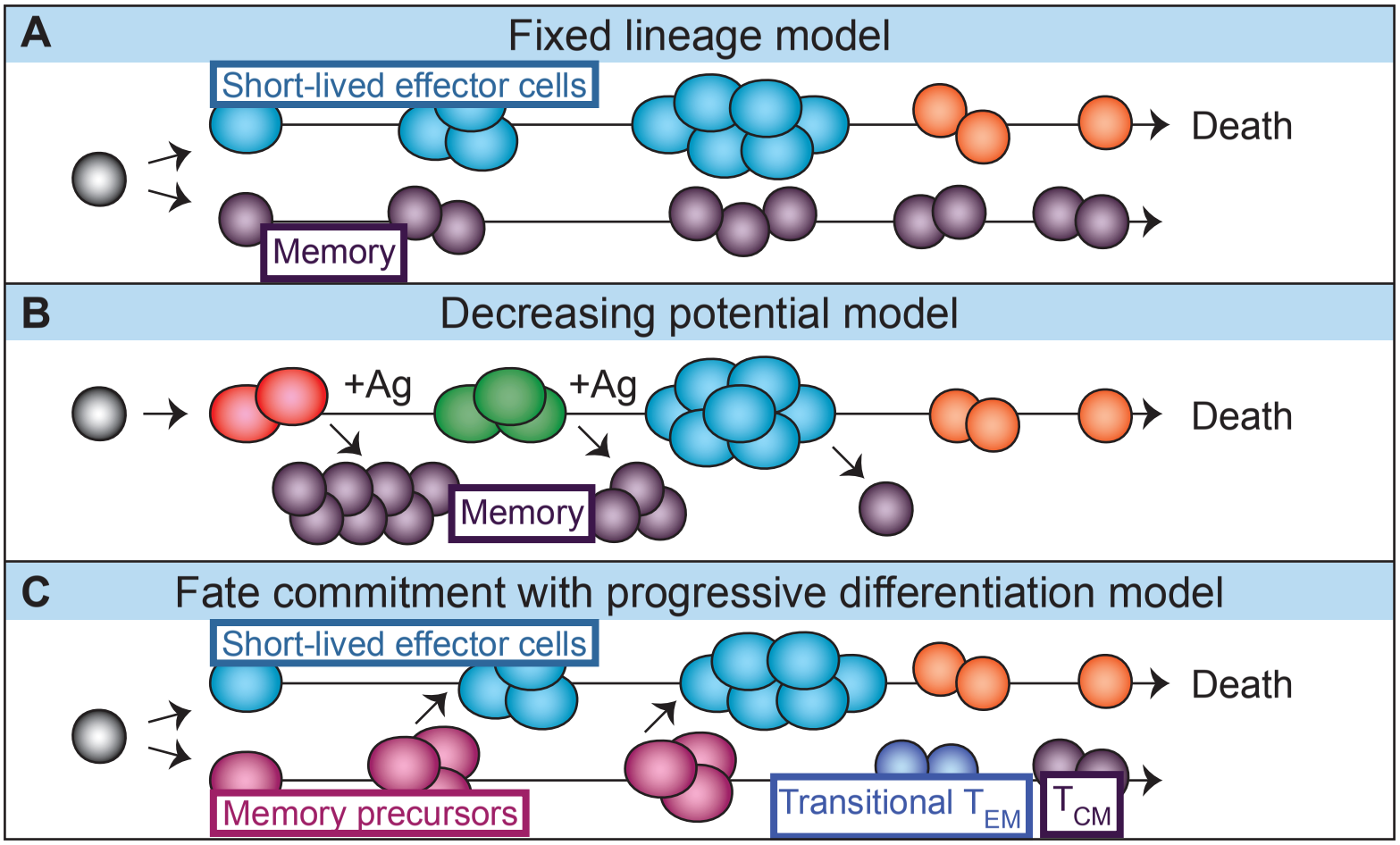
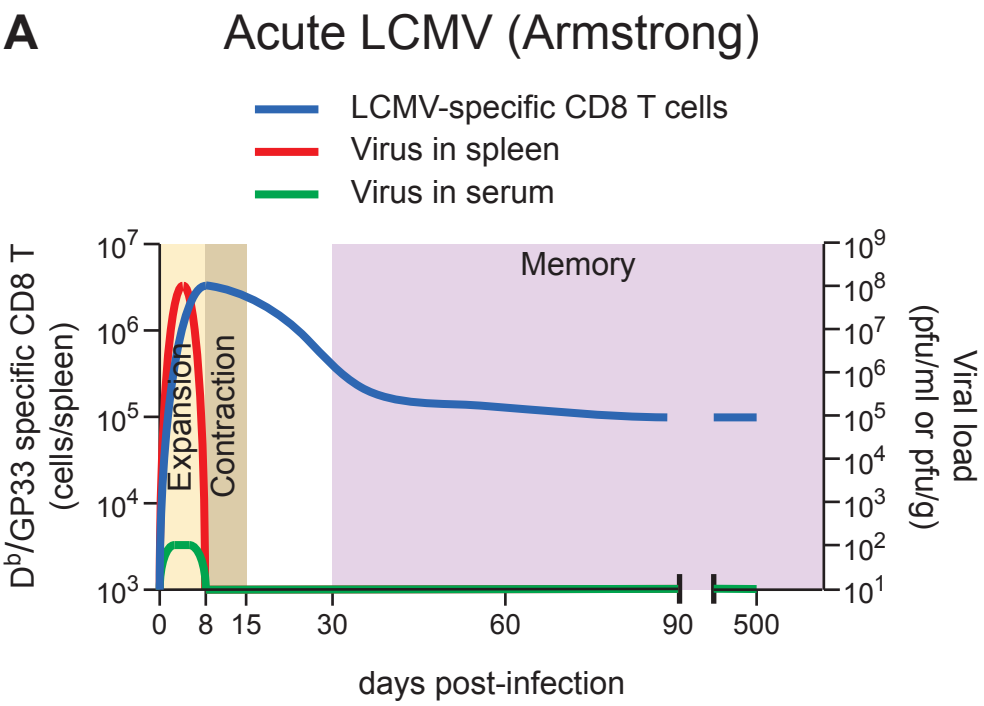
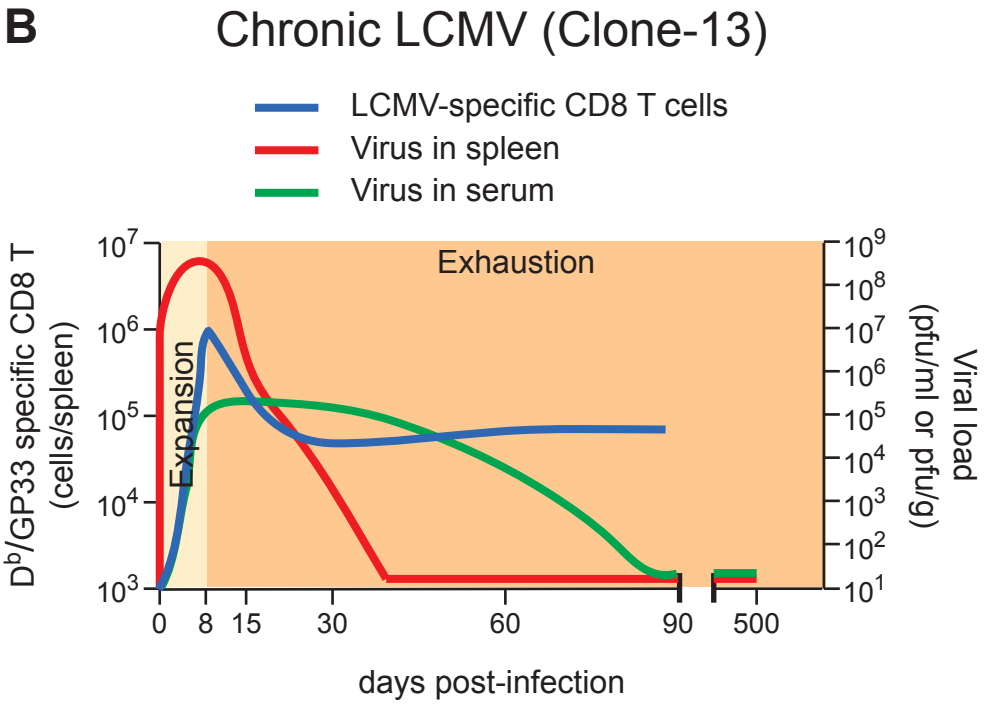

A
Fixed lineage model
Short-lived effector cells
Death
Memory
B
Decreasing potential model
+Ag
+Ag
Death
Memory
C
Fate commitment with progressive differentiation model
Short-lived effector cells
Death
Memory precursors
Transitional $T_{EM}$
$T_{CM}$

**Figure 3**

**Kinetics of CD8 T cell response during acute and chronic virus infection**

LCMV-specific CD8 T cell numbers (blue line) and viral loads in the spleen (red line) and serum (green line) during acute and chronic LCMV infection, adapted from article and review [80,146].


A
Acute LCMV (Armstrong)
LCMV-specific CD8 T cells
Virus in spleen
Virus in serum
Memory
Expansion
Contraction
$D^b$/GP33 specific CD8 T (cells/spleen)
Viral load (pfu/ml or pfu/g)
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^9$
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
0 8 15 30 60 90 500
days post-infection


B
Chronic LCMV (Clone-13)
LCMV-specific CD8 T cells
Virus in spleen
Virus in serum
Exhaustion
Expansion
$D^b$/GP33 specific CD8 T (cells/spleen)
Viral load (pfu/ml or pfu/g)
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^9$
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
0 8 15 30 60 90 500
days post-infection

# Chapter 2

# Expression of chemokine receptor CXCR3 on T cells affects the balance between effector and memory CD8 T-cell generation

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# Expression of chemokine receptor CXCR3 on T cells affects the balance between effector and memory CD8 T-cell generation

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**ABSTRACT:**

Generation of a robust immunological memory response is essential for protection upon subsequent encounters with the same pathogen. The magnitude and quality of the memory CD8 T cell population is shaped and influenced by the strength and duration of the initial antigenic stimulus as well as inflammatory cytokines. Although chemokine receptors have been established to play a role in recruitment of effector CD8 T cells to sites of inflammation, their contribution to determination of T cell fate and shaping of the long-lived memory

T cell population is not fully understood. Here we investigated whether reduced access to antigen and inflammation through alterations in expression of inflammatory and homeostatic chemokine receptors has an impact on generation of effector and memory CD8 T cells. We found that in mice infected with lymphocytic choriomeningitis virus, co-localization of virus specific CD8 T cells with antigen in spleen is dependent on expression of the inflammatory chemokine receptor, CXCR3. In addition, absence of CXCR3 expression on CD8 T cells leads to formation of fewer short-lived effector cells and more memory precursor cells. Furthermore, the memory CD8 T cell population derived from CXCR3 deficient cells has fewer cells of the effector memory phenotype and exhibits a recall response of greater magnitude than wild-type cells. These data demonstrate that CD8 T cell positioning relative to antigen and inflammatory cytokines in secondary lymphoid organs affects the balance of effector and memory T cell formation and has both a quantitative and qualitative impact on the long- lived memory CD8 T cell population.

**INTRODUCTION:**

In response to an infection with an intracellular pathogen, antigen specific CD8 T cells undergo rapid clonal expansion while differentiating into cytotoxic effector T cells that then control the infection through lysis of the infected cells and production of cytokines [1]. After the peak of the expansion, the majority of effector T cells undergo apoptosis leaving behind a small fraction that gives rise to long-lived self-renewing memory CD8 T cells with a superior

recall response to the same antigen. In the past decade major advances have been made in our understanding of the lineage relationship between short- lived effector cells and the precursor cells that give rise to long-lived memory CD8 T cells. Elegant cell tracing studies have shown that an individual naïve CD8 T cell can have multiple fates and give rise to both short-lived effector and long-lived memory cells [2]. However, the proportion of effector cells that give rise to long-lived memory cells can be affected by several factors including the strength and duration of antigenic stimulus as well as pro-inflammatory cytokines and other environmental factors. Progress in identification of terminal effector and memory precursor cells within the heterogeneous effector population at early times after infection, through their expression of markers such as KLRG-1 and IL-7R$\alpha$, has paved the way for elucidating how cell intrinsic and extrinsic factors, including positional cues, alter the balance between effector and memory T cell generation [3, 4].

Naïve CD8 T cells continuously circulate between blood and secondary lymphoid organs in search of their cognate antigen. They express high levels of CCR7 and thus, localize within the T zone areas of the spleen and lymph nodes where the CCR7 ligands CCL19 and CCL21 are homeostatically expressed. Upon activation by both antigen and innate stimuli, such as type I interferons, CD8 T cells alter their expression of chemokine receptors [5]. Receptors, such as CCR7 and S1PR1, are down-regulated and inflammatory chemokine receptors, such as CXCR3, CXCR6, and CCR5 are up- regulated [6-9]. These receptors then guide effector CD8 T cells towards activated dendritic

cells to receive further “help” from CD4 T cells or recruit them to inflamed tissues to control the infection. One particular receptor, CXCR3, has been shown to be important in recruitment of effector CD8 T cells to allografts and to sites of viral infection within the CNS and the genital tract where its ligands, CXCL9 and CXCL10 are induced by IFN-γ [10-15].

In addition to regulating the trafficking patterns of effector CD8 T cells, chemokine receptors also have an impact on the interaction of these cells with their microenvironment. How chemokine receptors influence these interactions and affect CD8 T cell differentiation is not completely understood. In this study we examined the role of CXCR3 in differentiation of activated CD8 T cells *in vivo* in response to infection of the host with lymphocytic choriomeningitis virus (LCMV). We found that absence of CXCR3 on CD8 T cells leads to less co-localization of effector T cells with antigen within the spleen and affects the balance between generation of short-lived effector and memory precursor cells. More strikingly, absence of CXCR3 leads to generation of more long-lived memory CD8 T cells with a qualitatively better recall response.

**RESULTS:**

**CXCR3 is gradually up-regulated on antigen specific CD8 T cells during the course of infection.**

To determine whether chemokine receptors play a role in CD8 T cell fate determination after activation, we analyzed expression of a number of these receptors on Ag-specific CD8 T cells following LCMV infection. One particular

receptor, CXCR3, was up-regulated approximately ten-fold as early as day 3 post-infection on P14 TCR Tg CD8 T cells specific for LCMV $D^bGP33$-41 (Fig. 1A). CXCR3 expression reached peak levels by day 5 but was then partially down-regulated on a small fraction of the cells by day 8 post-infection (Fig. 1A). Several recent studies have shown that within the heterogeneous effector CD8 T cell population level of expression of KLRG1 can distinguish those cells that are terminally differentiated and are destined to die ($KLRG1^{hi}$) from those that give rise to long-lived memory cells ($KLRG1^{lo}$) [3, 4]. Further analysis of $KLRG1^{hi}$ and $KLRG1^{lo}$ subsets of effector CD8 T cells showed that both populations expressed similar levels of CXCR3 at day 5 post-infection (Fig. 1B). However, the $KLRG1^{hi}$ terminally differentiated CD8 T cells had reduced levels of CXCR3 expression by day 8, whereas the $KLRG1^{lo}$ memory precursor cells maintained uniform and stable high levels of CXCR3 expression (Fig. 1B). Of the three known CXCR3 ligands, C57BL/6 mice express only two, CXCL9 and CXCL10 [16-19]. As determined by quantitative RT-PCR, both ligands were up-regulated in spleens of mice on day 1 post-infection and were maintained at somewhat lower levels throughout the course of infection (Fig. 1C). The continuous presence of CXCR3 ligands in the spleen suggested that lower expression of CXCR3 on $KLRG1^{hi}$ cells may be attributable to ligand induced receptor endocytosis as this population may be localized differently within the spleen than the $KLRG1^{lo}$ subset and encounter different amounts of CXCR3 ligands (20). To address this possibility we evaluated CXCR3 mRNA expression by quantitative RT- PCR in sorted $KLRG1^{hi}$ and $KLRG1^{lo}$ CD8 effector populations at various

times after *in vivo* activation. Consistent with cell surface CXCR3 expression, both populations had similar levels of CXCR3 mRNA at day 4 post-infection (Fig. 1D). However, by day 8, the KLRG1$^{lo}$ subset had approximately 5-fold higher CXCR3 mRNA expression than the KLRG1$^{hi}$ T cells. Similarly, in a functional chemotaxis assay, we did not find any differences in migration of KLRG1$^{hi}$ and KLRG1$^{lo}$ CD8 T cells to CXCL9 on day 5 post-infection (Fig. 1E). However, consistent with CXCR3 expression data, by day 8 post- infection, KLRG1$^{hi}$ cells had a somewhat lower chemotactic response to CXCL9 when compared to KLRG1$^{lo}$ cells (Fig. 1E).

**Absence of CXCR3 expression on CD8 T cells leads to the development of more memory precursor and fewer terminally differentiated effector CD8 T cells.**

CXCR3 and its ligands have been implicated to play a role in the trafficking of effector CD8 T cells to specific organs in several microbial infections and allograft rejection [10,11, 14, 15, 21-24]. To determine whether CXCR3 also influences the generation of effector and memory CD8 T cells, we co-transferred wild-type and CXCR3 deficient P14 T cells expressing different CD45 congenic alleles into wild-type mice and followed their expansion and phenotype in response to infection with LCMV. At the peak of CD8 T cell response, we consistently found a slightly higher proportion of wild-type P14 cells compared to those lacking CXCR3 (Fig. 2A). This was not due to a global alteration in CD8 T cell trafficking, since similar differences were observed in blood and other tissues

examined (Fig. 2A and data not shown). More strikingly, when compared to wild-type cells, a smaller proportion of CXCR3-deficient cells had phenotypic characteristics of short-lived effector cells (KLRG-1$^{hi}$ IL-7R$\alpha^{lo}$) and a greater proportion expressed markers of memory-precursor effector cells (KLRG-1$^{lo}$ IL-7R$\alpha^{hi}$) (Fig. 2A). This shift in effector population subsets became evident at the peak of the response on day 8 and corresponded to a modest decrease in the number of CXCR3-deficient short-lived effector cells. In contrast, the number of CXCR3-deficient memory precursor cells was increased (Fig. 2A and C). We did not observe any differences between wild-type and CXCR3-deficient cells at the peak of the response with respect to expression of several activation markers and the ability to make the effector cytokines IFN-$\gamma$ and TNF-$\alpha$ (Fig.2B). These data suggest that expression of CXCR3 favors the development of short-lived effector CD8 T cells as opposed to memory precursor cells.

**Restoration of CXCR3 expression in CXCR3 deficient CD8 T cells leads to development of more terminally differentiated effector cells and fewer memory precursor cells.**

To further determine whether expression of CXCR3 can drive differentiation of activated CD8 T cells towards short-lived effector cells rather than long-term memory cells, we utilized an MSCV based retroviral vector to express mouse CXCR3 in CXCR3-deficient P14 cells. CXCR3-deficient T cells were activated *in vivo* and then transduced *ex-vivo* with a construct containing mouse CXCR3 followed by an IRES-Thy1.1 that allows distinguishing between

transduced from non-transduced cells within the same starting population (Fig. 3A). Shortly after transduction, the cells were transferred into mice that were infected several hours earlier with LCMV and the fate of the cells was then followed *in vivo*. Expression of CXCR3 on transduced cells was slightly lower than on wild-type non-transduced P14 cells (Fig. 3B). Restoration of partial CXCR3 expression led to greater expansion of P14 T cells *in vivo* and the generation of more short-lived effector cells (KLRG-1$^{hi}$ IL-7R$\alpha^{lo}$) at the expense of long-lived memory precursor cells (KLRG-1$^{lo}$ IL-7R$\alpha^{hi}$) (Fig. 3C). Similar to what was observed when we compared wild-type and CXCR3 deficient P14 cells (Fig. 2C), the effect of CXCR3 expression on CD8 effector T cell differentiation was evident only at the peak of the response and during the contraction phase, but not at day 5 post-infection (Fig. 3D). These data suggest that although CXCR3 is not a major determinant of CD8 T cell activation and proliferation, its expression can affect the later stages of the CD8 response and have a negative impact on the generation of long-lived memory T cells.

In addition to affecting CD8 T cell fate, retroviral mediated expression of CXCR3 also affected expansion of effector CD8 T cells. At the peak of the response, we found a greater proportion of CXCR3-transduced cells to be Thy1.1+ when compared to those transduced with the control empty vector, despite similar initial transduction efficiencies (Fig. 3C and data not shown). Since CXCR3 is expressed only on a small subset of naïve P14 T cells (Fig. 1A), these results suggested that early retroviral expression of CXCR3 might facilitate exposure of CD8 T cells to antigen and further drive their expansion. We relied

on the observation that about 10-15% of naïve P14 T cells express CXCR3 to test this hypothesis by co-transferring either sorted CXCR3+ or CXCR3- WT P14 cells with CXCR3 KO cells and determining their expansion and differentiation (Fig. S1A). When CXCR3+ WT P14 cells were co-transferred with CXCR3 KO cells, WT cells expanded four to five-fold more in number. In contrast, when WT P14 cells that lacked CXCR3 expression were used, the WT cells expanded only two to three-fold more than CXCR3KO cells. (Fig. S1B). Regardless of whether the starting WT population expressed CXCR3 or not at the time of transfer, within the effector population at the peak of the response, there were proportionately more terminally differentiated effector cells (KLRG-$1^{hi}$ IL-$7R\alpha^{lo}$) and fewer memory precursor cells (KLRG-$1^{lo}$ IL-$7R\alpha^{hi}$) than in the CXCR3 KO population (Fig. S1B). These results suggest that the differences observed between WT and CXCR3-deficient P14 T cells are not due to expression of CXCR3 on a sub-population of naïve wild-type cells during the initial stages of activation.

**Absence of CXCR3 leads to more rapid phenotypic and functional maturation of memory CD8 T cells.**

To further examine the impact of CXCR3 expression on CD8 T cell memory development, we analyzed the phenotype and distribution of wild-type and CXCR3-deficient P14 TCR Tg T cells in various tissues within the same animal after day 40 and up to day 356 post-immunization. We consistently found ~2-4-fold more CXCR3 deficient memory CD8 T cells than wild type cells within the same recipient in all the examined tissues (Fig. 4A). At earlier times after

immunization, compared to wild-type, a greater proportion of CXCR3-deficient P14 memory cells were KLRG1$^{lo}$ IL-7R$\alpha^{hi}$, consistent with a faster transition to a more mature memory population (Fig. 4B) [25]. Similarly, when compared to wild-type memory cells, a smaller proportion of CXCR3- deficient cells had phenotypic characteristics of effector memory cells (CCR7$^{lo}$ CD62L$^{lo}$) (Fig. 4B) [26]. These differences were observed in all the examined tissues, ruling out a contribution from differential trafficking patterns, and gradually diminished over time, suggesting that the CXCR3-deficient cells had acquired a more mature memory phenotype more rapidly than wild-type cells (Fig. 4C) [27]. Alternatively, CXCR3-deficient memory precursor cells might be less likely to give rise to effector memory cells. The greatest difference we observed between wild-type and CXCR3-deficient memory CD8 T cells was within the CD62L$^{lo}$ population. In the absence of CXCR3, a greater proportion of this population was CCR7$^{hi}$ at all the examined times after infection. This CD62L$^{lo}$ CCR7$^{hi}$ population may represent a transitional state to the central memory phenotype since these cells express very high and uniform levels of CXCR3, similar to the KLRG1$^{lo}$ IL-7R$\alpha^{hi}$ and central memory (CD62L$^{hi}$ CCR7$^{hi}$) populations (Fig. S2). In contrast, effector memory cells, postulated to descend mostly from the KLRG1$^{hi}$ population express lower levels of CXCR3 consistent with expression on the KLRG1$^{hi}$ population (Fig. S2) [4].

In addition to cell surface phenotypic differences and consistent with a more mature functional memory phenotype, a greater proportion of CXCR3-deficient memory CD8 T cells were capable of producing IL-2 and were CD27$^{hi}$

compared to wild-type cells (Fig.4D). To determine whether there were any additional functional differences between wild-type and CXCR3-deficient memory CD8 T cells, CD62L$^{hi}$ P14 memory cells of each genotype were sorted and transferred either separately or together at a 1:1 ratio into groups of naïve mice. Recipient mice were challenged with LCMV, and at the peak of the secondary response we found that regardless of whether the two populations were transferred together or separately, there were about two-fold more CXCR3-deficient CD8T cells compared to wild-type cells (Fig. 4E). Similar differences were observed in the secondary response of sorted CD62L$^{lo}$ memory populations, although consistent with other reports, the extent of proliferation was much lower than that of CD62L$^{hi}$ memory cells (data not shown) [27]. These results indicate that the CXCR3-deficient memory CD8 T cells have a higher proliferative response and are thus functionally more mature than wild-type cells.

A major advantage of using a TCR Tg system is that the contribution of a specific molecule to T cell function can be evaluated by comparing cells that are sufficient and deficient for that molecule within the same organism. To determine whether our observations were restricted to a TCR Tg system or were more widely applicable to CD8 T cell biology, we generated mixed bone marrow chimeras by reconstituting lethally irradiated mice with a 1:1 mixture of wild-type and CXCR3-deficient bone marrow cells with CD45 congenic haplotypes distinct from each other and the recipient mice. As a control, a group of mice were reconstituted with a 1:1 mixture of wild-type bone marrow cells of differing CD45 congenic haploytpes as well. The ratio of CXCR3-deficient and sufficient CD8 T

cells was determined before immunization with LCMV. At the peak of the effector response and during the memory phase, the frequency of CD8 T cells specific for the major epitopes of LCMV in each population was determined by tetramer staining. Similar to the results obtained with the Tg system, there were fewer CXCR3-deficient effector cells compared to wild-type cells at the peak of the response, and relatively more CXCR3-deficient memory cells in the later stages (Fig. S3A and C). Consistent differences were not observed between CD8 T cells derived from either wild-type donor bone marrow cells in control chimeras (Fig. S3B and D). These results suggest that the effect of CXCR3 on generation of effector and memory CD8 T cells is not restricted to the TCR Tg system.

**Effector CD8 T cells co-localize in the spleen with antigen and CXCL9 in a CXCR3- dependent manner.**

The role of CXCR3 in trafficking of effector CD8 T cells to inflamed organs and especially the CNS has been well established. However, very little is known about the function of CXCR3 in localization of responding CD8 T cells within a lymphoid organ during the course of an immune response. We hypothesized that such differences in localization may contribute to effector CD8 T cell fate determination. To address this question, wild-type and CXCR3-deficient naïve P14 cells expressing distinct congenic alleles of CD45 and Thy1 were co-transferred in a 1:1 ratio into naïve mice. At different times after infection with LCMV, localization of the responding cells, as well as viral antigen

and CXCL9, a CXCR3 ligand, was determined by immunofluorescent staining of spleen sections. Consistent with previous reports, we found the majority of LCMV antigen localized within the marginal zone of the spleen and diminished in amount by day 5 post-infection (Fig. 5A) [28]. In addition, CXCL9 was localized in the same area as viral antigen, and marginal zone macrophages sorted from LCMV infected mice contained high levels of both viral RNA and mRNA for the CXCR3 ligands, CXCL9 and CXCL10 (Fig. 5A and B). Even though viral antigen was less readily visualized by day 5 post-infection, CXCL9 could still be detected in sections. Within the same spleen, wild-type P14 T cells co-localized with CXCL9 and viral antigen in the marginal zone area, whereas CXCR3-deficient P14 T cells were mostly found within the T zone area and were less readily found associated with CXCL9 and viral antigen (Fig.5A and C and Fig. S4). These results show that CXCR3 can affect localization of effector CD8 T cells within secondary lymphoid organs and provide more or prolonged access to antigen.

**CXCR3-deficient CD8 T cells show reduced proliferation during the later stages of infection.**

We have found that activated CXCR3-deficient CD8 T cells do not co-localize as effectively as wild-type cells with antigen *in vivo* and produce fewer terminally differentiated effector cells and more central memory cells. To delineate more specifically the stage at which CXCR3 affects CD8 T cell responses, we examined activation, proliferation, and survival of wild-type and CXCR3-deficient P14 T cells *in vivo* at various times after infection. CXCR3

expression was not required for early events of activation and proliferation, since there were no differences between WT and KO cells in up-regulation of CD25 on day 1 and CFSE dilution by day 2.5 post-infection (Fig. 6A and B). These results were not entirely unexpected since CXCR3 was not highly expressed on CD8 T cells during early stages of activation (Fig. 1A and D). Since the earliest effects of CXCR3 were observed between days 5 and 8 post-infection, we examined the survival and proliferation of activated CD8 T cells during this period. We did not observe any differences in the frequency of dying WT and KO cells, as measured by annexin V staining (Fig. 6C). Similarly, there was no effect of CXCR3 expression on the proportion of CD8 T cells that incorporated BrdU on day 5 post-infection. However, on days 6 and 7, approximately half as many CXCR3-deficient CD8 T cells incorporated BrdU relative to WT cells (Fig. 6D). Further phenotypic characterization of CD8 T cells WT and KO cells in BrdU incorporation was in the KLRG1$^{lo}$ IL-7R$\alpha^{lo}$ population (Fig.6E). This population has been shown to mostly give rise to long-lived memory CD8 T cells but also retain the potential to become terminally differentiated effector cells as well [3, 4]. Since the KLRG1$^{lo}$ IL-7R$\alpha^{lo}$ CD8 T cells also express CXCR3, our data suggest that further exposure to antigen and inflammatory stimuli driven by this chemokine receptor may affect the balance between generation of terminally differentiated effector cells and long-lived memory cells as well as the quality of the latter population. The effect of CXCR3 on the production of terminal effectors and memory precursors occurs towards the end of the CD8 T cell response and

is consistent with the fate commitment with progressive differentiation model [29, 30].

**Persistent CCR7 expression tips the balance towards the development of more memory precursors than terminally differentiated effector CD8 T cells.**

Our results suggest that inflammatory chemokine receptors, such as CXCR3, can affect differentiation of activated CD8 T cells by promoting encounters with antigen and inflammatory cues. These encounters may also be facilitated by down-regulation of homeostatic chemokine receptors, such as CCR7, that usually maintain T cells in the T zone areas. Expression of CCR7 in antigen specific CD8 T cells declined at both mRNA and protein levels early after *in vivo* activation (Fig. S5). However, the extent of down-regulation was higher in KLRG1$^{hi}$ when compared to KLRG1$^{lo}$ cells, and the latter, regained partial surface expression of CCR7 by day 8 post-infection (Fig. S5B). These results are consistent with reported observations demonstrating that KLRG1$^{lo}$ cells display a greater chemotactic response to the CCR7 ligand CCL19, and are localized within the T zone areas of the spleen at day 8 post-infection [20]. A previous study has shown that persistent transgenic expression of CCR7 in P14 cells leads to sequestration of effector CD8 T cells within the splenic T zone [31]. To determine whether this altered localization affects the differentiation of antigen experienced CD8 T cells, we generated P14 TCR Tg mice that were also transgenic for mouse CCR7 driven by a mouse CD4 promoter lacking the intronic transcriptional silencer that remains active in both CD4 and CD8 T cells [32]. In

contrast to wild-type cells, CCR7 Tg P14 CD8 cells maintained cell surface expression of CCR7 after *in vivo* activation (Fig. 7A). Furthermore, fewer CCR7 Tg cells were found in blood compared to wild-type, indicating that altered CCR7 expression led to sequestration within lymphoid tissues (Fig. 7B). More strikingly, in both blood and spleen, a greater proportion of CCR7 Tg P14 cells were KLRG1$^{lo}$ IL-7Rá$^{hi}$ when compared to wild-type cells (Fig. 7C and D), suggesting that the former had less exposure to signals that drive terminal effector differentiation, such as antigen and inflammation. Indeed, at day 4 post-infection, we found wild-type P14 T cells localizing with viral antigen in the spleen, whereas CCR7 Tg P14 T cells were found mostly in the T zone areas and away from viral antigen (Fig. 7E). These results suggest that in addition to the expression of inflammatory chemokine receptors, down-regulation of chemokine receptors that homeostatically position T cells within the T zone areas of the spleen contributes to their exposure to antigen and inflammatory signals and have an impact on their fate determination.

**DISCUSSION:**

A number of studies have established that the magnitude and quality of CD8 T cell effector and memory responses are influenced by T cell precursor frequencies as well as the extent and duration of antigen and inflammation [33-36]. In this study we demonstrated that chemokine receptors also affect CD8 T cell differentiation in response to an infection and influence the quality and quantity of long-lived memory CD8 T cells. Absence of an inflammatory

chemokine receptor, CXCR3, on CD8 T cells led to fewer short-lived effector cells and more memory precursor cells at the peak of the response. The long-lived memory CD8 T cells derived from this population had fewer cells with phenotypic characteristics of effector memory cells and displayed a more robust recall response than wild-type cells. Similarly, constitutive expression of a homeostatic chemokine receptor, CCR7, normally down-regulated upon T cell activation, led to development of more memory precursor cells. We found that in the absence of CXCR3, effector CD8 T cells were more localized in the T cell zone than with viral antigen present in the marginal zone areas of the spleen. Similarly, persistent expression of CCR7, a homeostatic chemokine receptor that is usually down-regulated upon T cell activation, led to sequestration of effector CD8 T cells within the T zone areas and away from antigen and skewed the CD8 T cell response towards more memory precursor cells. Since both of these changes in chemokine receptor expression affected localization of effector CD8 T cells, the observed effects on CD8 T cell fate are most likely due to alterations in the access and exposure of these cells to antigen and inflammatory stimuli.

Differential localization of effector and memory CD8 T cells within secondary lymphoid tissues has been well described. A number of studies using various infection models and approaches have found effector CD8 T cells mostly localized in the red pulp of the spleen with a small fraction residing in the T cell zone at the peak of the response [7, 20, 37, 38]. In contrast, memory CD8 T cells were found primarily in the T cell areas of the spleen. A recent study showed that this differential localization of effector and memory CD8 T cells could be

dictated by transcription factors that regulate CD8 T cell fate. Localization of terminally differentiated KLRG1$^{hi}$ IL-7Rá$^{lo}$ effector CD8 T cells in the red pulp was dependent on expression of T-bet and Blimp-1 [20]. In the absence of either of these transcription factors, CD8 T cells were predominantly in the T cell zone of the spleen and had phenotypic characteristics of long-lived memory CD8 T cells (KLRG1$^{lo}$ IL-7R$\alpha^{hi}$). As part of regulating distinct transcriptional programming of terminal effector and memory precursor CD8 T cells, T-bet and Blimp-1 may also regulate differential expression of various chemokine receptors leading to altered localization of these cells within the spleen [20, 39]. However, the molecular nature of the cues that guide effector CD8 T cells out of the T cell zone is not well understood. Although CXCR3 is up- regulated on activated CD8 T cells, we found that up to day 5 post-immunization, it was not differentially expressed in terminal effector and memory precursor CD8 T cells. Furthermore, up to this time after infection, CXCR3 expression did not have an impact onthe proportion of effector CD8 T cells that were KLRG1$^{hi}$ suggesting that this chemokine receptor is not a major determinant of CD8 T cell fate. However, absence of CXCR3 expression on CD8 effector T cells had three effects: 1) fewer cells became KLRG1$^{hi}$ terminal effectors between days 5 and 8 post-infection; 2) a smaller proportion of memory CD8 T cells had an effector memory phenotype; and 3) both the quality and quantity of long-lived memory CD8 T cells were increased. Since the KLRG1$^{hi}$ effector cells gradually down-regulated CXCR3 expression between days 5 and 8 post-infection through an unknown mechanism, it is most likely that these observed phenotypes are due to the presence of CXCR3 on the

KLRG1$^{lo}$ IL-7R$\alpha^{lo}$ CD8 T cell population. Several studies have shown that the majority of cells in this population are destined to become long-term memory cells of both central and effector phenotype [3, 4]. However, consistent with the fate commitment with progressive differentiation model, these cells retain the potential to become terminally differentiated effector cells as well when given the appropriate signals [3]. These results suggest that expression of CXCR3, particularly when the amount of antigen has diminished during the later stages of infection (between days 5-8), may facilitate encounter of the KLRG1$^{lo}$ IL-7R$\alpha^{lo}$ CD8 T cells with antigen and/or inflammatory signals that further drive their proliferation and differentiation intoKLRG1$^{hi}$ IL-7R$\alpha^{lo}$ effector cells and away from becoming long-lived memory cells. Repeated exposure of memory precursor cells to antigen or inflammation is known to diminish the quality of the memory CD8 T cells that are generated [40]. Therefore, it is likely that CXCR3 facilitates exposure of the KLRG1$^{lo}$ IL-7R$\alpha^{lo}$ CD8 T cells to antigen and inflammation, thus negatively impacting the quality and phenotype of the memory CD8 T cells that are generated. Because IL-7 is mostly expressed in the T zone areas of the spleen, it is also possible that the altered localization of CD8 T cells within these areas in the absence of CXCR3 or through constitutive expression of CCR7 may lead to better survival and eventual formation of long-lived memory cells. However, the fact that expression of IL-7R itself is not sufficient to drive differentiation of CD8 T cells towards memory argues against this possibility [41]. Therefore, expression of an inflammatory chemokine receptor can tip the balance

towards more terminal effector cells as well as have an impact on the quality of the memory CD8 T cells that are generated (Fig. S6).

Several recent studies on the role of the mTOR pathway in CD8 effector T cells have highlighted an important role for a metabolic switch in transitioning from memory precursor to mature long-lived CD8 T cells [25, 42, 43]. Treatment of mice with rapamycin, an inhibitor of mTOR, during the course of infection with LCMV led to a phenotype that is very similar to that of CXCR3-deficient CD8 T cells: generation of fewer short lived effector CD8 T cells and more memory precursor cells, as well as an increase in both the quantity and quality of long-lived memory cells. In T cells, mTOR is activated by various cytokines as well as engagement of T cell receptor by antigen, resulting in a switch from catabolic metabolism to anabolic metabolism [44, 45]. Generation of long-lived memory T cells requires a reversal of this metabolic switch to the homeostatic catabolic state. Inhibition of mTOR presumably accelerates this return to the baseline metabolic state, resulting in a more robust long-lived memory T cell population. Since CXCR3 deficiency phenocopies mTOR inhibition, it is possible that expression of CXCR3 leads to more encounters with antigen and inflammatory stimuli resulting in further activation of mTOR in effector CD8 T cells. Further studies are necessary to determine if there were any links between expression of inflammatory chemokine receptors and metabolic state of effector CD8 T cells.

Several pro-inflammatory cytokines have been shown to act as “signal 3” and affect CD8 T cell responses to various infections [46]. In particular,

absence of IFN-γ leads to a reduction in expansion of CD8 T cells in response to an LCMV infection [47]. Most of this effect is attributable to direct action of IFN-γ on CD8 T cells [48]. Nevertheless, because IFN-γ is an inducer of CXCR3 ligands, in light of our findings, some of the effects of this cytokine could be mediated indirectly through recruitment of effector CD8 T cells to antigen, leading to further expansion or decreased potential to form memory T cells. Furthermore, because production of CXCR3 ligands is most likely secondary to local production of IFN-γ by other activated T cells, recruitment of CXCR3 expressing effector CD8 T cells to these sites will also expose them directly to more IFN-γ. Thus, the IFN-γ and CXCR3 ligands together may provide a positive feedback loop for further exposure of effector CD8 T cells to antigen and inflammation. Although in this study we focused on localization of T cells within the spleen, it is also possible that in other infected tissues, IFN-γ and CXCR3 may play a similar role in exposure of effector CD8 T cells to antigen and pro-inflammatory cytokines.

Our study did not find a major role for CXCR3 in early recruitment, activation, and expansion of CD8 T cells, which is consistent with lack of expression of this receptor on the majority of naïve CD8 T cells. However, CXCR3 expression on a minor population of naïve P14 cells gave these cells a slight proliferative advantage. In addition, retroviral mediated forced expression of CXCR3 at an early time in T cell activation also led to an increase in the magnitude of the CD8 T cell response. Since CXCR3 is constitutively expressed at high levels on memory CD8 T cells and its ligands are also expressed shortly

after infection, we expected that wild-type memory CD8 T cells would have an advantage over CXCR3 deficient memory cells in a secondary recall response. Surprisingly, we found that CXCR3 KO memory CD8 T cells had a larger secondary response than wild-type cells, suggesting that absence of CXCR3 led to development of qualitatively superior memory CD8 T cells (Fig. S6). In addition, these results indicate that CXCR3 is not necessary for a recall response to LCMV infection. This could be attributable to redundancy in expression of inflammatory chemokine receptors, such as CXCR6, which may facilitate encounter of memory CD8 T cells with antigen-presenting cells. These results do not completely rule out a role for CXCR3 in a secondary response, because whatever competitive advantage expression of CXCR3 might have offered was overshadowed by qualitative differences between WT and CXCR3 KO memory CD8 T cells.

During the acute phase of infection, although CXCR3-deficient effector cells did not localize to antigen in the marginal zone areas of the spleen, they were readily found in blood and other tissues indicating that this receptor was not necessary for either egress from lymphoid tissues or recruitment to other sites of infection. One study has implicated a role for another inflammatory chemokine receptor, CCR5, in guiding CD8 effector T cells to sites of antigen-specific dendritic cell-CD4 T cell interactions to receive necessary help to form a functional memory population [49]. Since CXCR3- deficient memory CD8 T cells demonstrated a robust response to a secondary challenge, expression of this receptor on CD8 effector T cells is not necessary to receive CD4 T cell help.

These results suggest that each inflammatory chemokine receptor may play a unique role in development and function of CD8 T cells. Further investigation is necessary to address whether these differences can be generalized to other infections or immunization protocols and determine the role of other inflammatory chemokine receptors, such as CXCR6, that are up-regulated on activated CD8 T cells [50].

In this study we showed that alterations in chemokine receptor expression can alter distribution of effector CD8 T cells within lymphoid tissues and have an impact on their differentiation and generation of memory CD8 T cells. Further studies on the role of various chemokine receptors and other guidance cues will advance our understanding of the interplay between these signals, localization within tissue microenvironments, and shaping of the effector and memory T cell responses. In addition, it will be interesting to determine the role of chemokine receptors in repeated exposure of CD8 T cells to antigen in a chronic viral infection and their impact on T cell exhaustion. Our findings suggest that targeting chemokine receptors, such as CXCR3, as part of vaccination protocols for protection against infections or cancer, may provide an approach to generate a more robust memory CD8 T cell response.

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## DISCLOSURES

The authors have no financial conflicts of interest.

## MATERIALS AND METHODS Mice

C57BL/6 (CD45.2), B6.BoyJ (CD45.1), and Thy1.1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or the National Cancer Institute (Fredrick, MD). CD45.1/CD45.2 P14 and Thy1.1 P14 mice bearing the $D^bGP33$-specific TCR were maintained in our animal colony. *Cxcr3*$^{-/-}$ mice were a generous gift from Dr. C. Gerard (Children's Hospital, Harvard Medical School, Boston, MA). CCR7 transgenic mice were a generous gift from Dr. N. Killeen (University of California, San Francisco, CA). All animals were housed in the specific pathogen–free facility at the University of California, San Francisco, and were treated according to protocols approved by university animal care ethics

and veterinary committees in accordance with guidelines of the US National Institutes of Health.

**Infection, Adoptive transfer and Bone marrow Chimeras**

LCMV Armstrong was propagated on BHK cells and titered on Veros cells as described [27]. Unless indicated below, P14 chimeric mice were generated by adoptive transfer of $2x10^4$ wild-type (WT) and $2x10^4$ CXCR3 KO, or $2x10^4$ WT and $2x10^4$ CCR7 transgenic(Tg) P14 T cells into C57BL/6 recipients that were infected i.p. 1-3 days later with $2x10^5$ PFU LCMV Armstrong. $2x10^5$ P14 T cells were adoptively transferred for the sorting of $KLRG^{hi}$ and $KLRG1^{lo}$ cells and for the retroviral transductions; $2x10^5$ to $1x10^6$ P14 T cells were adoptively transferred for localization experiments; $1x10^6$ P14 cells were transferred for CFSE dilution assays. Mice were infected i.v. with $2x10^6$ PFU LCMV for secondary infections, $2x10^6$ PFU LCMV i.p. for immunofluorescence. To generate mixed bone marrow chimeras, 6-8-week-old C57BL/6 mice were lethally irradiated by $\gamma$ irradiation from a Cesium source. Bone marrow cells from WT and *Cxcr3*$^{-/-}$ mice were mixed in a 1:1 ratio and used to reconstitute the irradiated mice. The mice were maintained on antibiotics for 6 weeks during reconstitution and then bled to determine the proportion of CD8 T cells derived from each donor. Mice were infected i.p. with $2x10^5$ PFU LCMV Armstrong and LCMV-specific CD8 T cells were analyzed after infection.

**Isolation of lymphocytes from non-lymphoid organs**

Mice were perfused with PBS before removal, mincing, and passage of liver and lung through a 70 μm filter mesh. Lymphocytes were enriched from the homogenates at the interface of a 40-60% discontinuous Percoll (GE Healthcare Bio-Sciences AB) gradient.

**Cell sorting**

Marginal zone macrophages and marginal metallophilic macrophages were sorted from uninfected and LCMV-infected spleens at one day post-infection. Macrophages were enriched by negative selection using a biotinylated antibody cocktail (CD4, CD19, and Thy1.2), streptavidin microbeads, and an autoMACS instrument (Miltenyi Biotec). Enriched cells were sorted using antibodies to CD11b, CD169, CD209b, and DAPI on a FACSAria (BD Biosciences). For some experiments, naïve, effector, or memory P14 T cells were enriched by negative selection using a biotinylated antibody cocktail (CD4,CD19, and I-$A^b$), streptavidin microbeads, and an autoMACS instrument. Enriched cells were stained with antibodies to CD8 and CXCR3 to sort for naïve cells; antibodies to CD8, CD45.1, and KLRG1 to sort for effector cells; or antibodies to CD8, CD45.1, CD90.1, and CD62L to sort for memory cells. The purity of FACS-sorted T cells was ~99% and the purity of enriched T cells isolated by AutoMACS was ~90%.

**Antibodies and reagents**

Anti-LCMV serum and MHC I tetramers were generous gifts from Dr. R. Ahmed (Emory University School of Medicine, Atlanta, GA). CCL19-Fc was a generous gift from Dr. J. Cyster (University of California, San Francisco, CA). All antibodies were purchased from Biolegend except for anti-mouse KLRG1 (Southern Biotech and eBioscience); anti- mouse CXCL9 (R&D Systems); anti-mouse CD25, CD45.1, CD62L, CD90.1, CD90.2, CD169, and CD209b (eBioscience); anti-mouse CD45R and CD122 (BD Pharmingen); anti-mouse CD4, CD11b, CD16/32, CD19, and Ly6G (UCSF Antibody Core Facility); anti-human Granzyme B (Invitrogen); anti-human LFA3 (Biogen); and anti-human IgG, anti-guinea pig IgG, and anti-goat IgG (Jackson Immunoresearch).

**Quantitative RT-PCR**

Previously snap frozen tissues were homogenized and RNA was isolated using TRIzol Reagent (Invitrogen) per manufacturer's instructions. Total RNA was reverse- transcribed using a M-MLV reverse transcriptase kit (Invitrogen). The resulting cDNA was analyzed for expression of different genes by quantitative PCR using FastStart Universal SYBR Green Master (ROX) (Roche) or AmpliTaq Gold DNA polymerase (Applied Biosystems) on an ABI 7300 Real Time PCR System (Applied Biosystems). Relative gene expression was normalized to the housekeeping genes, hypoxanthine phosphoribosyltransferase (*Hprt)* or glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). PCR primer pairs were as follows: *Hprt*: 5'-AGGTTGCAAGCTTGCTGGT and 5'-TGAAGTACTCATTATAGTCAAGGGCA,

probe 5'- TGTTGGATACAGGCCAGACTTTGTTGGAT; *Gapdh*: 5'-GGTCTACATGTTCCAGTATGACTCCAC and 5'-GGGTCTCGCTCCTGGAAGAT; *Cxcl9*: 5'-CCCAAGCCCCAATTGCA and 5'-ATTTGCCGAGTCCGGATCTAG, probe 5'-CAAAACTGAAATCATTGCTACACTGAAGAACGGAG; *Cxcl10*: 5'-AGCACCATGAACCCAA and 5'- CAGTTGCAGCGGACCGTC, probe 5'-TGGGACTCAAGGGATCCCTCTCGC; and LCMV *S segment*: 5'-CGTCATTGAGCGGAGTCTGT and 5'-AGATCATGAGGTCTGAAAGGC.

**Retroviral transduction**

The mouse stem cell virus (MSCV)-IRES-Thy1.1 vector was a generous gift from Dr. A. Abbas (University of California, San Francisco, CA). cDNA was made from LCMV- infected spleens and *Cxcr3* was amplified by PCR using the following primers: 5'-TAGTAGGCGGCCGCACCATGTACCTTGAGGTTAGTGAACGTCAA and 5'TAGTAGATCGATGAATTACAAGCCCAGGTAGGAGGC. The amplified product was cloned into NotI and ClaI restriction sites of the MSCV-IRES-Thy1.1 vector and the sequence was verified by automated sequencing. Retroviruses were packaged by transient transfection of Phoenix cells using Lipofectamine 2000 (Invitrogen) per manufacturer's instructions. For retroviral transduction of P14 T cells, CD45.1 CXCR3 KO P14 mice were infected i.v. with $2x10^6$ PFU LCMV Armstrong. One day later, P14 T cells from infected spleens were transduced with empty MSCV-IRES-Thy1.1 vector or MSCV-CXCR3-IRES-

Thy1.1 vector by spin infection (2000 rpm for two hours at 30°C). C57BL/6 recipients were i.p. infected with $2x10^5$ PFU LCMV Armstrong one to two hours before receiving $2x10^5$ transduced CXCR3 KO P14 T cells.

**CFSE, BrdU, Annexin V, Chemokine receptors and Chemotaxis**

Spleens and lymph nodes were enriched for naïve P14 T cells as described above. Enriched P14 T cells were incubated in PBS with 7 μM CFSE (5-(and-6)- carboxyfluorescein diacetate, succinimidyl ester; Invitrogen) for 20 minutes at room temperature. The cells were quenched with FBS and washed in RPMI 1640 + 10% FBS. $1x10^6$ CFSE-labelled P14 T cells were adoptively transferred into naïve C57BL/6 mice. The next day, recipients were infected i.v. with $2x10^5$ PFU LCMV Armstrong. Mice were injected i.p. with 1 mg BrdU (5-bromo-deoxyuridine; BD Pharmingen) at 5, 6, and 7 days post-infection. Spleens were harvested 1 hour later and the FITC BrdU Flow Kit (BD Pharmingen) was used per manufacturer's instructions. Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen) was used per manufacturer's instructions. To stain for chemokine receptors, cells were first incubated for thirty minutes at 37° C. For CCR7, cells were stained with CCL19-Fc or hLFA3-Fc as a control for thirty minutes. Chemotaxis assays were performed as described (50).

**Intracellular staining**

Spleen cells were cultured in media as described in the presence of 1 μg/ml GolgiPlug(BD Biosciences) and 0.2 μg/ml of LCMV GP33-41 peptide (27).

After 5 h of culture, cells were stained for surface markers, washed and fixed with 2% formaldehyde, and then stained for intracellular cytokines in the presence of 0.5% saponin.

**Immunofluorescence and Imaging**

Mice were euthanized and spleens were embedded in optimum cutting temperature embedding compound (Sakura Finetek) and then frozen. Six μm-thick sections were cut with a cryomicrotome (Leica Microsystems) and collected onto Superfrost/Plus microscope slides (Fisher Scientific). Acetone-fixed sections were blocked with 5% non- fat dry milk and stained with fluorescent antibodies for 45 minutes in tris-buffered saline containing 0.1% BSA, 1% normal mouse serum, and 1% normal donkey serum. Spleen sections were imaged using the Zeiss Axio Imager M1 upright microscope (Carl Zeiss Microimaging, Inc.), a Zeiss objective with a 20x magnification and a 0.8 aperture, and an AxioCam MRc camera. Axiovision software program MosaiX was used to generate a stitched image from individual tiles.

**Statistical Analysis**

Prism software (GraphPad Software, Inc.) was used for all statistical analyses. All results are expressed as mean ± SEM. Two groups were compared using the two-tailed Student's *t* test or two-way ANOVA as noted in figure legends. $P < 0.05$ was considered significant.

## REFERENCES


1. Williams MA, Bevan MJ (2007) Effector and memory CTL differentiation. *Annu Rev Immunol* 25:171–192.
2. Gerlach C, van Heijst JW, Swart E, Sie D, Armstrong N, Kerkhoven RM, Zehn D, Bevan MJ, Schepers K, Schumacher TN (2010) One naive T cell, multiple fates in CD8+ T cell differentiation. *J Exp Med* 207:1235–1246.
3. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, Gapin L, Kaech SM (2007) Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27:281–295.
4. Sarkar S, Kalia V, Haining WN, Konieczny BT, Subramaniam S, Ahmed R (2008) Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J Exp Med* 205:625–640.
5. Bromley SK, Peterson DA, Gunn MD, Dustin ML (2000) Cutting edge: hierarchy of chemokine receptor and TCR signals regulating T cell migration and proliferation. *J Immunol* 165:15–19.
6. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, Cyster JG (2004) Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* 427:355–360.
7. Potsch C, Vohringer D, Pircher H (1999) Distinct migration patterns of naive and effector CD8 T cells in the spleen: correlation with CCR7 receptor expression and chemokine reactivity. *Eur J Immunol* 29:3562–3570.
8. Sallusto F, Kremmer E, Palermo B, Hoy A, Ponath P, Qin S, Forster R, Lipp M, Lanzavecchia A (1999) Switch in chemokine receptor expression upon TCR stimulation reveals novel homing potential for recently activated T cells. *Eur J Immunol* 29:2037–2045.
9. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A (1998) Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* 187:875–883.
10. Campanella GS, Tager AM, El Khoury JK, Thomas SY, Abrazinski TA, Manice LA, Colvin RA, Luster AD (2008) Chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 are required for the development of murine cerebral malaria. *Proc Natl Acad Sci U S A* 105:4814–4819.
11. Hancock WW, Lu B, Gao W, Csizmadia V, Faia K, King JA, Smiley ST, Ling M, Gerard NP, Gerard C (2000) Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *J Exp Med* 192:1515–1520.
12. Nakanishi Y, Lu B, Gerard C, Iwasaki A (2009) CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. *Nature* 462:510–513.
13. Thapa M, Carr DJ (2009) CXCR3 deficiency increases susceptibility to genital herpes simplex virus type 2 infection: Uncoupling of CD8+ T-cell effector function but not migration. *J Virol* 83:9486–9501.

14. Wuest TR, Carr DJ (2008) Dysregulation of CXCR3 signaling due to CXCL10 deficiency impairs the antiviral response to herpes simplex virus 1 infection. *J Immunol* 181:7985–7993.
15. Zhang B, Chan YK, Lu B, Diamond MS, Klein RS (2008) CXCR3 mediates region- specific antiviral T cell trafficking within the central nervous system during West Nile virus encephalitis. *J Immunol* 180:2641–2649.
16. Farber JM (1990) A macrophage mRNA selectively induced by gamma-interferon encodes a member of the platelet factor 4 family of cytokines. *Proc Natl Acad Sci U S A* 87:5238–5242.
17. Luster AD, Ravetch JV (1987) Genomic characterization of a gamma-interferon- inducible gene (IP-10) and identification of an interferon-inducible hypersensitive site. *Mol Cell Biol* 7:3723–3731.
18. Ohmori Y, Hamilton TA (1990) A macrophage LPS-inducible early gene encodes the murine homologue of IP-10. *Biochem Biophys Res Commun* 168:1261–1267.
19. Vanguri P, Farber JM (1990) Identification of CRG-2. An interferon-inducible mRNA predicted to encode a murine monokine. *J Biol Chem* 265:15049–15057.
20. Jung YW, Rutishauser RL, Joshi NS, Haberman AM, Kaech SM (2010) Differential localization of effector and memory CD8 T cell subsets in lymphoid organs during acute viral infection. *J Immunol* 185:5315–5325.
21. Christensen JE, de Lemos C, Moos T, Christensen JP, Thomsen AR (2006) CXCL10 is the key ligand for CXCR3 on CD8+ effector T cells involved in immune surveillance of the lymphocytic choriomeningitis virus-infected central nervous system. *J Immunol* 176:4235–4243.
22. Hsieh MF, Lai SL, Chen JP, Sung JM, Lin YL, Wu-Hsieh BA, Gerard C, Luster A, Liao F (2006) Both CXCR3 and CXCL10/IFN-inducible protein 10 are required for resistance to primary infection by dengue virus. *J Immunol* 177:1855–1863.
23. Kohlmeier JE, Cookenham T, Miller SC, Roberts AD, Christensen JP, Thomsen AR, Woodland DL (2009) CXCR3 directs antigen-specific effector CD4+ T cell migration to the lung during parainfluenza virus infection. *J Immunol* 183:4378–4384.
24. Stiles LN, Liu MT, Kane JA, Lane TE (2009) CXCL10 and trafficking of virus- specific T cells during coronavirus-induced demyelination. *Autoimmunity* 42:484–491.
25. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, Larsen CP, Ahmed R (2009) mTOR regulates memory CD8 T-cell differentiation. *Nature* 460:108–112.
26. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708–712.
27. Wherry EJ, Teichgraber V, Becker TC, Masopust D, Kaech SM, Antia R, von Andrian UH, Ahmed R (2003) Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 4:225–234.

28. Seiler P, Aichele P, Odermatt B, Hengartner H, Zinkernagel RM, Schwendener RA (1997) Crucial role of marginal zone macrophages and marginal zone metallophils in the clearance of lymphocytic choriomeningitis virus infection. *Eur J Immunol* 27:2626–2633.
29. Ahmed R, Gray D (1996) Immunological memory and protective immunity: understanding their relation. *Science* 272:54–60.
30. Kaech SM, Wherry EJ (2007) Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity* 27:393–405.
31. Unsoeld H, Voehringer D, Krautwald S, Pircher H (2004) Constitutive expression of CCR7 directs effector CD8 T cells into the splenic white pulp and impairs functional activity. *J Immunol* 173:3013–3019.
32. Kwan J, Killeen N (2004) CCR7 directs the migration of thymocytes into the thymic medulla. *J Immunol* 172:3999–4007.
33. Badovinac VP, Porter BB, Harty JT (2002) Programmed contraction of CD8(+) T cells after infection. *Nat Immunol* 3:619–626.
34. Kaech SM, Ahmed R (2001) Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat Immunol* 2:415–422.
35. Mercado R, Vijh S, Allen SE, Kerksiek K, Pilip IM, Pamer EG (2000) Early programming of T cell populations responding to bacterial infection. *J Immunol* 165:6833–6839.
36. Zehn D, Lee SY, Bevan MJ (2009) Complete but curtailed T-cell response to very low-affinity antigen. *Nature* 458:211–214.
37. Dauner JG, Williams IR, Jacob J (2008) Differential microenvironment localization of effector and memory CD8 T cells. *J Immunol* 180:291–299.
38. Khanna KM, McNamara JT, Lefrancois L (2007) In situ imaging of the endogenous CD8 T cell response to infection. *Science* 318:116–120.
39. Rutishauser RL, Martins GA, Kalachikov S, Chandele A, Parish IA, Meffre E, Jacob J, Calame K, Kaech SM (2009) Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 31:296–308.
40. Wherry EJ, McElhaugh MJ, Eisenlohr LC (2002) Generation of CD8(+) T cell memory in response to low, high, and excessive levels of epitope. *J Immunol* 168:4455–4461.
41. Hand TW, Morre M, Kaech SM (2007) Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. *Proc Natl Acad Sci U S A* 104:11730–11735.
42. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang LS, Jones RG, Choi Y (2009) Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* 460:103–107.
43. Prlic M, Bevan MJ (2009) Immunology: A metabolic switch to memory. *Nature* 460:41–42.
44. Jones RG, Thompson CB (2007) Revving the engine: signal transduction fuels T cell activation. *Immunity* 27:173–178.
45. Sinclair LV, Finlay D, Feijoo C, Cornish GH, Gray A, Ager A, Okkenhaug K, Hagenbeek TJ, Spits H, Cantrell DA (2008) Phosphatidylinositol-3-OH

kinase and nutrient-sensing mTOR pathways control T lymphocyte trafficking. *Nat Immunol* 9:513–521.
46. Haring JS, Badovinac VP, Harty JT (2006) Inflaming the CD8+ T cell response. *Immunity* 25:19–29.
47. Badovinac VP, Tvinnereim AR, Harty JT (2000) Regulation of antigen-specific CD8+ T cell homeostasis by perforin and interferon-gamma. *Science* 290:1354–1358.
48. Whitmire JK, Tan JT, Whitton JL (2005) Interferon-gamma acts directly on CD8+ T cells to increase their abundance during virus infection. *J Exp Med* 201:1053–1059.
49. Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, Germain RN (2006) Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature* 440:890–895.
50. Matloubian M, David A, Engel S, Ryan JE, Cyster JG (2000) A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nat Immunol* 1:298–30

**FIGURE LEGENDS:**

**Figure 1. CXCR3 is gradually up-regulated on antigen specific CD8 T cells during the course of infection.**

C57BL/6 mice containing CD45.1/CD45.2 WT P14 T cells were infected with LCMV. **(A)** Expression of CXCR3 on splenic P14 T cells was determined by flow cytometry at the indicated times after infection. Plots are gated on $CD8^+$ $CD45.1^+$ P14 T cells. **(B)** Cell surface expression of CXCR3 on $KLRG1^{hi}$ and $KLRG1^{lo}$ subsets of P14 T cells was determined at the indicated times after infection. (A-B) Data shown are representative of three independent experiments (n=6 for each time). **(C)** Expression of mRNA for CXCR3 ligands CXCL9 and CXCL10 in spleen samples was measured by quantitative RT-PCR at the indicated times after infection. Data from one representative experiment are shown (n=2-3 for each time). **(D)** Expression of CXCR3 mRNA in sorted $KLRG1^{hi}$ and $KLRG1^{lo}$ P14 T cells was measured by quantitative RT-PCR at the indicated times. Data shown are averages of three independently sorted samples. (C-D) Graphs show mean data ± SEM. **(E)** Chemotactic response of splenic $KLRG1^{hi}$ and $KLRG1^{lo}$ WT and CXCR3 deficient P14 T cells to 1 $\mu g\ ml^{-1}$ CCL21, 1 $\mu g\ ml^{-1}$ CXCL9, and 0.3 $\mu g\ ml^{-1}$ CXCL12 was determined at the indicated times after infection. Data shown are averages of duplicate samples with bars representing the range.

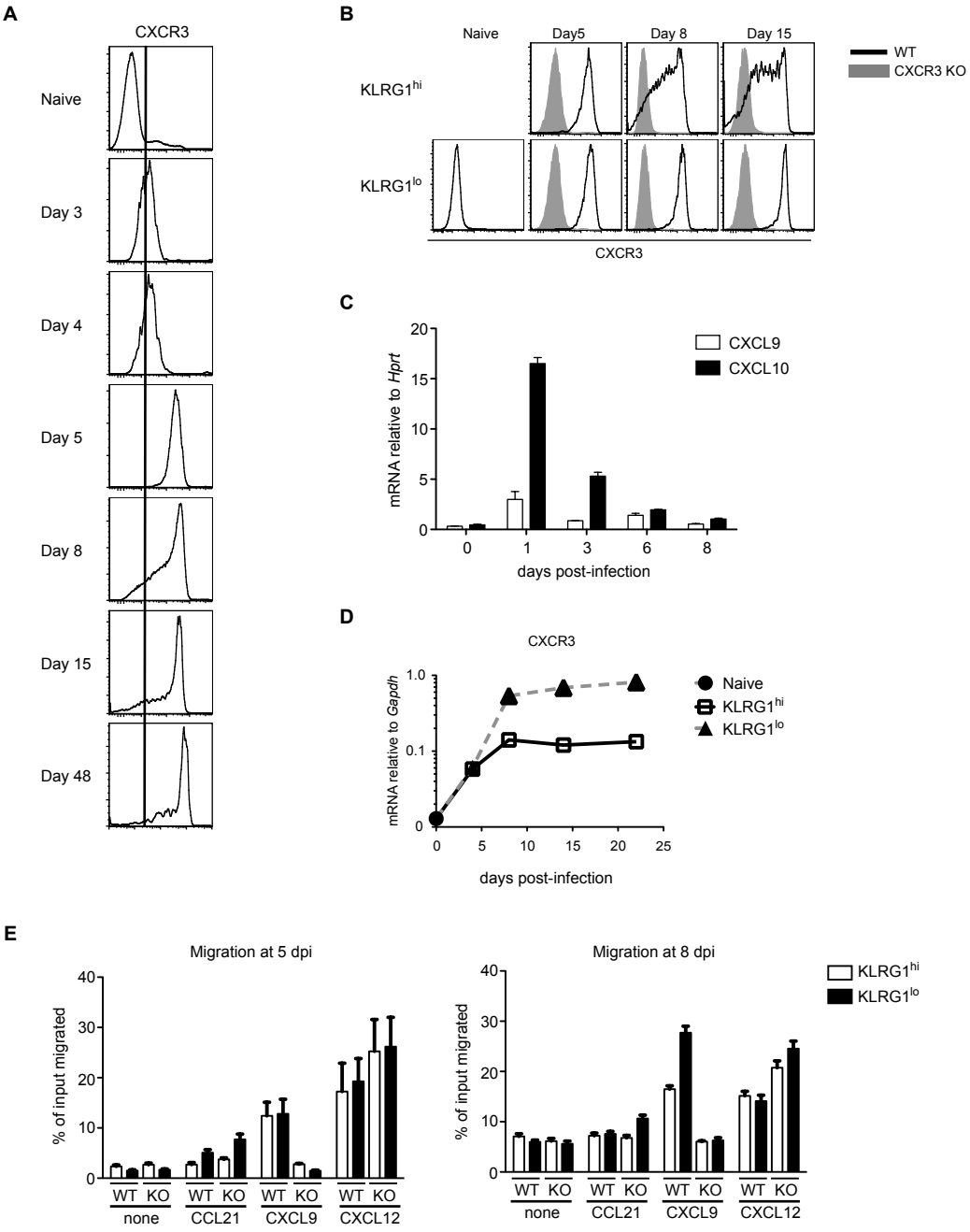



**Figure 1. Hu et al.**

**Figure 2. Absence of CXCR3 expression on CD8 T cells leads to the development of more memory precursor and fewer terminally differentiated effector CD8 T cells.**

Mice containing equal numbers of CD45.1/CD45.2 WT and CD45.1/CD45.1 CXCR3 KO P14 T cells were infected with LCMV. **(A)** Dot plots on the left show frequencies of WT and CXCR3 KO P14 T cells within the CD8 T cell population in blood and spleen on day 8 post-infection. Density plots on the right show surface expression of KLRG1 and IL-7R$\alpha$ on WT and CXCR3 KO P14 T cells in spleen at the indicated times after infection. **(B)** Expression levels of cell surface markers and intracellular cytokines in WT and CXCR3 KO P14 T cells was determined on day 8 post-infection. **(C)** Total number of WT and CXCR3 KO P14 T cells and their KLRG1$^{hi}$IL-7R$^{lo}$ and KLRG1$^{lo}$IL-7R$^{hi}$ subsets in spleen were determined at the indicated times after infection. (A-C) Data are representative of three independent experiments (n=4-8 for each time). Graphs show mean data ± SEM. Statistics were done using a two-tailed unpaired Student's *t* test.

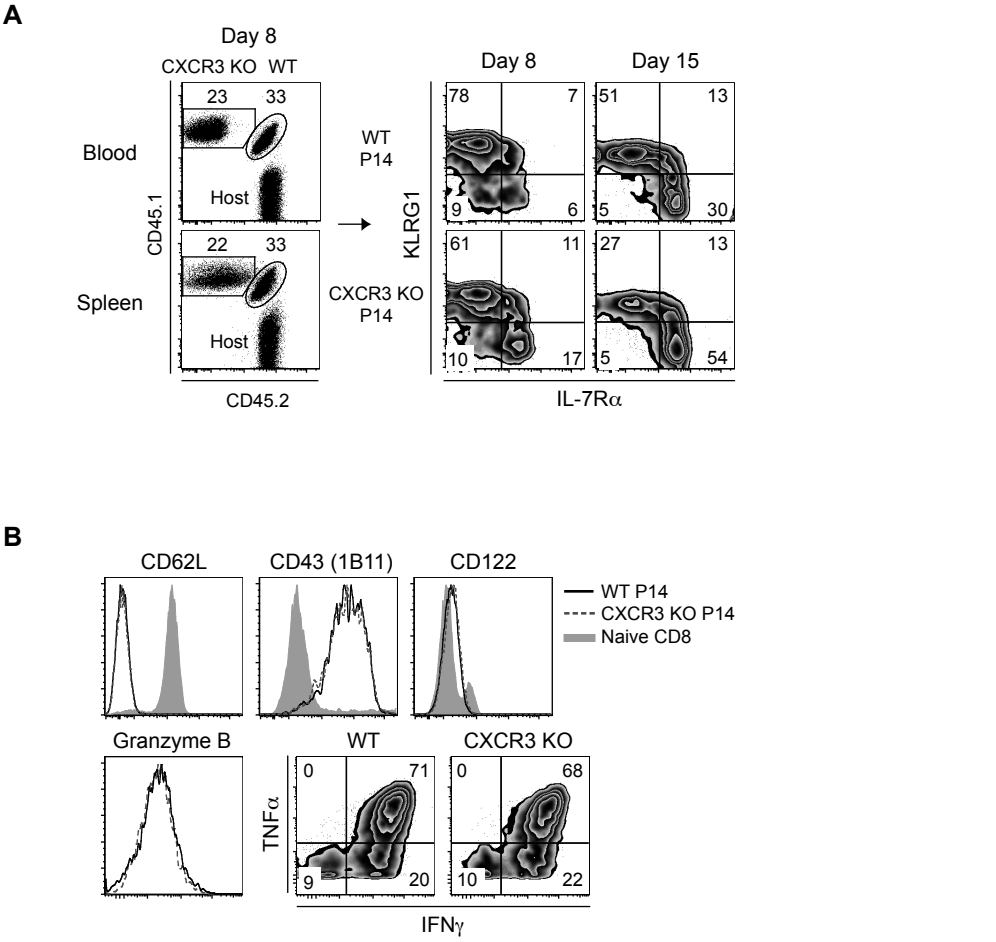



**Figure 2. Hu et al.**

**Figure 3. Restoration of CXCR3 expression in CXCR3 deficient CD8 T cells leads to development of more terminally differentiated effector and fewer memory precursor cells.**

**(A**) CD45.1+ CXCR3 deficient P14 T cells were activated *in vivo* and, the next day, transduced *ex-vivo* with retroviral preparations expressing either nothing (empty vector) or mouse CXCR3 followed by IRES-Thy1.1. C57BL/6 mice that were infected a few hours earlier with LCMV received $2x10^5$ transduced CXCR3 KO P14 T cells and were analyzed at the indicated times after infection. **(B)** Surface expression of CXCR3 on transduced CXCR3 KO P14 T cells in spleens of recipients was determined at the indicated times after infection. Histograms are gated on $CD8^+$ $CD45.1^+$ $Thy1.1^+$ transduced or $CD8^+$ $CD45.1^+$ $Thy1.1^-$ non-transduced CXCR3 KO P14 T cells within the same recipient mouse. Dotted histograms show CXCR3 expression on WT P14 T cells at the indicated times. **(C)** Transduced ($Thy1.1^+$) and non-transduced ($Thy1.1^-$) cells were gated from adoptively transferred CXCR3 KO P14 T cells and analyzed for surface expression of KLRG1 and IL-7R$\alpha$. **(D**) Frequencies of $KLRG1^{hi}IL\text{-}7R^{lo}$ and $KLRG1^{lo}IL\text{-}7R^{hi}$ effector cell subsets in transduced (black bars) and non-transduced (white bars) CXCR3 KO P14 T cells in spleens of recipient mice were determined at the indicated times after infection. **(A-D)** Data shown are representative of three independent experiments (n=2-8 per group per day). Graphs show mean ± SEM. Statistics were done using a two-tailed unpaired Student's *t* test.

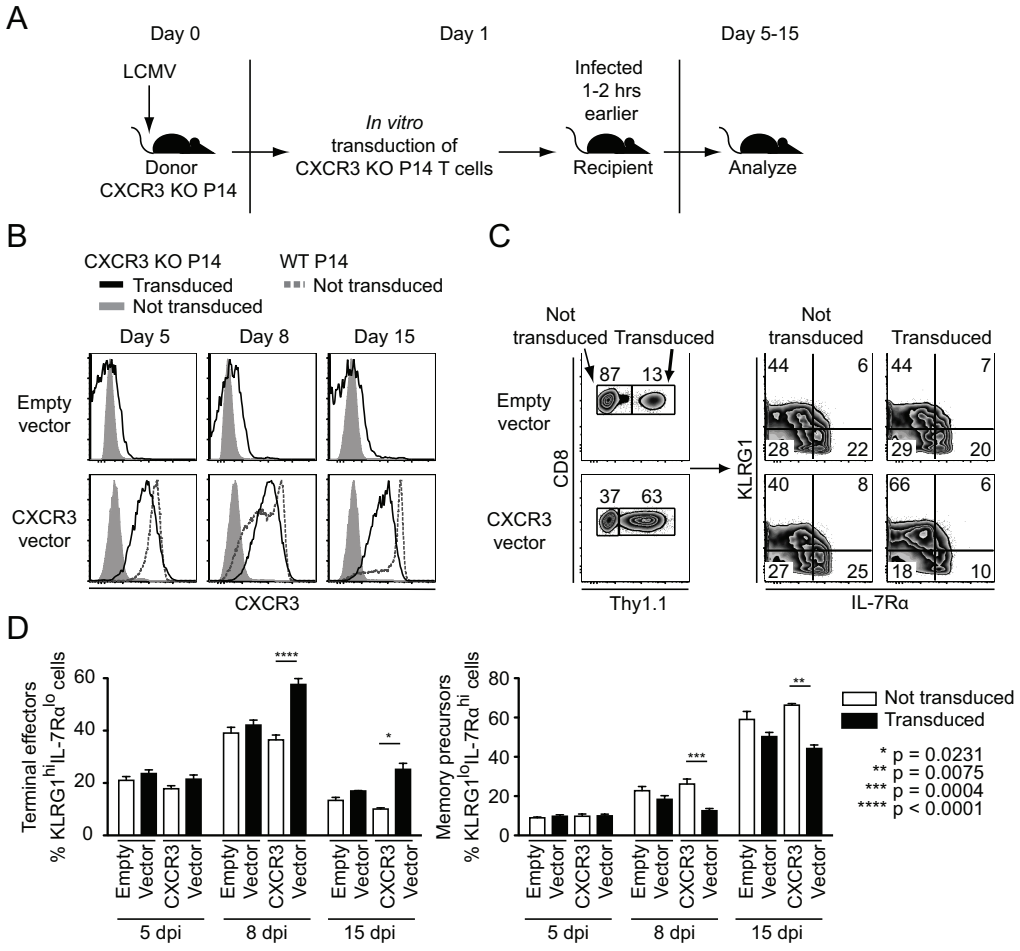



**Figure 3. Hu et al.**

**Figure 4. Absence of CXCR3 leads to more rapid phenotypic and functional maturation of memory CD8 T cells.**

**(A-C)** C57BL/6 mice containing equal numbers of WT and CXCR3 KO P14 T cells were infected with LCMV. **(A)** Ratio of CXCR3 KO to WT memory P14 T cells in blood or the indicated tissues. Data are combined for days 40 and 60 post-infection (n=2 mice for each time) and are representative of three experiments. **(B)** At the indicated times after infection, WT and CXCR3 KO memory P14 T cells in blood were analyzed by flow cytometry for expression of KLRG1 and IL-7R$\alpha$ (left panel) and CD62L and CCR7 (right panel). Plots show data from one representative experiment (n=2-4 mice for each time). **(C)** Frequencies of effector memory ($CD62L^{lo}$ $CCR7^{lo}$), central memory ($CD62L^{hi}$ $CCR7^{hi}$), and $CCR7^{hi}$ cells in WT and CXCR3 KO P14 T cell populations in various tissues were determined at the indicated times after infection. Plots show combined data from three independent experiments (n=2-4 for each time). **(D)** Historgrams show proportion of WT and CXCR3 KO memory P14 T cells in the spleen that express IL-2 or CD27 on days 60 and 40 post-infection, respectively. Gray historgrams correspond to unstimulated P14 T cells for IL-2 and non-TCR Tg naïve CD8 T cells for CD27. Corresponding bar graphs show the frequencies of IL-2 expressing cells and $CD27^{hi}$ cells in the spleen. Data are from two independent experiments (n=4 mice per group). Statistics were done using a two-tailed unpaired Student's *t* test. **(E)** Recall response of WT and CXCR3 KO memory P14 T cells to secondary infection. B6.BoyJ (CD45.1) mice containing CD45.2/CD45.2 WT P14 T cells and C57BL/6 mice containing CD45.1/CD45.1

CXCR3 KO P14 T cells were infected with LCMV. After 110 days post-infection, memory cells were sorted and $5x10^3$ $CD62L^+$ WT and $5x10^3$ $CD62L^+$ CXCR3 KO memory P14 T cells were transferred either separately (single transfer) or together in a 1:1 ratio (co-transfer) into naïve mice. Two days later, recipients were infected i.v. with $2x10^6$ PFU LCMV and the number of WT and CXCR3 KO P14 T cells in blood and spleen was determined at day 8 post-infection. Data shown are representative of three experiments (n=7 mice per group). Graphs show mean data ± SEM. Statistics were performed using two way ANOVA.

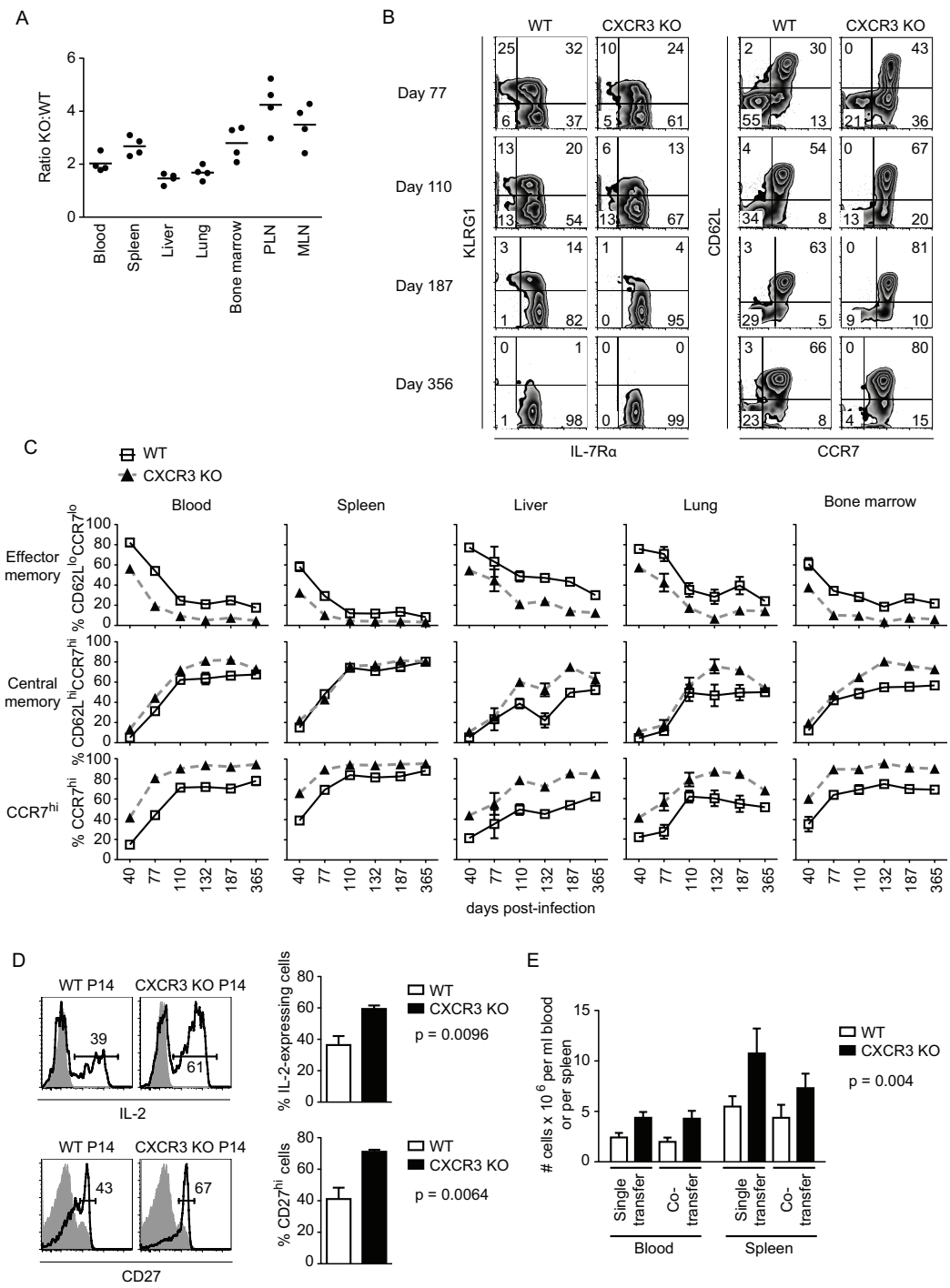



**Figure 4. Hu et al.**

**Figure 5. Effector CD8 T cells co-localize in the spleen with antigen and CXCL9 in a CXCR3-dependent manner.**

**(A and C)** Equal numbers of Thy1.1$^+$ WT and CD45.1/CD45.1 CXCR3 KO P14 T cells were co-transferred into C57BL/6 recipients that were then infected with LCMV. Consecutive sections were prepared from spleens taken at the indicated times after infection and were stained for viral antigen, CXCL9, WT P14 (Thy1.1), CXCR3 KO P14 (CD45.1), and B220. Data are representative of two independent experiments (n=4 for each time). Scale bars are 100 μm in length**.** **(B)** Expression of LCMV RNA or mRNA for CXCR3 ligands, CXCL9 and CXCL10, in marginal zone macrophages sorted from naïve mice or those infected a day earlier with LCMV was determined by quantitative RT-PCR. Expression is shown relative to *Gapdh* for LCMV RNA and relative to *Hprt* for CXCL9 and CXCL10. Data shown are averages of two independent sorted samples (n=4-5 mice for each sample).

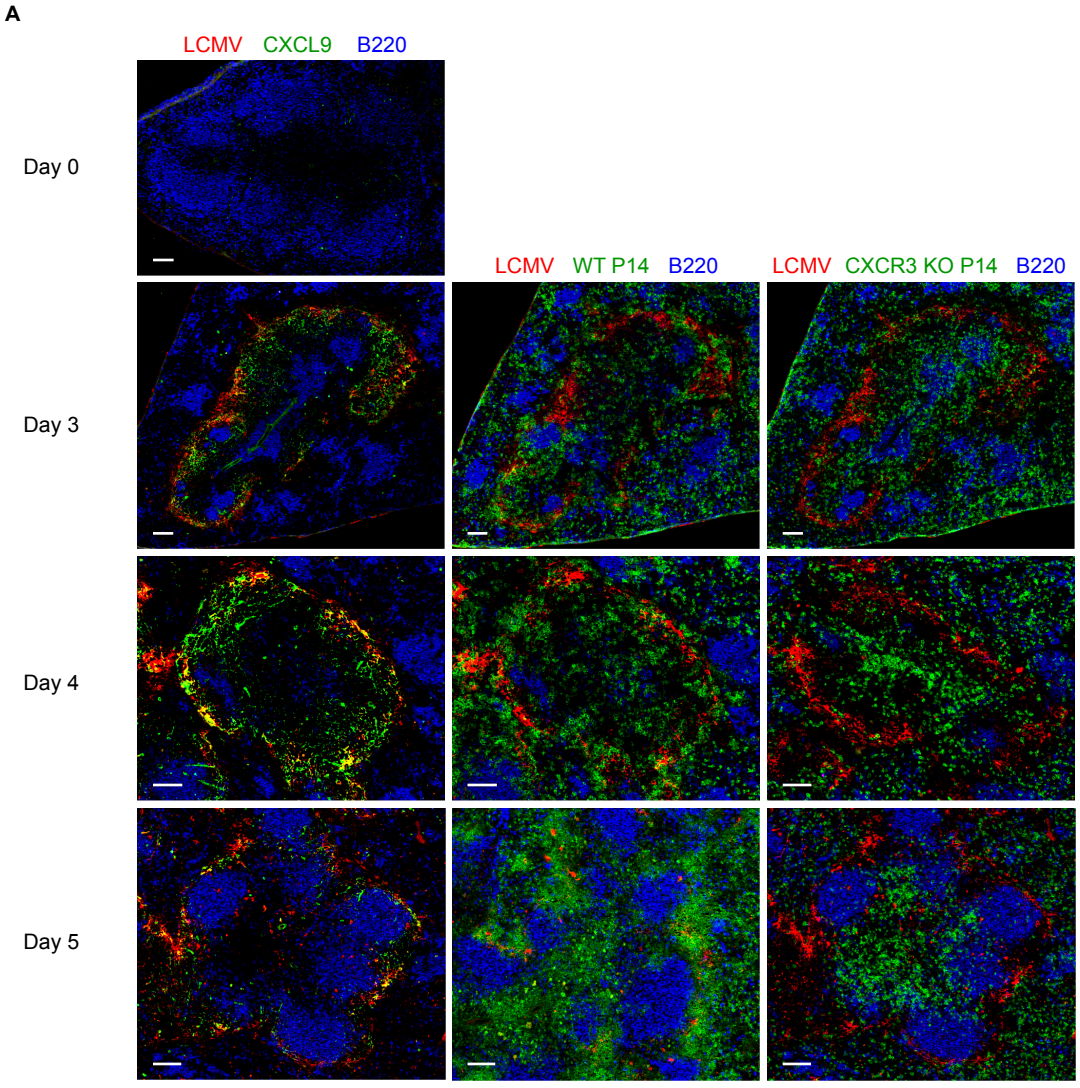



**Figure 5. Hu et al.**

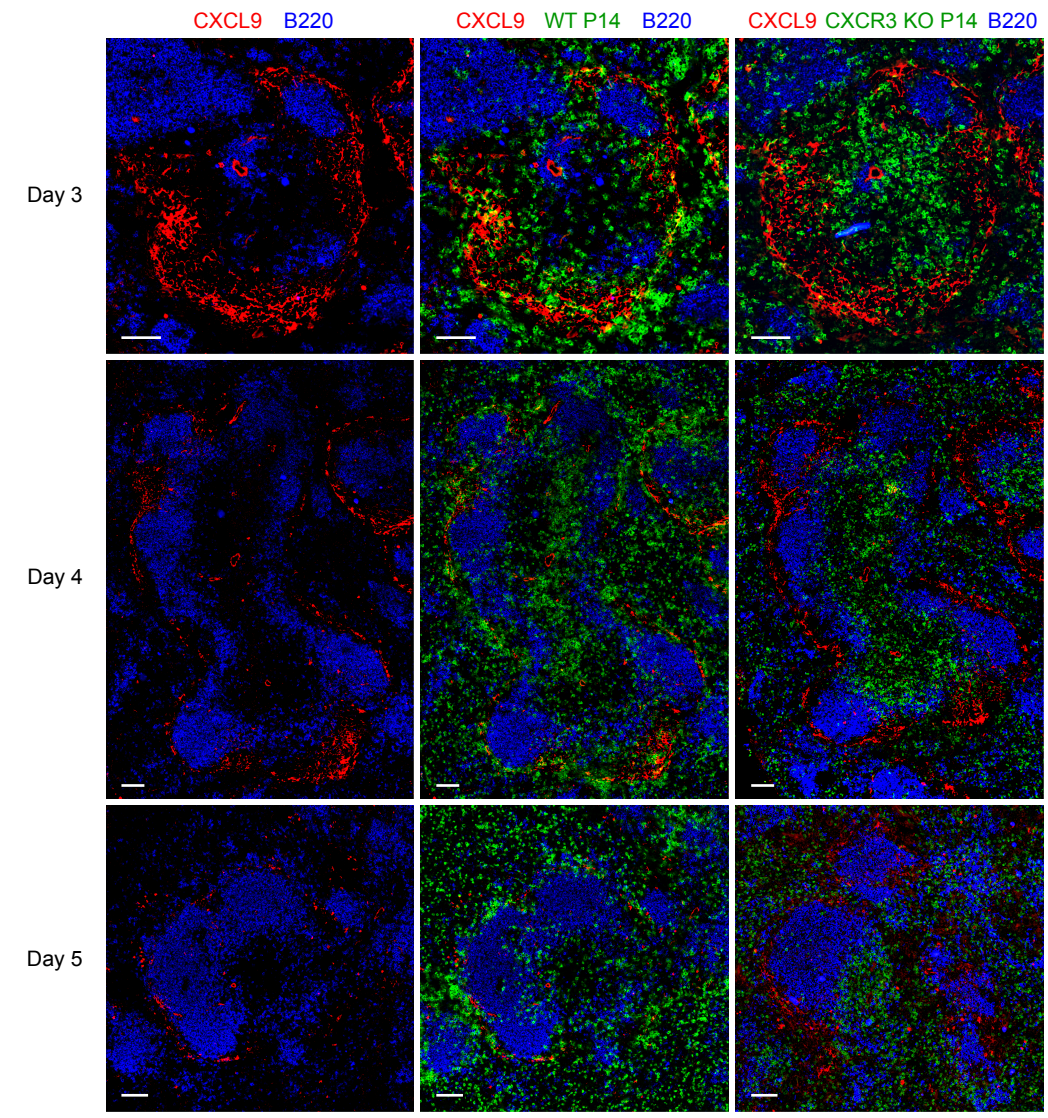



**Figure 5. Hu et al.**

**Figure 6. CXCR3 deficient CD8 T cells show reduced proliferation during the later stages of infection.**

**(A**) Mice containing equal numbers of CD45.1/CD45.2 WT and CD45.1/CD45.1 CXCR3 KO P14 T cells were infected with LCMV. Histograms show CD25 expression on WT and CXCR3 KO P14 T cells in spleen at one day post-infection. **(B**) Equal numbers of CFSE labeled CD45.1/CD45.2 WT and CD45.1/CD45.1 CXCR3 KO P14 T cells were transferred into C57BL/6 mice and then infected a day later with LCMV. Histograms show CFSE dilution of the indicated population within the spleen 2.5 days after infection. Data shown are representative of two independent experiments (n=4). **(C**) C57BL/6 mice containing equal numbers of CD45.1/CD45.2 *Cxcr3*$^{+/-}$ and CD45.1/CD45.1 *Cxcr3*$^{-/-}$ P14 T cells were infected with LCMV and the proportion of apoptotic cells in each population within the spleen was determined by flow cytometry at the indicated times. *Cxcr3*$^{+/-}$ and *Cxcr3*$^{-/-}$ P14 T cells were gated from CD8$^{+}$ PI$^{-}$ cells to determine the frequency of AnnexinV$^{+}$ apoptotic cells in each population. Data shown are representative of two mice at each time. **(D-E)** C57BL/6 mice containing equal numbers of CD45.1/CD45.2 WT and CD45.1/CD45.1 CXCR3 KO P14 T cells were infected with LCMV and at the indicated times after infection, were pulsed with BrdU for one hour and then analyzed by flow cytometry. (**D**) Dot plots show BrdU incorporation of WT and CXCR3 KO P14 T cells in spleen at the indicated times after infection. **(E**) Proportion of BrdU+ cells in KLRG1$^{lo}$ IL-7R$^{lo}$, KLRG1$^{hi}$ IL-7R$^{lo}$, and KLRG1$^{lo}$ IL-7R$^{hi}$ subsets of WT and CXCR3 KO P14 T cell populations was determined by flow cytometry at the

indicated times post-infection. Averages and SEM are shown. Data shown are representative of two independent experiments (n=4 for each time).

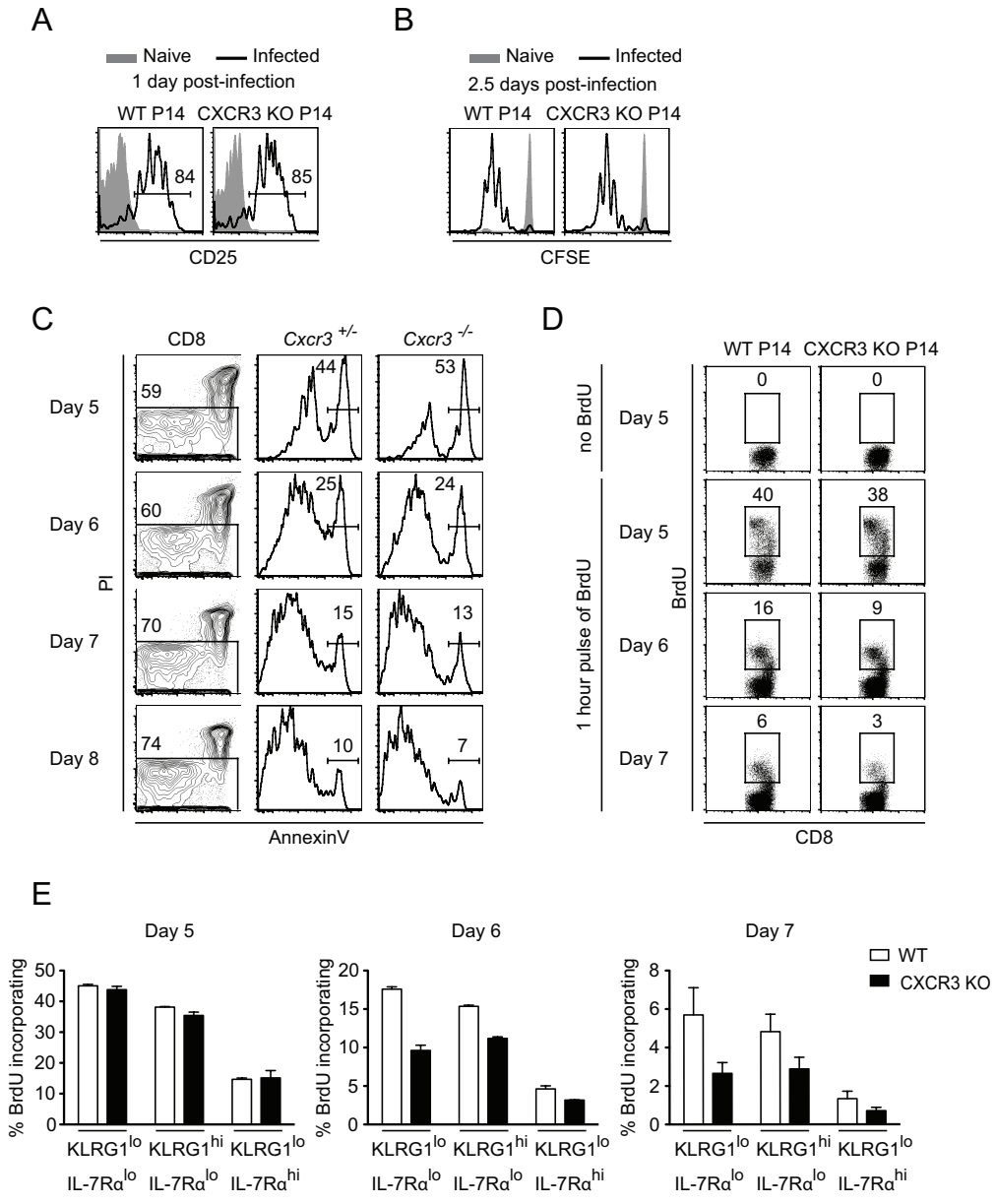



**Figure 6. Hu et al.**

**Figure 7. Persistent CCR7 expression tips the balance towards development of more memory precursors than terminally differentiated effector CD8 T cells.**

**(A-D**) B6.BoyJ (CD45.1) mice containing equal numbers of Thy1.1$^+$, CD45.2$^+$ WT and Thy1.2$^+$, CD45.2$^+$ CCR7 transgenic (Tg) P14 T cells were infected with LCMV and analyzed at the indicated times. **(A)** Histograms show surface expression of CCR7 on WT and CCR7 transgenic (Tg) P14 T cells in spleen at the indicated times after infection. Gray histograms show the isotype control. **(B)** Frequencies of WT and CCR7 Tg P14 T cells in the blood and spleen were determined on day 8 post-infection. Plots are gated on CD8$^+$ T cells. **(C)** Density plots show proportion of WT and CCR7 Tg CD8$^+$ P14 T cells that express KLRG1 and IL-7R$\alpha$ at the indicated times after infection. **(D)** Frequencies of KLRG1$^{lo}$ IL-7R$\alpha^{hi}$ memory precursor CD8 T cells within WT and CCR7 Tg P14 T cell populations in blood and spleen were determined by flow cytometry at the indicated times after infection. Data are representative of three independent experiments (n=9-11 mice per indicated time). Graphs show mean data ± SEM. Statistics were done using a two-tailed unpaired Student's *t* test. **(E)** C57BL/6 mice containing equal numbers of Thy1.1$^+$, CD45.2$^+$ WT and Thy1.2$^+$, CD45.1$^+$ CCR7 transgenic (Tg) P14 T cells were infected with LCMV. Sections were prepared from spleens at day 4 post-infection and stained for viral antigen, B220, and WT P14 (Thy1.1) and CCR7 Tg P14 (CD45.1) cells. Scale bars are 100 $\mu$m in length**.**

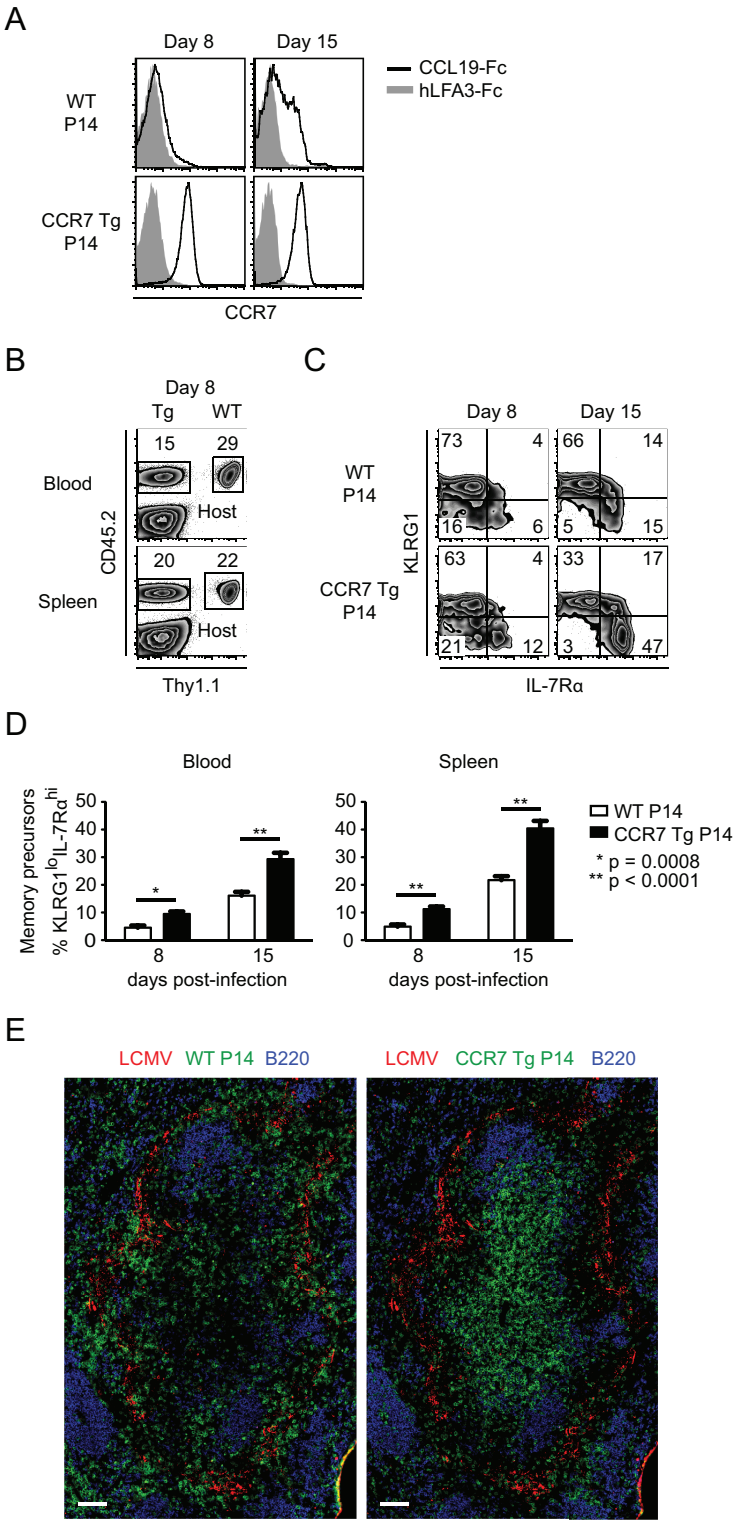
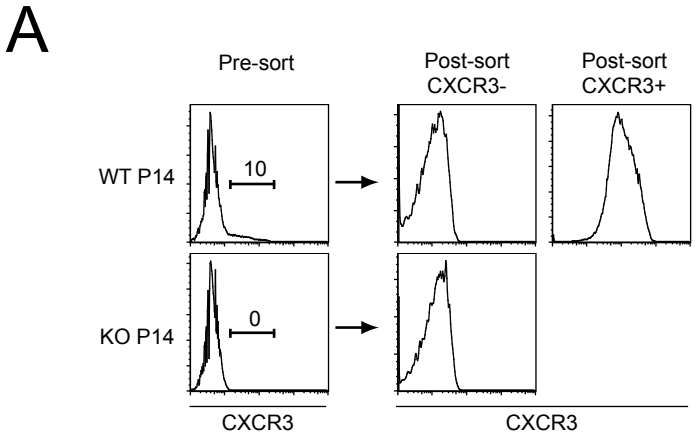
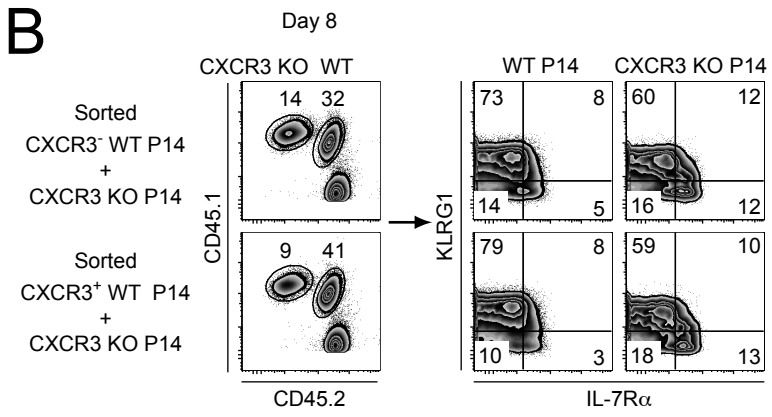



**Figure 7. Hu et al.**

**Supporting Figure Legends**

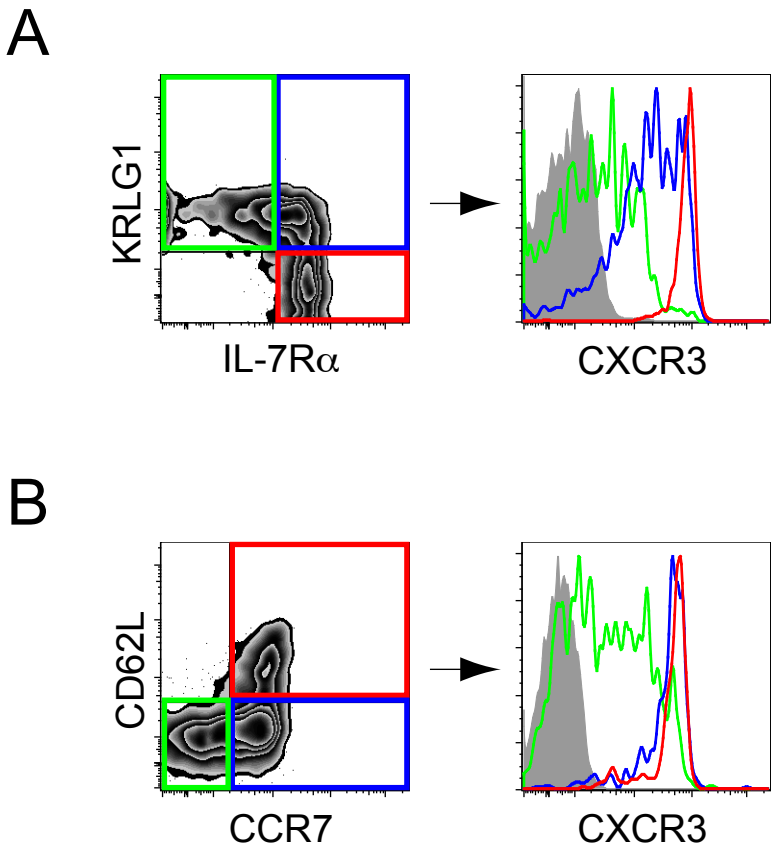
**Supplementary Figure 1. Early expression of CXCR3 leads to generation of more CD8$^+$ T cells at the peak of expansion. (A)** Naïve WT P14 T cells were sorted based on expression of CXCR3. Histograms are gated on CD8$^+$ TCR V$\alpha$2$^+$ P14 T cells and show expression of CXCR3 on pre-sort and post-sort populations. As a control, naïve CXCR3 deficient cells were sorted using the same cocktail of antibodies used for WT cells. **(B)** Equal numbers of either CXCR3$^-$ or CXCR3$^+$ CD45.1/CD45.2 WT P14 cells were co- transferred with CD45.1/CD45.1 CXCR3 KO P14 T cells into C57BL/6 mice that were then infected with LCMV. Left panel shows the proportion of each population within the CD8$^+$ gate in spleen at day 8 post-infection. Right panel shows proportion of cells within each P14 population that express KLRG1 and IL-7R$\alpha$. Data shown are representative of two independent experiments (n=4 for each time).



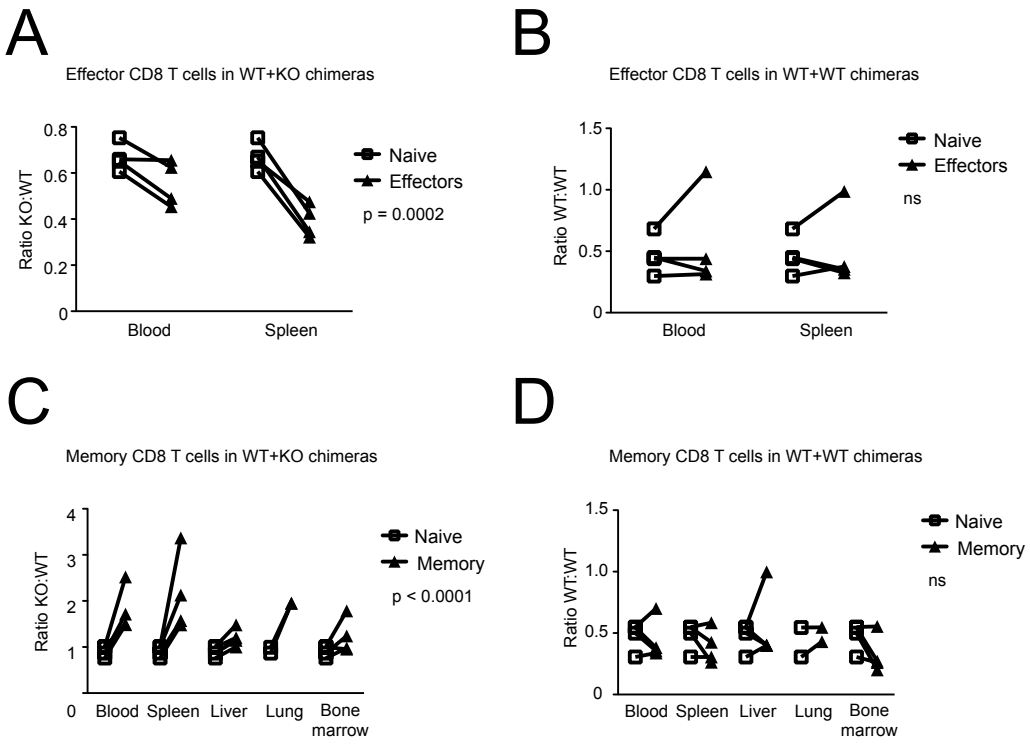

**Supplementary Figure 1. Hu et al.**

**Supplementary Figure 2. CXCR3 is differentially expressed on subsets of memory P14 T cells.** Memory cell subsets defined by the expression of **(A)** KLRG1 and IL-7Rα or **(B)** CD62L and CCR7 were gated for expression of CXCR3 in the spleen at day 60 post-infection. **(A-B)** Colored histograms on the right show CXCR3 expression within memory cell subsets designated by boxes of the same color on the left.



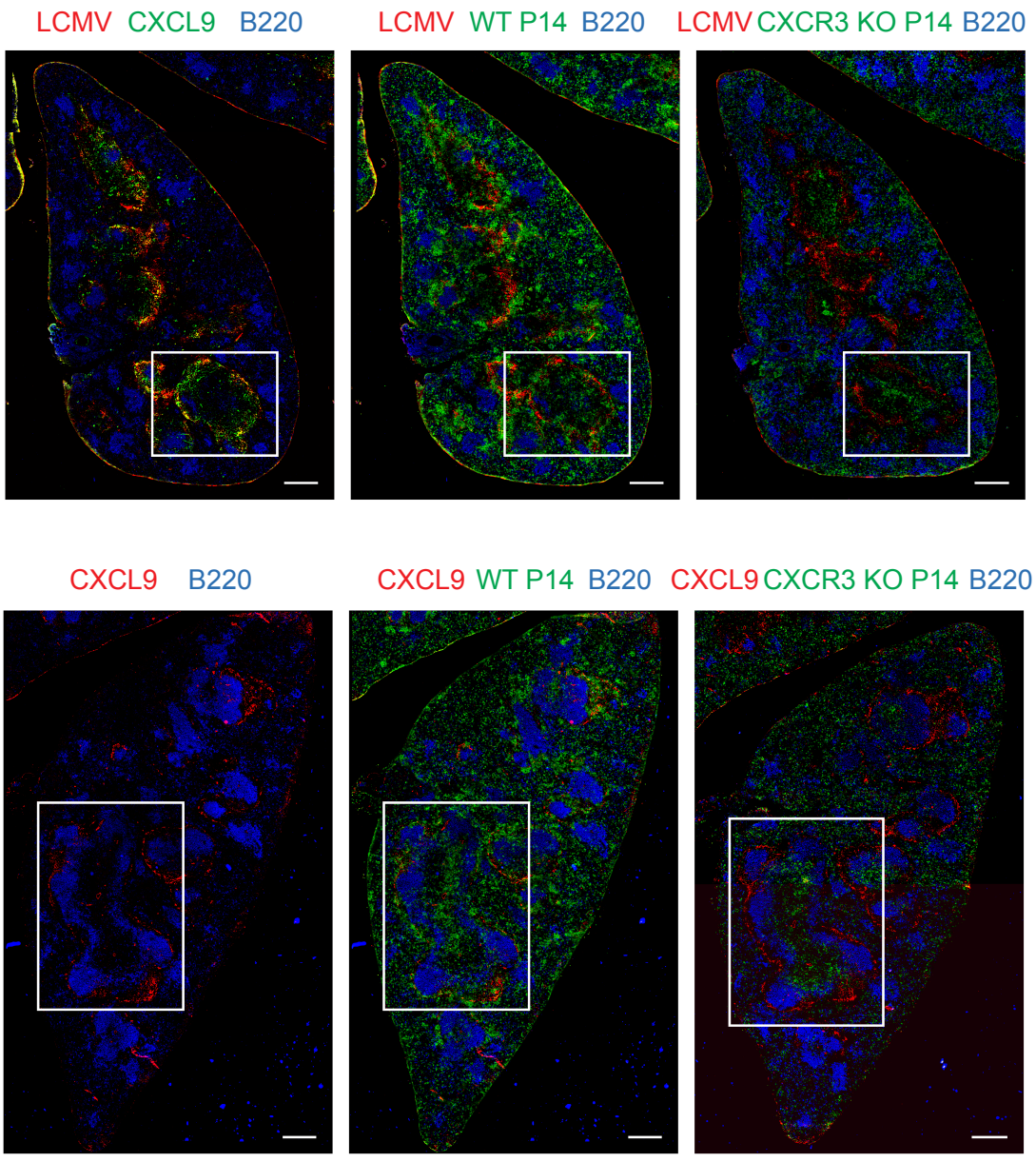

**Supplementary Figure 2. Hu et al.**

**Supplementary Figure 3. Non-TCR-transgenic CXCR3 deficient CD8 T cells generate fewer antigen specific effector cells and more memory cells after infection. (A-D)** Irradiated C57BL/6 mice were reconstituted with a 1:1 mixture of bone marrow cells from CD45.1/CD45.2 WT and CD45.1/CD45.1 CXCR3 KO mice or CD45.1/CD45.2 WT and CD45.1/CD45.1 WT mice as a control. Following reconstitution, chimeric mice were bled to determine the proportions of CD8+ T cells derived from each bone marrow donor population (labeled “Naïve”). The mice were then infected with LCMV and analyzed at either day 8 (effectors) or day 220$^{+}$ (memory) after infection to determine the ratio of tetramer$^{+}$ antigen specific CD8+ T cells derived from each bone marrow donor population in the indicated tissues. Empty squares show data before infection and filled triangles show tetramer$^{+}$ (GP33, GP276, NP396) effector or memory CD8 T cells after infection. A line connects data from individual mice. Data from one set of chimeras is shown (n=4 for each group). Statistics were done using two- way ANOVA.




**Supplementary Figure 3. Hu et al.**

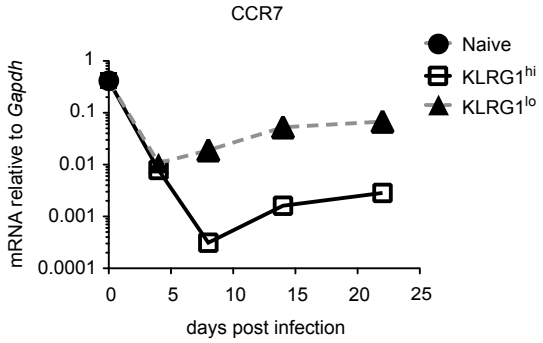
**Supplementary Figure 4. Effector CD8 T cells co-localize with antigen and CXCL9 in a CXCR3 dependent manner.** Equal numbers of Thy1.1$^+$ CD45.2/CD45.2 WT and Thy1.2+ CD45.1/CD45.1 CXCR3 KO P14 T cells were co-transferred into C57BL/6 recipients that were then infected with LCMV. Consecutive sections were prepared from spleens taken on day 4 post-infection and were stained for viral antigen, CXCL9, WT P14 (Thy1.1), CXCR3 KO P14 (CD45.1), and B220. Scale bars are 300 μm in length. Data are representative of two independent experiments (n=4 for each time). Boxes delineate areas shown at higher magnification in Fig. 5.



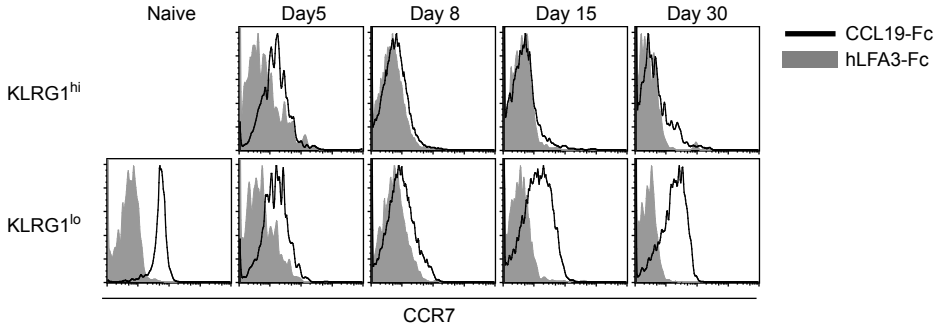

**Supplementary Figure 4. Hu et al.**

**Supplementary Figure 5. CCR7 is differentially expressed on KLRG1$^{hi}$ and KLRG1$^{lo}$ effector CD8 T cells. (A)** Expression of CCR7 mRNA in sorted KLRG1$^{hi}$ and KLRG1$^{lo}$ effector P14 CD8 T cells at the indicated times after infection was determined by qRT-PCR and is expressed relative to *Gapdh* mRNA. Data shown are averages of three independently sorted samples. **(B)** Cell surface expression of CCR7 on KLRG1$^{hi}$ and KLRG1$^{lo}$ CD8$^{+}$ effector P14 T cells at the indicated times after infection was determined by flow cytometry. Histograms are gated on P14 T cells or total CD8 T cells for the naïve mouse with isotype control shown as a shaded histogram. Data shown are representative of two independent experiments (n=4 for each time).

A

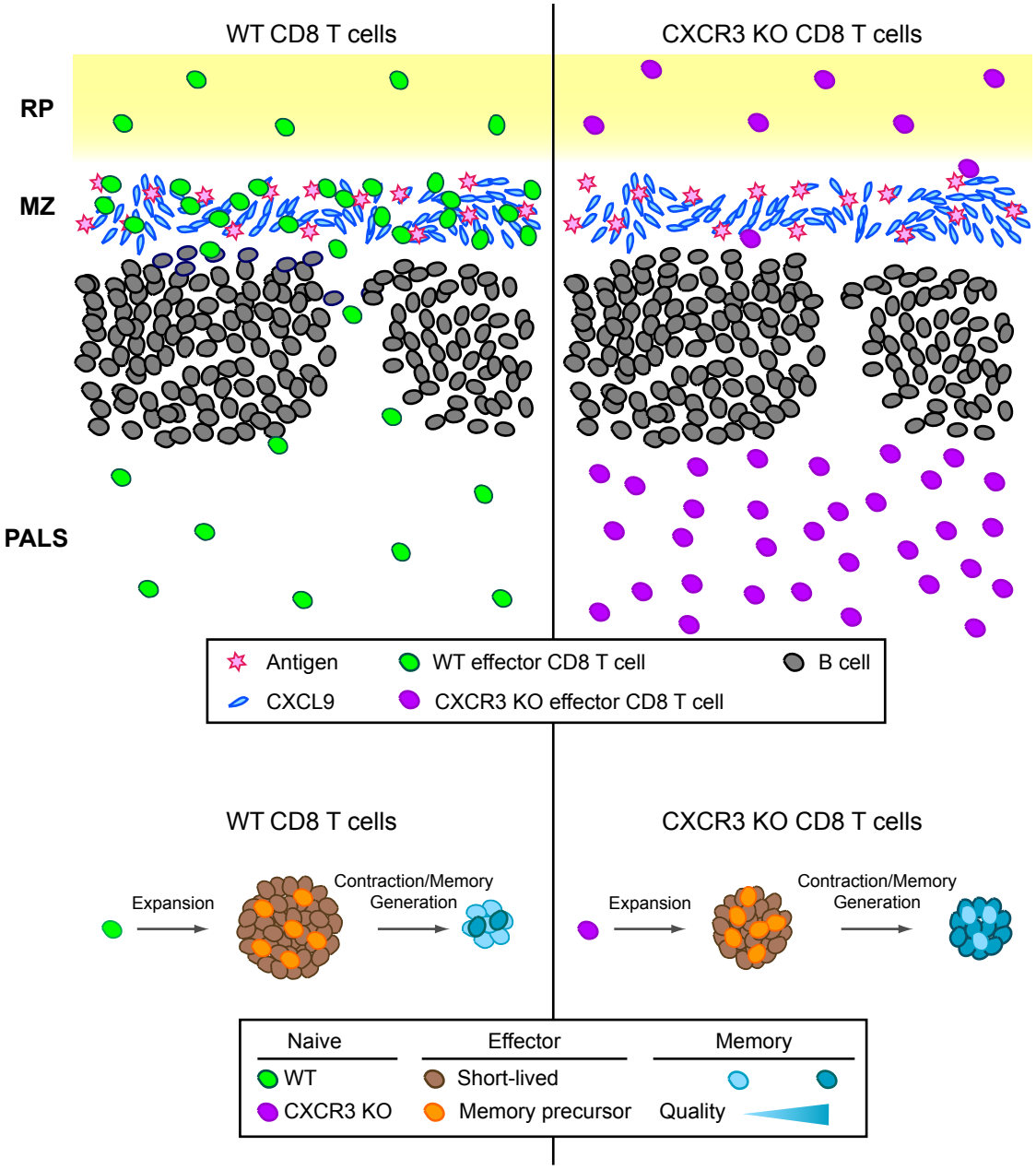



B




**Supplementary Figure 5. Hu et al.**

**Supplementary Figure 6. Model of the contribution of CXCR3 to CD8 T cell differentiation.** In the spleen, marginal zone macrophages and marginal metallophilic macrophages are infected with LCMV and express the CXCR3 ligand CXCL9. Activated effector CD8 T cells express CXCR3 and move towards CXCL9 in marginalzone areas where they are exposed to antigen. Consequently, WT CD8 T cells encounter more antigen and inflammation than CXCR3 deficient cells. As a result, CXCR3 expressing cells tend to become short-lived effector cells instead of long-lived memory precursors. In contrast, CD8 T cells that lack CXCR3 localize away from marginal zone areas and, as a result, are less exposed to antigen and inflammatory stimuli. This leads to generation of more long-lived memory CD8 T cells that are qualitatively better than wild- type cells.




**Supplementary Figure 6. Hu et al.**

# Chapter 3

# Conclusions and Discussion

This work demonstrated that expression of the inflammatory chemokine receptor CXCR3 on CD8 T cells promoted the generation of more terminally differentiated effector cells and fewer memory precursor cells. This difference occurred between 5 to 8 days post-infection (dpi), suggesting that CXCR3 was not a major determinant of CD8 T cell fate. Rather, it shifted the balance towards the generation of more terminal effectors later during infection. The resulting CXCR3-expressing cells formed fewer memory precursor cells and 2- to 4-fold fewer memory cells. These memory cells generated from wild-type (WT) and CXCR3 knockout (KO) cells were phenotypically and functionally different. WT memory cells contained a greater proportion of effector memory cells and a smaller proportion of central memory cells, as delineated by the markers L-selectin/CD62L and CCR7. Central memory cells are associated with increased IL-2 expression [1], which is required for increased expansion during the recall response [2]. Indeed, WT memory cells displayed decreased expression of IL-2. When compared on a per cell basis, central memory cells from the WT population did not expand as well as central memory cells from the CXCR3 KO population during secondary infection. This work demonstrated that CXCR3 promoted CD8 T cell differentiation into terminal effectors and decreased the quantity and quality of memory cells that were formed.

Importantly, this work showed that T cell differentiation was influenced by chemokine receptor-mediated localization in splenic microenvironments. After LCMV infection, CXCL9 protein was expressed in marginal zone areas of the spleen at 1-5 dpi. CXCL9 mRNA and viral RNA were detected in macrophages

in the marginal zone, demonstrating that macrophages that were infected or had taken up viral RNA were a source of CXCL9. Because previous reports have shown that CXCL9 can be expressed by both macrophages [3] and dendritic cells [4], it is important to consider that there may be other cellular sources of CXCL9 in the MZ. By staining LCMV-specific TCR transgenic WT and CXCR3 KO P14 T cells within the same spleens, I found that CXCR3-expressing cells formed clusters in marginal zone (MZ) areas near CXCL9 at 3-5 dpi. In contrast, most CXCR3 KO CD8 T cells were concentrated in the T zone away from marginal zone areas. Spleen sections further showed that LCMV antigen was abundant in the MZ at 3-4 dpi and detectable at 5 dpi. Consequently, WT cells that clustered in MZ areas were more exposed to antigen. Interestingly, it was previously shown that the maintenance of CD8 T cell clusters in the MZ largely depended on antigen-presentation [5]. This suggested that antigen presentation contributed to the formation of CD8 T cell clusters in MZ areas. In addition to increased exposure to antigen, CD8 T cells in the MZ may be more exposed to inflammatory factors. Type I interferons (IFN-$\alpha/\beta$) are a family of important early antiviral factors that are expressed in the MZ by marginal metallophilic macrophages (MMM), marginal zone macrophages (MZM), and plasmacytoid dendritic cells (pDCs) [6-11]. IFN-$\alpha/\beta$ act directly on CD8 T cells and dramatically increase their survival during T cell expansion [12]. However, because differences in WT and CXCR3 KO T cell clustering in the MZ occur after type I IFN expression has declined, this family of cytokines may not contribute to CXCR3-mediated effects on CD8 T cell differentiation. Instead, other

inflammatory factors may contribute to these differences. Another inflammatory cytokine, interferon-γ (IFN-γ), has been shown to act directly on CD8 T cells to increase CD8 T cell expansion during LCMV infection [13]. Interestingly, activated CD8 T cells express IFN-γ and consequently, CD8 T cells that cluster in the MZ may receive both autocrine and paracrine IFN-γ stimulation. Moreover, IFN-γ expression by CD8 T cells may induce the expression of more interferon-inducible CXCR3 ligands that recruit more activated CD8 T cells. This further increases CD8 T cell exposure to IFN-γ in the MZ. In conclusion, CXCR3 increases CD8 T cell access and exposure to antigen and inflammation in the MZ that likely influences differentiation.

The interpretation of the role of CXCR3 on differentiation requires careful examination of the kinetics of CXCR3-mediated clustering in the MZ. Because greater precursor frequencies accelerate the kinetics of expansion and contraction [14] and because I transferred high frequencies of CD8 T cells (0.2-1x$10^6$) to examine localization, it is possible that WT exposure to antigen and inflammation in the MZ occurs later during infection around 5 dpi. A previous report visualized the movements of endogenous antigen-specific CD8 T cells in the spleen during acute *Listeria monocytogenes* (Lm) and vesicular stomatitis virus (VSV) infection [5]. In this report, endogenous antigen-specific CD8 T cells were found at T-B boundaries and in the MZ at 3 dpi, localized to the periarteriolar lymphoid sheath (PALS) at 4 dpi, and formed clusters around B cell follicles and in the MZ at 5 dpi. Similarly, visualizing endogenous WT and CXCR3 KO CD8 T cells in the spleen is an important way to examine the

modulation and kinetics of T cell movement within different splenic compartments and to dissect the role of CXCR3 in these movements. Furthermore, determining the precise timing of CXCR3-mediated movements of activated CD8 T cells to the MZ influences our interpretation of when and how CXCR3 influences CD8 T cell differentiation. A recent report similarly demonstrated that CXCR3 promoted differentiation into terminal effector cells and reduced the quantity of memory cells in acute Lm and vaccinia virus (VV) infections [15]. However, when they transferred 1-3x$10^6$ CD8 T cell precursors, significant differences in WT clustering in MZ areas occurred only at 2 dpi when IFN-$\alpha/\beta$ was still detectable. Although they commented that transferring lower precursor frequencies led to delayed clustering in the MZ, they concluded that CXCR3 was a key factor that influenced early programming of CD8 T cell differentiation. Further studies will be required to determine if CXCR3 is a key determinant in the early programming of CD8 T cells at 1-2 dpi or if CXCR3 influences differentiation by altering T cell exposure at later times during infection.

Because CXCR3 promotes CD8 T cell differentiation into terminal effectors, one may hypothesize that activated cells that express more CXCR3 have a greater potential to differentiate into terminal effector cells while those that express less CXCR3 have a greater potential to generate memory precursors. However, upon examination of mRNA and protein expression of CXCR3 among effector subsets, it was clear that terminally differentiated cells downregulated CXCR3 while memory precursors retained high levels of CXCR3 expression. Instead, it would be important to examine how multiple receptors controlled the

movement of activated CD8 T cells to different splenic microenvironments and how these movements alter the type and amount of stimulation T cells received. For example, CCR7 expression mediates T cell movement to the T zone and this can be seen when transferring CCR7 Tg P14 T cells into mice and infecting them. Examination of static spleen sections conveys the notion that CD8 T cells appear to remain in particular splenic microenvironments over days. However, T cell movements are dynamic and continuous movements of each cell to different microenvironments may lead to differences in the overall stimulation received. We hypothesize that CXCR3 increases movement towards the inflamed MZ whereas CCR7 mediates T cell movement to the T zone. Dynamic movements to different microenvironments contribute to the overall stimulation received by a cell. At 4 dpi, KLRG1$^{lo}$ cells express CXCR3, which increases exposure to antigen and inflammation. This may induce more T-bet expression and promote differentiation into KLRG1$^{hi}$ terminal effectors instead of IL-7R$\alpha^{hi}$ memory precursors. Although CXCR3 and CCR7 are key receptors that mediate T cell localization in the spleen during infection, there may be additional receptors that influence T cell exposure to antigen and inflammation.

To better understand how CXCR3 increases the quantity of terminal effectors by 8 dpi, I examined CD8 T cell proliferation and death. There were no significant differences between WT and CXCR3 KO P14 T cell apoptosis between 5 to 8 dpi. However, at 6 and 7 dpi, WT cells proliferated more than CXCR3 KO cells. The greatest differences in proliferation occurred in the KLRG1$^{lo}$IL-7R$\alpha^{lo}$ effector subset. Between 5 to 8 dpi, this subset can further

differentiate into KLRG1$^{hi}$IL-7R$\alpha^{lo}$ terminally differentiated cells or into KLRG1$^{lo}$IL-7R$\alpha^{hi}$ memory precursor cells. With further antigenic and inflammatory stimulation, these KLRG1$^{lo}$IL-7R$\alpha^{lo}$ cells may proliferate more and differentiate into KLRG1$^{hi}$IL-7R$\alpha^{lo}$ cells, leading to greater numbers of terminally differentiated cells.

In addition to influencing the differentiation of effector cell subsets, CXCR3 influences the proportion of memory cell subsets as identified by L-selectin/CD62L and CCR7. During the formation of T cell memory in the contraction phase, KLRG1$^{lo}$ memory precursors upregulate CCR7 whereas KLRG1$^{hi}$ terminally differentiated cells do not. The expression of CCR7 on memory cells localizes them to the T zone of lymphoid tissues [16,17]. CXCR3 deficiency increased the proportion of memory cells that expressed L-selectin/CD62L and CCR7, which led to the increased accumulation of CXCR3 KO memory cells in lymph nodes compared to other tissues. Although it was shown that decreasing the availability of antigen during CD8 T cell priming increased the proportion of CD62L-expressing memory cells [18], it is not known whether decreasing the availability of antigen later during infection influences CD62L re-expression in memory cells. Our data suggests that antigen stimulation acquired after T cell priming has an impact on the expression of CD62L and CCR7 on memory cells.

This work supports both the “decreasing potential model” and “fate commitment with progressive differentiation model”. It suggests that increased access to antigenic and inflammatory stimulation promotes the formation of

terminally differentiated effector cells at the expense of the formation of memory precursors. Moreover, it shows that the stimulation experienced later during infection affects differentiation. It may do this either by reducing a CD8 T cell's potential to become a memory cell as in the "decreasing potential model" or by stimulating memory precursors to differentiate into terminal effectors as in the "fate commitment with progressive differentiation model". Interestingly, this work suggests that the duration of stimulation a CD8 T cell receives also influences the proportion of memory cell subsets formed. Increased stimulation generates more effector memory cells in both lymphoid organs and peripheral tissues. These effector memory cells may arise from longer-lived, but still terminally differentiated cells or from memory precursor cells as a transitional stage to central memory cells, or both. Over time, there are proportionately more central memory cells, suggesting that either the effector memory cells die over time or that effector memory cells convert to central memory cells over time, or both. However, the amount of conversion to central memory cells may be overrepresented in these experiments due to the high precursor frequency of P14 T cells ($1x10^4$), which reduces the formation of a stable lineage of effector memory cells [19]. Further examination of effector and central memory T cells within endogenous CD8 T cell populations will aid in our understanding of the maintenance of these subsets over time. This work provides support for current CD8 T cell differentiation models and emphasizes the importance of T cell movement and localization to the process of differentiation.

In conclusion, CXCR3 increases CD8 T cell exposure to antigen and inflammation in the MZ, which affects T cell differentiation and impacts the quantity and quality of memory cells formed. Additional studies of other homeostatic and inflammatory chemokine receptors may elucidate how coordinated T cell movements in lymphoid organs impacts T cell differentiation during infection. Furthermore, future studies in chronic infections will add to our understanding of the role of chemokine receptors in T cell expansion, differentiation, and exhaustion in situations were antigen persists for prolonged periods.

## References


1. Wherry EJJ, Ahmed R (2004) Memory CD8 T-Cell Differentiation during Viral Infection. *Journal of virology* 78: 5535.
2. Feau S, Arens R, Togher S, Schoenberger SP (2011) Autocrine IL-2 is required for secondary population expansion of CD8(+) memory T cells. *Nature immunology*.
3. Farber J (1997) Mig and IP-10: CXC chemokines that target lymphocytes. *J. Leukoc. Biol.* 61: 246-257.
4. Eberlein J, Nguyen TT, Victorino F, Golden-mason L, Rosen HR, et al. (2010) Technical advance Comprehensive assessment of chemokine expression profiles by flow cytometry. 120.
5. Khanna KM, McNamara JT, Lefrançois L (2007) In situ imaging of the endogenous CD8 T cell response to infection. *Science (New York, N.Y.)* 318: 116-20.
6. Louten J, Rooijen N van, Biron C a (2006) Type 1 IFN deficiency in the absence of normal splenic architecture during lymphocytic choriomeningitis virus infection. *Journal of immunology (Baltimore, Md. : 1950)* 177: 3266-72.
7. Montoya M, Dawes R, Reid D, Lee LN, Piercy J, et al. (2006) Innate immunity CD45 is required for type I IFN production by dendritic cells. 2150-2158.
8. Malmgaard L, Salazar-mather TP, Lewis CA, Biron CA (2002) Promotion of Alpha / Beta Interferon Induction during In Vivo Viral Infection through Alpha / Beta Interferon Receptor / STAT1 System-Dependent and - Independent Pathways. *Society* 76: 4520-4525.
9. Mebius RE, Kraal G (2005) Structure and function of the spleen. *Nature reviews. Immunology* 5: 606-16.
10. Asselin-Paturel C, Brizard G, Chemin K, Boonstra A, O'Garra A, et al. (2005) Type I interferon dependence of plasmacytoid dendritic cell activation and migration. *The Journal of experimental medicine* 201: 1157-67.
11. Scheu S, Dresing P, Locksley RM (2008) Visualization of IFN ℕL production by plasmacytoid versus conventional dendritic cells under specific stimulation conditions in vivo. *PNAS*.
12. Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K (2005) Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *The Journal of experimental medicine* 202: 637-50.
13. Whitmire JK, Tan JT, Whitton JL (2005) Interferon-gamma acts directly on CD8+ T cells to increase their abundance during virus infection. *The Journal of experimental medicine* 201: 1053-9.
14. Badovinac VP, Haring JS, Harty JT (2007) Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity* 26: 827-41.

15. Kurachi M, Kurachi J, Suenaga F, Tsukui T, Abe J, et al. (2011) Chemokine receptor CXCR3 facilitates CD8+ T cell differentiation into short-lived effector cells leading to memory degeneration. *The Journal of experimental medicine*.
16. Unsoeld H, Voehringer D, Krautwald S, Pircher H (2004) Constitutive expression of CCR7 directs effector CD8 T cells into the splenic white pulp and impairs functional activity. *Journal of immunology (Baltimore, Md. : 1950)* 173: 3013-9.
17. Jung YW, Rutishauser RL, Joshi NS, Haberman M, Kaech SM, et al. (2010) Differential Localization of Effector and Memory CD8 T Cell Subsets in Lymphoid Organs during Acute Viral Infection. *Journal of immunology (Baltimore, Md. : 1950)*.
18. Obar J (2010) Early signals during CD8+ T cell priming regulate the generation of central memory cells. *The Journal of Immunology*.
19. Marzo AL, Klonowski KD, Bon A Le, Borrow P, Tough DF, et al. (2005) Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nature immunology* 6: 793-9.

# Appendix

**Figure 1**

**In lymph nodes, WT P14 T cells cluster at T-B boundaries, around B cell follicles, and in the subcapsular sinus (SCS) while CXCR3 KO P14 T cells are spread out in T zone areas.**

$5x10^5$ Thy1.1$^+$CD45.2$^+$ WT and Thy1.2$^+$CD45.1$^+$ CXCR3 KO P14 T cells were transferred into Thy1.2$^+$CD45.2$^+$ mice that were infected with $2x10^6$ PFU LCMV (Armstrong) i.p. the next day. At 4 days post-infection, axillary lymph nodes (A), brachial lymph nodes (B), and inguinal lymph nodes (C) were sectioned and stained for localization of WT (red) and CXCR3 KO (green) P14 T cells.

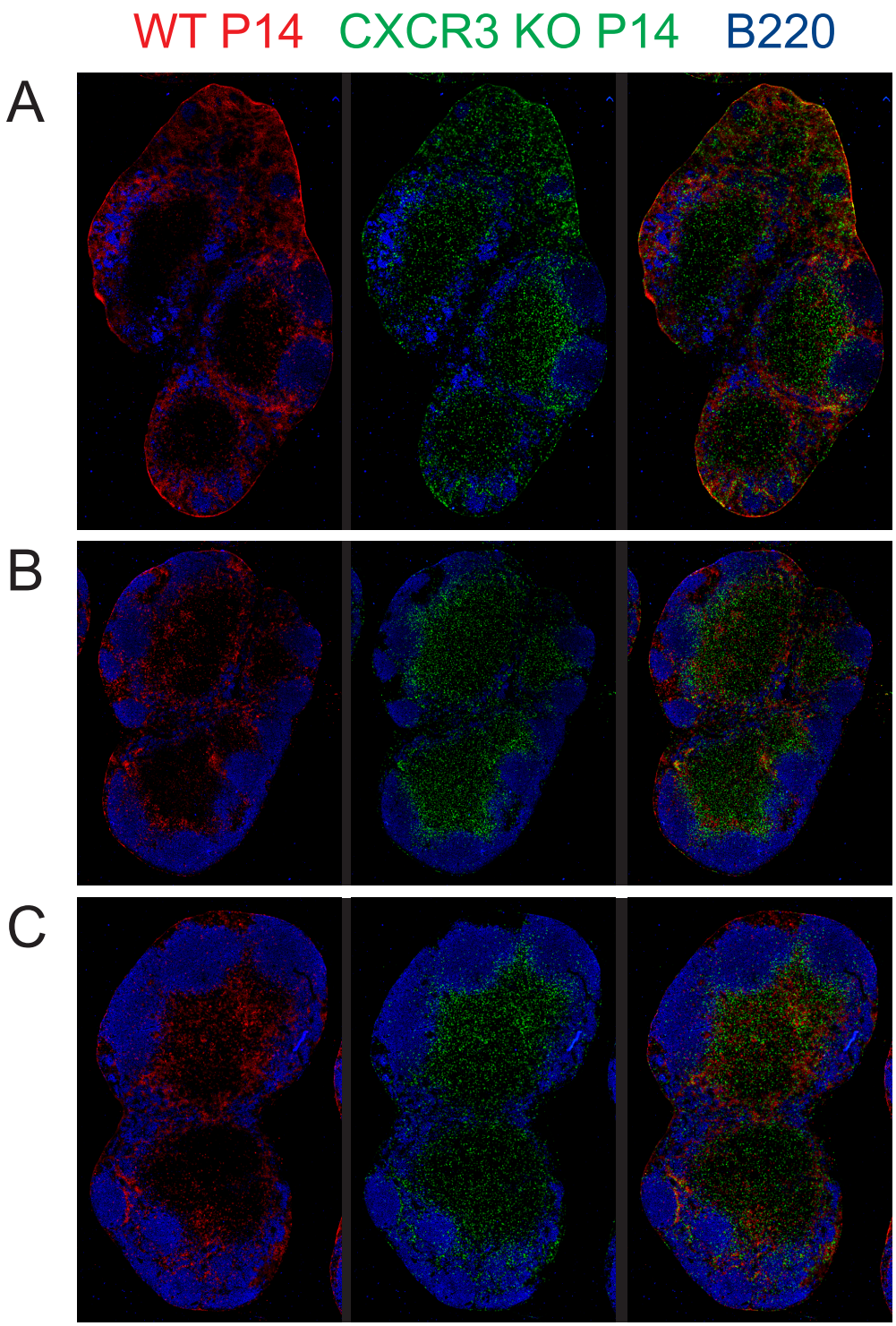



**Figure 1**

**Figure 2**

**Most WT and CXCR3 KO P14 T cells are found in the red pulp of the spleen at 8 days post-infection.**

$1x10^3$ $Thy1.1^+CD45.2^+$ WT and $Thy1.2^+CD45.1^+$ CXCR3 KO P14 T cells were transferred into $Thy1.2^+CD45.2^+$ mice that were infected with $2x10^5$ PFU LCMV (Armstrong) i.p. the next day. At 8 days post-infection, spleens were sectioned and stained for localization of WT (green) and CXCR3 KO (red) P14 T cells.

WT P14 CXCR3 KO P14

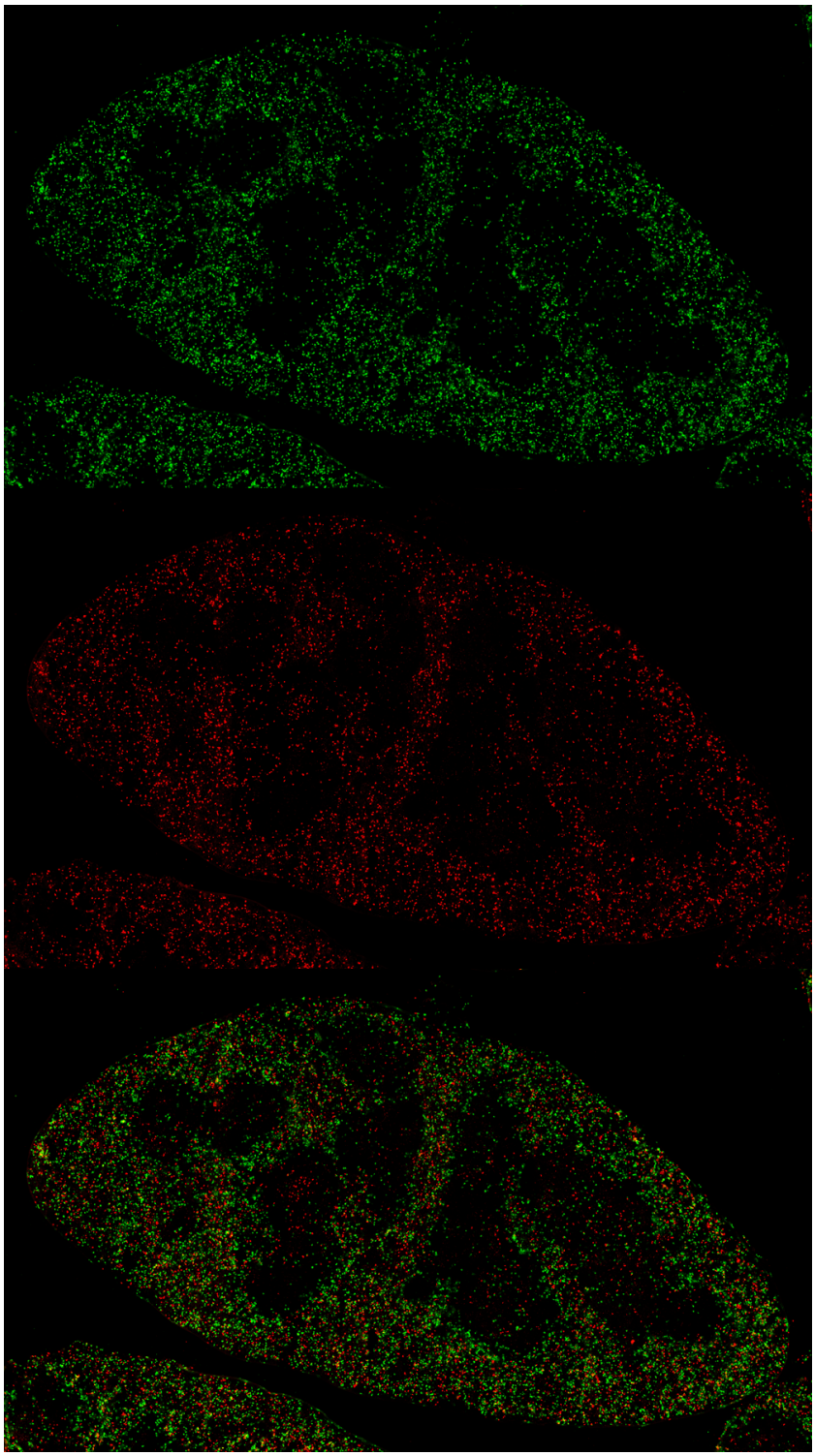


**Figure 2**

**Figure 3**

**CXCR3 affects effector and memory differentiation in recombinant *Listeria monocytogenes*-GP33 infection.**

C57BL/6 mice containing equal numbers of CD45.1/CD45.2 WT and CD45.1/CD45.1 CXCR3 KO P14 T cells were infected with $1x10^3$ CFU recombinant *Listeria monocytogenes*-GP33. Plots are gated on WT or CXCR3 KO P14 T cells and show KLRG1 and IL-7R$\alpha$ expression at 8 days post-infection in the indicated tissues.

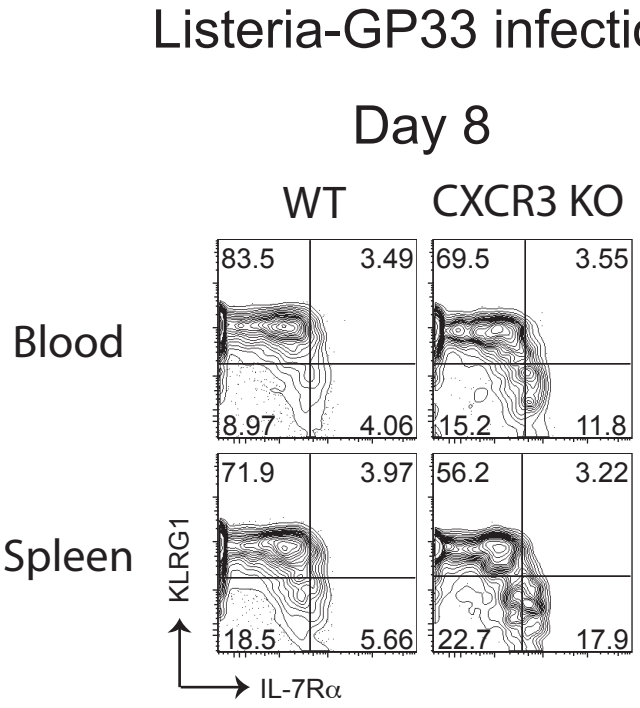

Listeria-GP33 infection
Day 8
WT
CXCR3 KO
Blood
Spleen
83.5
3.49
8.97
4.06
69.5
3.55
15.2
11.8
71.9
3.97
18.5
5.66
56.2
3.22
22.7
17.9
KLRG1
IL-7Rα

**Figure 3**

**Figure 4**

**Interferon-γ (IFN-γ) prolongs expression of CXCR3 ligands and influences CD8 T cell differentiation.**

Transcriptional expression of the CXCR3 ligands, CXCL9 and CXCL10, was measured in wild-type (WT) and IFNγR knock-out (KO) mice infected with $2x10^5$ PFU LCMV (Armstrong) i.p. (A). (B) WT and IFNγR KO mice containing equal numbers of CD45.1/CD45.2 WT and CD45.1/CD45.1 CXCR3 KO P14 T cells were infected with $2x10^5$ PFU LCMV (Armstrong) i.p. and T cell differentiation was examined at 5, 6, and 7 days post-infection (dpi). (C) KLRG1 and IL-7Rα expression was examined on WT and CXCR3 KO P14 T cells transferred into WT and IFNγR KO mice at the indicated times after infection.

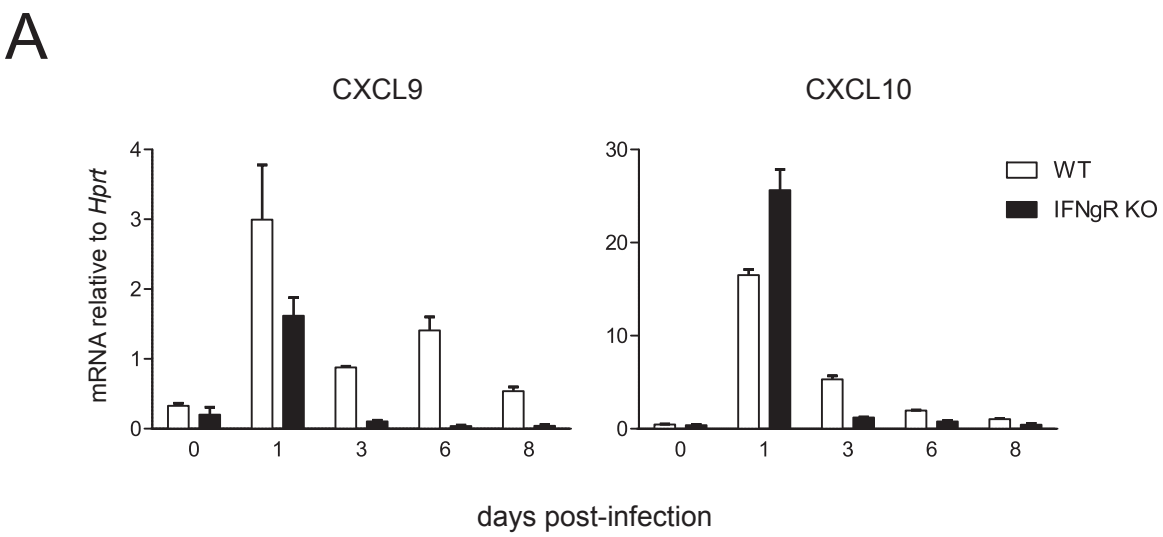

A
CXCL9
CXCL10
mRNA relative to Hprt
WT
IFNgR KO
days post-infection
B
WT recipients
WT P14 +
CXCR3 KO P14
1:1
LCMV
Days 5, 6, 7
Ifngr1-/- recipients
LCMV
Days 5, 6, 7
C
Day 5
Day 6
Day 7
WT P14
KO P14
WT
Recipients
Ifngr1-/-
Recipients
KLRG1
IL-7R

**Figure 4**

**Figure 5**

**IL-15 affects the proportion of KLRG1$^{hi}$IL-7R$^{lo}$ WT and CXCR3 KO P14 T cells at 8 and 15 days post-infection.**

WT and IL-15$^{-/-}$ KO mice containing equal numbers of CD45.1/CD45.2 WT and CD45.1/CD45.1 CXCR3 KO P14 T cells were infected with 2x10$^5$ PFU LCMV (Armstrong) i.p. KLRG1 and IL-7R$\alpha$ expression was examined on WT and CXCR3 KO P14 T cells transferred into WT and IL-15$^{-/-}$ KO mice at the indicated times after infection (A). (B) Proportions of KLRG1$^{hi}$IL-7R$^{lo}$ WT and CXCR3 P14 T cells in WT and IL-15$^{-/-}$ KO hosts were plotted.

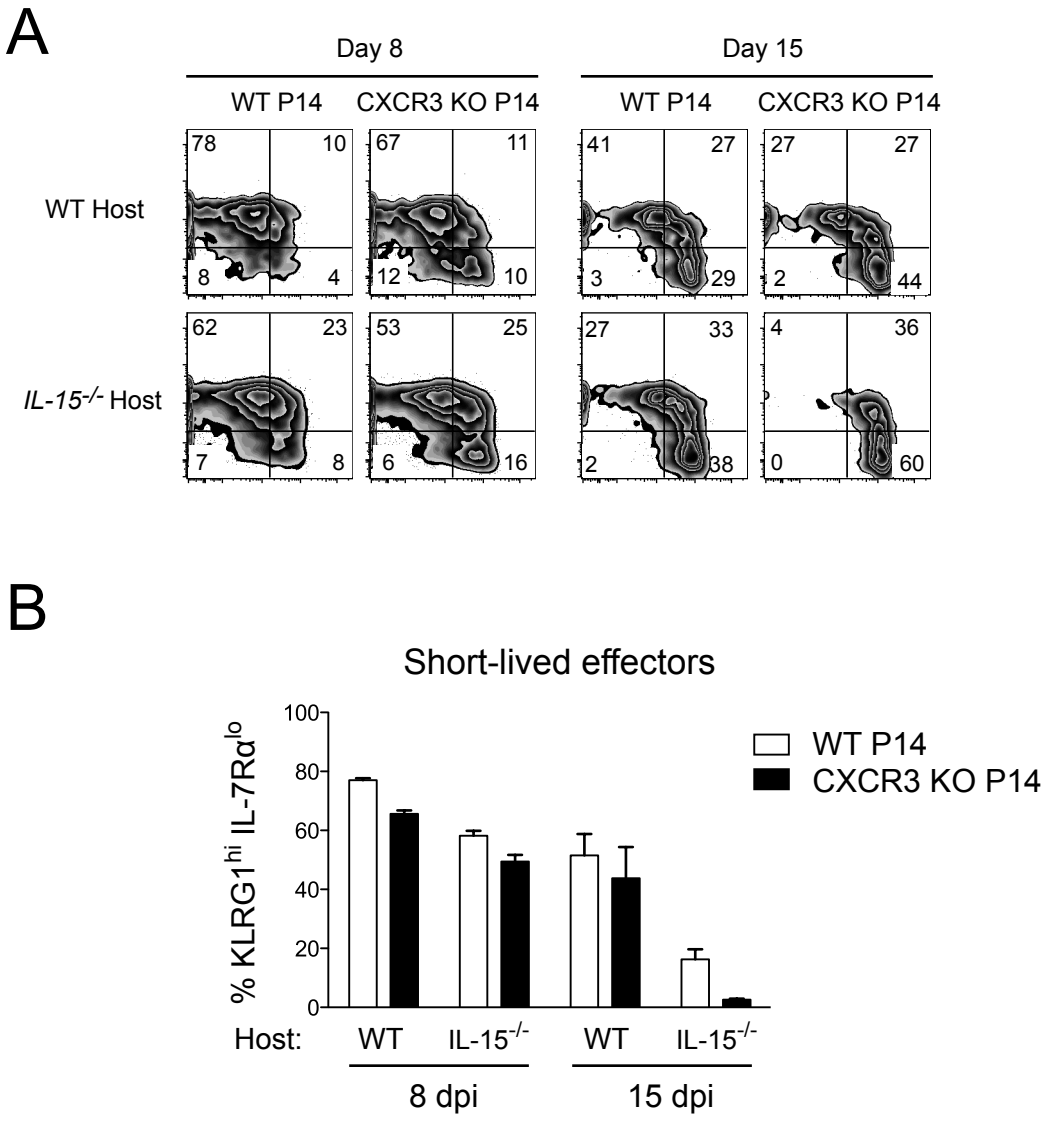

A
Day 8
Day 15
WT P14
CXCR3 KO P14
WT P14
CXCR3 KO P14
WT Host
IL-15-/- Host
78
10
8
4
67
11
12
10
41
27
3
29
27
27
2
44
62
23
7
8
53
25
6
16
27
33
2
38
4
36
0
60
B
Short-lived effectors
% KLRG1hi IL-7Rαlo
100
80
60
40
20
0
WT P14
CXCR3 KO P14
Host:
WT
IL-15-/-
WT
IL-15-/-
8 dpi
15 dpi

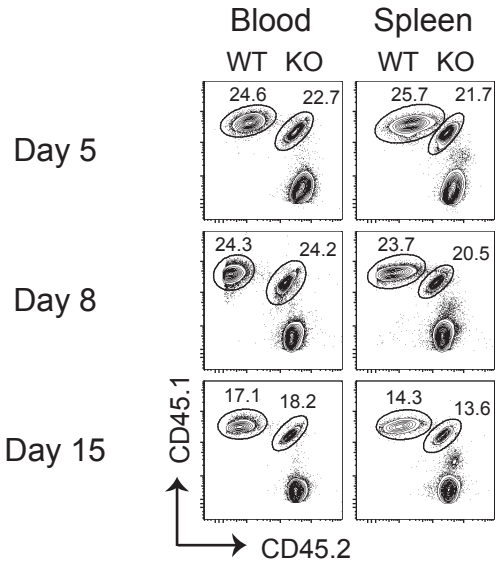
**Figure 5**

**Figure 6**

**CCR5 does not affect CD8 T cell differentiation into short-lived effector cells and memory precursor cells.**

C57BL/6 mice containing equal numbers of CD45.1/CD45.1 WT and CD45.1/CD45.2 CCR5 KO P14 T cells were infected with $2x10^5$ PFU LCMV (Armstrong) i.p. (A) Frequencies of WT and CCR5 KO CD8 T cells were measured in the indicated tissues at the indicated days post-infection. (B) KLRG1 and IL-7R$\alpha$ expression was examined on WT and CCR5 KO P14 T cells in the indicated tissues at the indicated days post-infection.

A


Blood
Spleen
WT KO
WT KO
Day 5
Day 8
Day 15
24.6 22.7
25.7 21.7
24.3 24.2
23.7 20.5
17.1 18.2
14.3 13.6
CD45.1
CD45.2

B

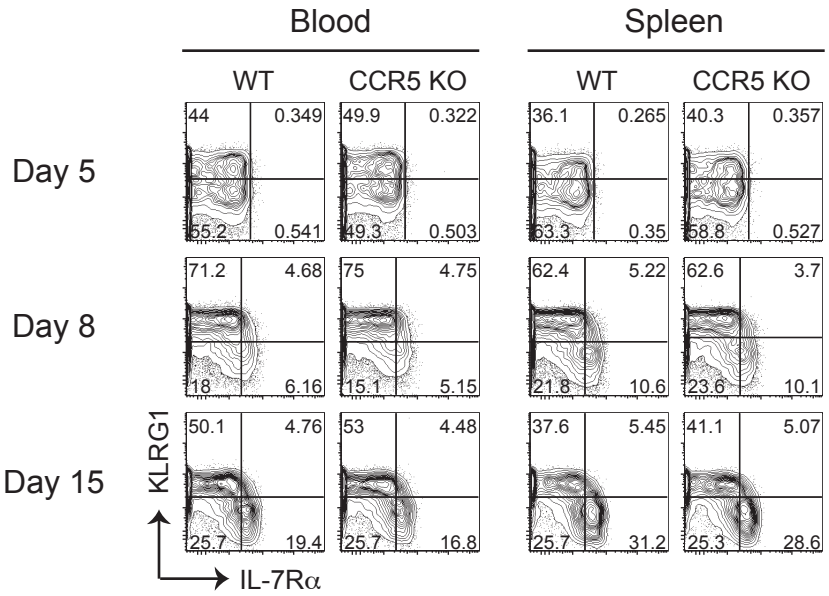

Blood
Spleen
WT
CCR5 KO
WT
CCR5 KO
Day 5
Day 8
Day 15
44 0.349 55.2 0.541
49.9 0.322 49.3 0.503
36.1 0.265 63.3 0.35
40.3 0.357 58.8 0.527
71.2 4.68 18 6.16
75 4.75 15.1 5.15
62.4 5.22 21.8 10.6
62.6 3.7 23.6 10.1
50.1 4.76 25.7 19.4
53 4.48 25.7 16.8
37.6 5.45 25.7 31.2
41.1 5.07 25.3 28.6
KLRG1
IL-7Rα

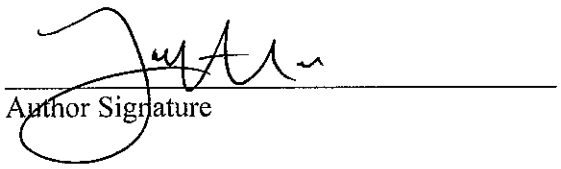
**Figure 6**

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