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Generation and Regulation of Virus-specific Memory CD8 T Cells

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Generation and Regulation of Virus-specific Memory CD8 T Cells

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Abstract

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Differentiation of CD8 T cells into cytotoxic T lymphocytes and subsequently into long-lived memory cells, which help provide protective immunity, is an important component of an effective adaptive immune response. This concept of "immunological memory" has resulted in the successful development of vaccines toward many human diseases. Despite this progress, there remain diseases (e.g. HIV and cancer) to which vaccines have yet to be successfully developed. Therefore, there is a continual need to better understand the requirements for the generation and maintenance of memory CD8 T cells. It is also crucial to further comprehend how these cells confer life-long protective immunity. We began our studies by addressing the role of helper CD4 T cells in memory CD8 T cell development. Contrary to current dogma, we determined that CD4 T cell help was not required for either the "programming" or "maintenance" of memory cells. Instead, CD4 T cells were essential for the complete clearance of antigen and thereby, creating an ideal environment for memory development to occur. Next, we quantified the homeostatic turnover of memory CD8 T cells and addressed the role of helper CD4 T cells in the maintenance of the overall quality of memory cells. We found that all memory cells underwent turnover that was stochastic and independent of both CD4 T cell help and antigen-specificity. This turnover occurred at an average rate of 0.02 divisions/day (intermiotic time of ~50 days). Moreover, the overall quality (phenotypic profile and recall capability) was unaffected by the absence of CD4 T cell help during memory maintenance. Our final study focused on the migratory properties of CD8 T cells after LCMV infection. Utilizing FTY720 as a tool, we observed that the egress of effector cells from the peripheral lymph nodes, unlike their naïve predecessors, occurred independently of sphingosine-1-phosphate signaling. In addition, the pool of memory cells consisted of both tissue-resident and re-circulating cells that collectively worked to confer protective immunity. These studies should shed more light on the generation and regulation of "immunological memory" and hopefully will aid in the development of novel strategies toward diseases to which there are no current vaccines.

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Chapter 1: Introduction

Tenets of CD8 T cell mediated immunological protection

A hallmark of the adaptive immune system is its ability to mount a rapid and potent CD8 T cell response upon re-exposure to the same antigen in vivo (1-4). This enhanced protection is the result of both quantitative and qualitative changes that occur as CD8 T cells differentiate from naïve to effector to memory cells upon activation. For example, acute lymphocytic choriomeningitis virus (LCMV) infection results in an overall net increase in the number of antigen-specific CD8 T cells (~1000-fold increase) and this increase in number alone could suffice for the rapid secondary response (5).

However, subsequent studies have shown that memory CD8 T cells are intrinsically more efficient in their responsiveness toward antigen compared to naïve CD8 T cells. In support, memory CD8 T cells require shorter duration of antigenic stimulation and are less dependent on co-stimulatory molecules for activation compared to naïve CD8 T cells (6-10). This allows for the rapid induction of memory CD8 T cell response as memory cells can be activated directly within the peripheral tissues (both lymphoid and non-lymphoid) without having to first migrate to the secondary lymphoid tissues. In addition, memory cells express a unique pattern of tissue-specific homing receptors that enables them to traffick through peripheral tissues that are not normally surveyed by naïve CD8 T cells (11-17). Memory CD8 T cells also produce effector molecules (e.g. IFN-γ, granzyme B, and perforin) much more quickly (within just a few hours of activation and even before cell division has occurred) (18-23). This ability to rapidly display effector function is due to the epigenetic inheritance that occurs as

effector CD8 T cells differentiate into long-lived memory CD8 T cells. In support, it has been shown that DNA methylation negatively regulates gene expression (24, 25). Accordingly, Fitzpatrick et al. observed that the IFN-γ locus of naïve CD8 T cells was extensively methylated at several CpG sites, whereas in effector CD8 T cells, this locus was largely demethylated, explaining the efficient IFN-γ production by activated CD8 T cells, but not by naïve cells (26). As memory cells differentiate from effector CD8 T cells, they partly inherit the demethylated IFN-γ locus of effector CD8 T cells; therefore, this locus in memory CD8 T cells is only partially methylated (27, 28). This allows memory CD8 T cells to demethylate their IFN-γ locus and produce IFN-γ much more quickly compared to naïve CD8 T cells. Collectively, this combination of increased frequency and faster responsiveness enables memory CD8 T cells to play an important role in both limiting the extent of infection and reducing the severity of disease upon secondary challenge. The subsequent sections of this chapter will discuss in more detail our current understanding of the different aspects of memory CD8 T cells.

Different stages of CD8 T cell immune response

As depicted in Fig 1, CD8 T cell immune response toward most acute infections can be broken down into three distinct stages: expansion, contraction, and maintenance (4).

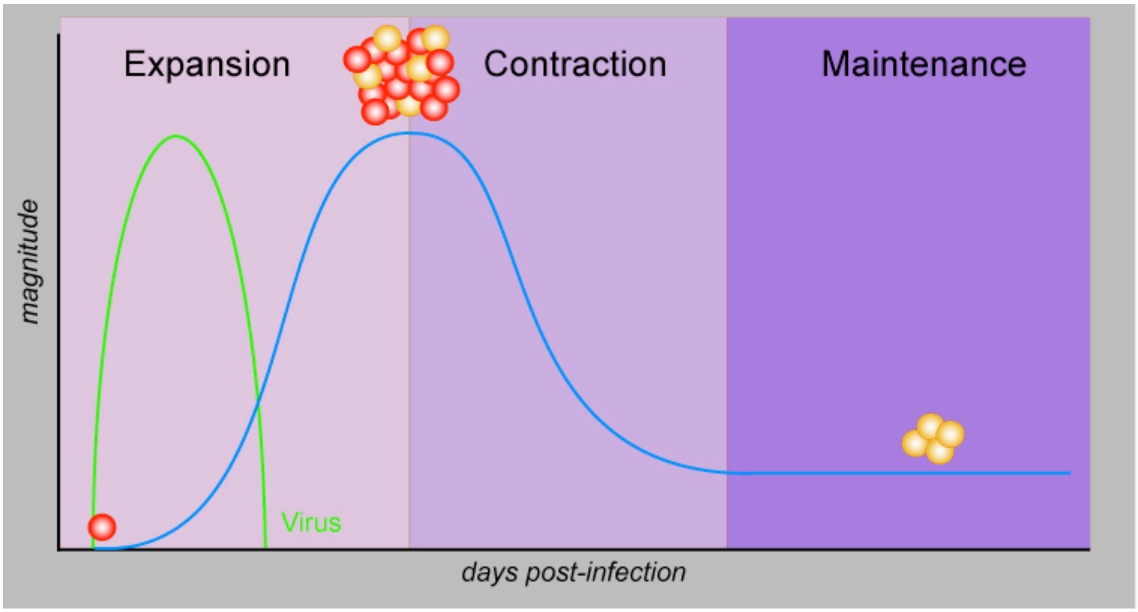


Figure 1. Kinetics of CD8 T cell immune response during an acute infection. Upon infection, antigen-specific CD8 T cells undergo rapid proliferation (>1000-fold) resulting in the generation of a large pool of effector CD8 T cells. Upon antigen clearance, approximately 90-90% of these cells undergo contraction resulting in the generation of a small pool of memory CD8 T cells that persist for the life of the host.

Expansion: generation of a protective CD8 T cell immune response

Upon primary immunization/infection, antigen-specific naïve CD8 T cells interact with dendritic cells (DCs) presenting antigen in the context of major histocompatibility complex (MHC) class I molecules within the secondary lymphoid tissues (29, 30). Restricting this interaction within the lymphoid tissues is important for several reasons. First, there are only about 100-200 CD8 T cells specific for a given antigen within an uninfected mouse (31, 32). Therefore, if this interaction between DCs and CD8 T cells are allowed to occur within random peripheral tissues, CD8 T cells are not likely to interact with cells expressing their cognate antigen. Second, naïve CD8 T cells lack the necessary tissue-specific homing receptors to survey the many different peripheral tissues in search of antigen. For instance, naive cells express primarily CD62L (L-selectin) and

CCR7, in terms of tissue-specific homing receptors; as a result, their migration is largely limited to the peripheral blood, spleen, lymph nodes (LNs), and the bone marrow (15, 33). Due to this restriction in migratory capability, naïve CD8 T cells require DCs to "sample" antigen from the periphery and migrate to the secondary lymphoid tissues for their activation (34). Thus, limiting the priming of CD8 T cells within the secondary lymphoid tissues ensures that naïve T cells will interact with DCs expressing their cognate antigen.

If the above-described interaction occurs in the presence of both co-stimulatory molecules (e.g. CD80/CD86 – CD28, CD70 – CD27, OX40L – OX40, etc.) and different cytokines (e.g. IL-12 and Type I IFN), antigen-specific CD8 T cells rapidly proliferate, resulting in a large pool of effector CD8 T cells (35-45). Experiments with CFSE-labeled transgenic CD8 T cells have shown that CD8 T cells divide approximately every 6-8 hours, so that by the peak of the immune response, there is a 10,000- to 100,000-fold increase in the total number of antigen-specific CD8 T cells (46, 47). Some of the earlier studies had suggested that much of this expansion was a result of either bystander activation or cross-reactive stimulation of nonspecific cells, as only a small fraction (~1-5%) of these proliferating CD8 T cells were observed to be antigen-specific by limiting dilution assays (46, 48-50). However, with the introduction of more advanced technologies (e.g. MHC class I tetramers and more sensitive intracellular cytokine staining techniques), subsequent studies have shown that large majority (~80-95%) of the rapidly proliferating CD8 T cells are indeed antigen-specific (46, 51-54).

In addition to this dramatic increase in frequency, with each round of division, antigen-specific CD8 T cells undergo various changes both in their phenotypic

expression and in their function. For example, as activated CD8 T cells proliferate and differentiate into effector cells, they down-regulate the expression of tissue homing markers associated with naïve CD8 T cells (e.g. CD62L/L-selectin and CCR7) and up-regulate the expression of different peripheral homing receptors (e.g. $\alpha 4\beta 7$ and CCR9 – homing to the gut; ELAM-1 – homing to the skin) (13, 16, 53, 55). In addition, activated CD8 T cells begin to express different effector molecules (e.g. perforin and granzyme B) and inflammatory cytokines (e.g. IFN-γ and TNF-α) enabling them with various cytolytic functions (56-61). Recent experiments have demonstrated that an elaborate network of transcriptional factors tightly regulates this acquisition of effector function by CD8 T cells. For example, it has been reported that the expression of different attributes of effector CD8 T cells (e.g. IFN-γ, perforin, and granzyme B production) was dependent on the expression of two members of the T-box transcription factor family (T-bet and Eomesodermin) (62-64). Subsequent studies have implicated even more transcription factors in regulating effector CD8 T cell function by demonstrating that Notch and Runx3 act up-stream of Eomesodermin and regulate its expression. Deletion of any of these transcription factors has been shown to result in impaired cytolytic activity by effector CD8 T cells (65, 66).

Contraction: termination of CD8 T cell immune response

For most acute infections, the immune response is self-limiting as antigen is cleared within 5-8 days post infection and the peak number of effector CD8 T cells is reached shortly there after (4, 47, 67-70). Soon after antigen clearance, approximately 90-95% of the effector CD8 T cells undergo contraction resulting in a small pool of long-

lived memory CD8 T cells (4, 5). This contraction is obviously critical for T cell homeostasis, as the entire immune system would be encumbered with an overabundance of cells if a large fraction of the activated CD8 T cells had failed to undergo contraction at the end of each immune response. In addition, prolonged exposure to the different effector functions (e.g. inflammatory cytokine production and cytolytic activity) could result in T cell mediated immunopathologies of the peripheral tissues. However, too large of a contraction can be counterproductive as the extent of CD8 T cell contraction also directly determines the size of the resulting memory CD8 T cell pool. Hence, there is an obvious fine balance between the benefits and the detriments of CD8 T cell contraction on the host. Accordingly, much research has been done to help decipher the mechanisms that regulate this contraction of activated CD8 T cells.

Activation-induced cell death (AICD; also known as antigen-driven apoptosis) model

AICD is mediated via CD95 (Fas), which is a member of the tumor necrosis factor (TNF)-super family that contains an intracellular death domain in its cytoplasmic tail, which transmits a death signal to the cell upon interaction with its ligand (CD95L/FasL) (71, 72). In support of this model, in vitro experiments have demonstrated that effector CD8 T cells are more sensitive to AICD compared to both naïve and memory CD8 T cells (73). In addition, loss of either CD95 or CD95L in mice results in the increased accumulation of both B and T cells, and the onset of autoimmune disorders (74, 75). However, in contrast to these studies, other groups have reported normal CD8 T cell contraction in CD95 and/or CD95L deficient animals after acute LCMV infection (76, 77). Furthermore, as the name suggests, AICD requires re-

stimulation with antigen, but for most acute infections, effector CD8 T cell contraction occurs after the complete clearance of antigen (78). Collectively, these latter studies question the importance of AICD and suggest that other mechanisms may also be involved in mediating CD8 T cell contraction in the context of acute infections.

Activated T cell autonomous death (ACAD; also known as cytokine withdrawl-induced or growth factor deprivation-induced passive apoptosis)

ACAD occurs independently of both CD95- and TNF-mediated cell-death pathways and instead, relies on the activation of the BCL-2 family member BIM (BCL-2-interacting mediator of cell death) and PUMA (p53-upregulated modulator of apoptosis) (72, 79). Stress signals associated with cytokine or growth factor deprivation induce BIM and PUMA activation, which in turn, results in the permeabilization of the mitochondrial wall, the activation of caspases, and the eventual apoptosis of the cells (80, 81). In support of ACAD as the primary mechanism of effector CD8 T cell contraction, various groups have reported that activated CD8 T cells are more prone to death upon cytokine withdrawal compared to resting lymphocytes (72). In addition, Yajima et al. showed that the contraction of effector OT-I cells was much more profound in IL-15 -/- animals compared to WT controls and that the administration of rIL-15 during the contraction phase reduced the magnitude of this enhanced contraction (82). Similarly, Blattman et al. demonstrated that therapeutic administration of IL-2 during the contraction phase resulted in reduced contraction of LCMV-specific effector CD8 T cells (83). Lastly, Pellegrini et al. noted significant accumulation of activated herpes simplex virus (HSV) specific CD8 T cells in Bim -/- animals long after viral clearance (84).

Collectively, these studies imply that ACAD is the primary mechanism by which activated CD8 T cells undergo contraction after immune response.

In reality, however, CD95-mediated (AICD) and BIM-mediated (ACAD) cell-death pathways, most likely, have important complementary roles in the termination of immune response. For instance, in vivo studies have demonstrated that the loss of both BIM and CD95 results in much more severe autoimmune disease compared to single mutants (85, 86). Moreover, Weant et al. reported a drastic increase (>100-fold) in the number of LCMV-specific effector CD8 T cells in the LNs of animals deficient in both CD95 and BIM compared to mice deficient in only one of the two death pathways (87). These studies suggest that in the context of most acute infections, both AICD and ACAD death pathways play important non-redundant roles in the contraction of effector CD8 T cells.

Maintenance: conferment of lifelong protective immunity

Once generated, memory CD8 T cells persist for extended periods with a half-life in excess of one year (88, 89). Early studies had suggested that the long-term maintenance of memory CD8 T cells was dependent on the persistence of small amounts of antigen (perhaps trapped by follicular dendritic cells), as both the survival of memory cells and the overall magnitude of the secondary responses was greatly diminished in mice, in which the memory cells were maintained in the absence of antigen (90-92). In contrast, subsequent studies have shown that memory CD8 T cells adoptively transferred into naïve animals are stably maintained for long periods of time (93, 94). Similarly, Murali-Krishna et al. observed normal maintenance of LCMV-specific memory CD8 T

cells transferred into MHC class I deficient animals (95). Recent experiments by Leignadier et al. also showed that the ablation of the CD8 T cell receptor (TCR) on memory cells had minimal effect on long-term survival of the memory cells (96). These studies demonstrate that the continued persistence of memory CD8 T cells long after the resolution of infection is independent of continual antigenic stimulation.

Various studies have since observed that the long-term persistence of memory CD8 T cells is dependent on IL-7 mediated survival of the memory cells. In support, blocking IL-7 signaling on memory CD8 T cells results in the loss of these memory cells; it has been shown that IL-7 promotes memory survival by inducing the up-regulation of Bcl-2 expression via STAT5 signaling (97-102). Similarly, it was demonstrated that LCMV-specific memory cells expressed higher level of Bcl-2 compared to both naïve and effector CD8 T cells and that this increase in Bcl-2 expression correlated with greater resistance to apoptosis (103, 104). Moreover, Wojciechowski et al. showed that Bcl-2 promoted cell survival by counteracting the pro-apoptotic effects of Bim expression in activated CD8 T cells (105, 106). All in all, these studies highlight the importance of IL-7 mediated increase in survival for the long-term maintenance of memory CD8 T cells. However, experiments with mice deficient in IL-15 signaling have demonstrated that the homeostatic turnover of memory cells is also a critical component of the mechanism that regulates memory CD8 T cell maintenance (107-110).

IL-15 is a member of the common γ-chain (CD132) family of cytokines that also includes IL-2, IL-4, IL-7, IL-9, and IL-21 (111). In addition to the common γ-chain, the IL-15 receptor consists of the IL-2/IL-15 receptor β-chain (CD122) and an IL-15 specific α-chain, with the latter playing a very unique role in mediating IL-15 signaling. Unlike

other members of this family of cytokines that require all components of the receptors to bind their respective cytokines, the IL-15 receptor α-chain (IL-15Rα) alone binds IL-15 with great affinity (112, 113). This understanding has led to the discovery of the unique mechanism by which IL-15 signaling is regulated.

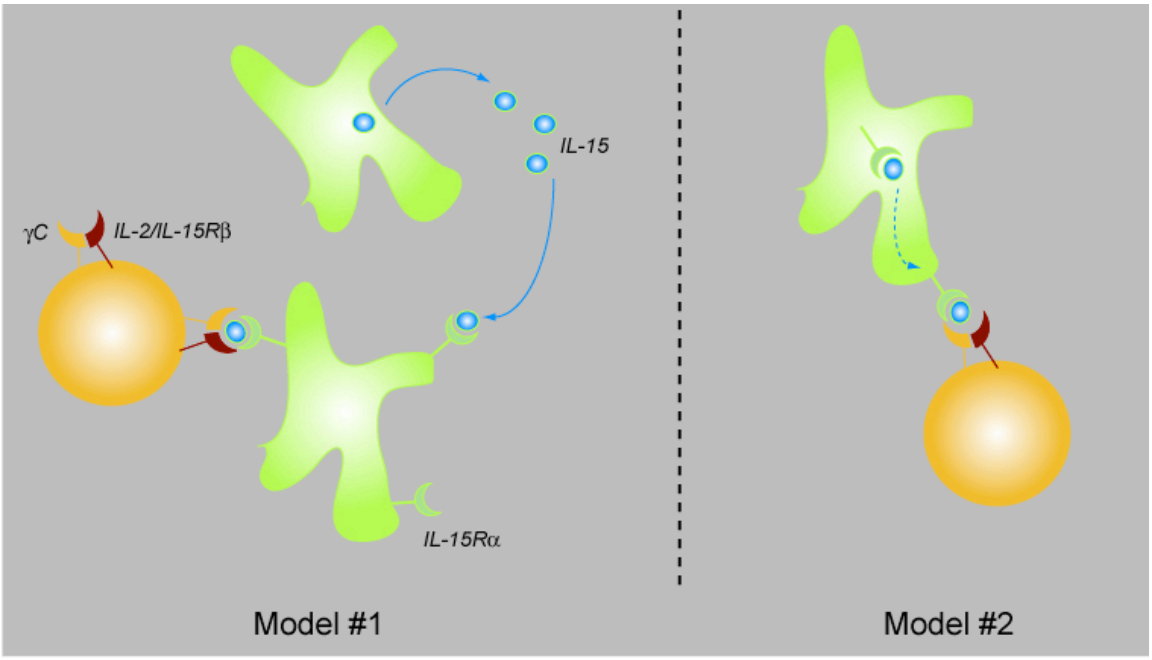


Figure 2. Different models of IL-15 trans-presentation. Model 1: IL-15 secreted extracellulary binds IL-15Rα expressed on nearby accessory cells. These cells, then, present in trans the IL-15Rα/IL-15 complex to responding cells (e.g. memory CD8 T cells) expressing the β- and γ-chains of the IL-15 receptor. Model 2: IL-15 binds IL-15Rα intracellularly and is transported to the surface as a single complex. Hence, the cytokine and the α-chain of the receptor must be synthesized by the same cell.

Unlike most other cytokines that are first secreted extracellularly and then act on distant cells, IL-15 is presented in trans through direct cell-to-cell interaction between IL-15/IL-15 receptor α-chain complex bearing accessory cells (e.g. DCs) and the responding cell types (e.g. memory CD8 T cells) (114, 115). In vivo experiments have shown that memory CD8 T cells expressing all three components of the IL-15 receptor are non-

responsive to IL-15 signaling when adoptively transferred into IL-15Rα deficient animals, highlighting the importance of this trans-presentation of IL-15 by IL-15Rα expressing accessory cells (116-118). Based on these initial studies, it was originally thought that upon their secretion, IL-15 bound IL-15Rα chain expressed on nearby accessory cells, which either responded to IL-15 themselves (and produced some other unknown factor) or trans-presented the cytokine to other nearby cells (Figure 2 – model #1). However, IL-15 has been shown to be extremely difficult to measure both in serum and in supernatant of cell culture. Due to the complex and multifaceted controls at the levels of message transcription, message translation, and protein translocation and secretion, even cells expressing significant level of IL-15 mRNA secrete negligible level of the cytokine, suggesting that IL-15 is not readily secreted in vivo (119-123). Along these lines, independent studies by Burkett et al. and Sandau et al. reported that the trans-presentation of IL-15 required the same population of cells to coordinately synthesize both IL-15 and the α-chain of its receptor (124, 125). Using mixed bone marrow chimeric animals, these studies showed that both IL-15 deficient cells expressing normal level of IL-15Rα and IL-15Rα deficient cells expressing normal level of IL-15 mRNA failed to support IL-15 mediated signaling to the responding cells. Subsequent experiments have provided an explanation for this impairment in mediating IL-15 signaling by demonstrating that IL-15 binds the IL-15 receptor α-chain within the endoplasmic reticulum/Golgi complex and is brought to the surface of the cell preassembled as a single complex (Figure 2 – model #2). In the absence of the α-chain, IL-15 remains sequestered within the intracellular compartments of the cell (125-127). Once on the surface, the IL-15/IL-15Rα complex remains bound to the cell surface,

although there have been reports of the induction of soluble form of this complex under certain inflammatory conditions (128, 129).

The importance of IL-15 in the long-term maintenance of memory CD8 T cells was initially observed through works by Tough et al., in which they found that injecting either Poly (I:C) or LPS without antigen into immune animals resulted in increased turnover of the memory CD8 T cells (49, 130). This increase in turnover has been linked to IL-15 as studies have shown that Poly (I:C) and LPS can elicit the production of type I IFN, which in turn elicits the production of IL-15 by the DCs (131). Subsequent work by various groups has since confirmed these observations and has shown that IL-15 mediated homeostatic turnover of memory CD8 T cells is critical for the long-term maintenance of memory CD8 T cells (98, 99, 107, 132). For instance, Becker et al. observed that although IL-15 deficient animals generated a very robust primary CD8 T cell immune response to LCMV infection, there was a slow decline in the number of LCMV-specific memory CD8 T cells. They determined that this impaired maintenance was not due to some intrinsic defect associated with the memory cells as these cells underwent normal homeostatic turnover and were maintained indefinitely when adoptively transferred into wild-type mice. Hence, Becker et al. concluded that the observed loss of memory CD8 T cells was a direct result of the impaired homeostatic turnover of the memory cells in the absence of IL-15 signaling, highlighting the importance of IL-15 mediated homeostatic turnover for the long-term maintenance of memory CD8 T cells.

Different models of memory CD8 T cell differentiation

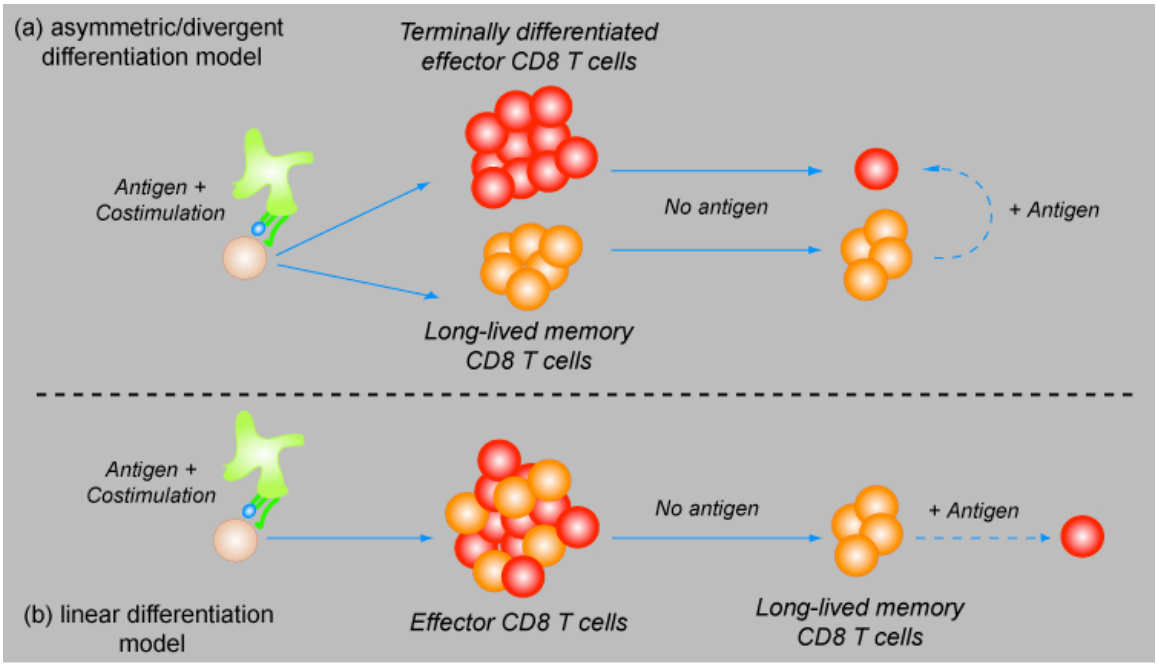


Figure 3. Models of memory differentiation. (a) Asymmetric/divergent differentiation model argues that effector and memory CD8 T cells are generated independent of one another. In this model, CD8 T cells can bypass the effector stage and differentiate directly into memory cells. (b) Linear differentiation model suggests that memory CD8 T cells are direct descendants of effector CD8 T cells. In this model, memory differentiation does not occur until the clearance of antigen.

Due to their critical role in providing secondary protection, there has been much interest over the years in better deciphering the precise lineage of memory CD8 T cells. Based on the understanding that a single, antigen-specific CD8 T cell can give rise to primary effector cells and both types ("central" and "effector") of memory cells (133), two main models have been proposed to explain the differentiation pathway followed by memory CD8 T cells: divergent vs. linear model of memory differentiation (Figure 3).

Divergent/Asymmetric model of memory differentiation

The divergent model proposes that the generation of memory and effector CD8 T cells occurs independently of one another. Upon T cell activation, two separate lineages of CD8 T cells are generated – one that differentiates into terminal effector CD8 T cells and the other that develops into long-lived memory cells. In support, various studies have shown that memory CD8 T cells can be generated without ever having to go through an effector stage. For instance, vaccination with heat-killed Listeria monocytogenes results in the generation of a large pool of memory CD8 T cells without having ever generated effector CD8 T cells (134). Similarly, Manjunath N et al. demonstrated that activation of CD8 T cells in vitro with cognate peptide and recombinant IL-15 resulted in memory cells that were capable of rapid-recall response without having gone through an effector stage (135). This divergent/asymmetric model of memory differentiation has been shown to also occur after infection with vaccinia or Listeria monocytogenes in vivo. Approximately 5 days after infection, Laouar et al. observed a small, but significant population of $CD44^{hi}$, $CD62L^{hi}$, and granzyme B^{lo} antigen-specific CD8 T cells that conferred protective immunity when adoptively transferred into new recipients and challenged with secondary exposure (136). Lastly, Chang et al. provided data demonstrating that CD8 T cell activation resulted in the unequal partitioning of cellular proteins during cell division (137). They proposed that this led to the generation of two distinct daughter cells: one that was destined to differentiate into effector CD8 T cells (e.g. expression of IFN-γ receptor, T-bet, granzyme B, and CD43) and one that had the attributes of a memory cell (e.g. lower expression of granzyme B, T-bet, and CD43; higher expression of CD127 and CD62L).

Linear model of memory differentiation

The linear model of memory differentiation, on the other hand, argues that memory CD8 T cells are generated directly from a pool of effector CD8 T cells (138). In this model, all activated CD8 T cells first develop into potent effector cells. Upon antigen clearance, a subset of these effector CD8 T cells (memory precursors) persists to become long-lived memory CD8 T cells. In support, Jacob and Baltimore utilized a system in which they induced the expression of the reporter gene PLAP (human placental alkaline phosphatate) in CD8 T cells that express (or had expressed in the past) granzyme B (139). They observed that great majority of the memory CD8 T cells were positive for PLAP expression, suggesting that these memory cells had once been granzyme B expressing effector CD8 T cells. Bannard et al. also observed similar findings using a transgenic mouse line that permits the mapping of the fate of granzyme B expressing CD8 T cells and their progeny by permanently marking them with enhanced yellow fluorescent protein (EYFP) (140). They found that influenza-specific CD8 T cells that became $EYFP^+$ during the primary infection, rapidly expanded upon secondary challenge.

Along these lines, studies have demonstrated that memory precursors could be distinguished from the rest of the pool of effector CD8 T cells based on their unique phenotypic expression (5, 141, 142). For example, Kaech et al. observed that at the peak of the CD8 T cell immune response (day 8 post acute LCMV infection), a small subset of effector CD8 T cells expressed higher level of CD127 (IL-7Rα) in comparison to the rest of the effector population; these cells preferentially survived to become long-lived memory CD8 T cells (5). Similarly, Sarkar et al. reported that activated CD8 T cells expressed varying level of KLRG-1 at day 4.5 post-LCMV infection and that the KLRG-

1^{lo} CD8 T cells preferentially survived to become long-lived memory CD8 T cells (143). Kalia et al. found that these memory precursors could be identified even as early as day 2.5 post-LCMV infection based on their IL-2Rα/CD25 expression. The $CD25^{lo}$ CD8 T cells preferentially up-regulated CD127 and CD62L and gave rise to functional long-lived memory CD8 T cells (144). On the surface, these studies seem to argue in favor of the divergent/asymmetric model of memory differentiation. However, it should be emphasized that in all these studies, despite the minor differences in phenotypic expression, these memory precursors resembled other effector CD8 T cells in regard to their overall function (e.g. granzyme B and perforin expression; cytolytic activity).

Despite this progress, it remains a mystery as to whether this unique phenotypic expression is merely a set of "markers" that identify the pool of effector CD8 T cells that survive to become long-lived memory cells, or whether these cell-surface proteins play a functional role in providing these cells with a selective advantage in differentiating into long-lived memory CD8 T cells. In support of the former, over-expression of CD127 on CD8 T cells does not result in decreased contraction and generation of a larger pool of memory CD8 T cells (145-148). However, this may be due to the availability of IL-7 within a given animal, as in vivo administration of IL-7 during the contraction phase of CD8 T cell immune response has been shown to result in decreased contraction and increased number of memory cells (149). Similarly, studies have shown that excessive or prolonged IL-2 signaling early on in the immune response results in impaired memory differentiation (144, 150). Although the importance of IL-2 both for the normal accumulation of effector CD8 T cells and for the programming of memory CD8 T cells to rapidly respond upon secondary infection in vivo has been well documented (151-155),

work by Pipkin et al. demonstrated that prolonged IL-2 stimulation early on during CD8 T cell expansion resulted in the induction of the transcription factor eomesodermin (Eomes), the up-regulation of perforin (Prf1) transcription, and the repression of the re-expression of memory CD8 T cell markers Bcl6 and IL-7Rα (150). These results argue in favor of the latter and suggest that the unique phenotypic profile (e.g. $CD25^{lo}$) of the memory precursors plays a functional role in providing these cells with a selective advantage over other activated cells to become long-lived memory CD8 T cells. All in all, these studies collectively support the linear model of memory CD8 T cell differentiation.

Regulation of memory CD8 T cell differentiation

Regardless of the pathway taken by CD8 T cells to become long-lived memory cells, it is still not completely understood how and why a population of CD8 T cells develops into long-lived memory cells whereas others fail to do so. Studies have demonstrated that the initial antigen encounter triggers a cell-intrinsic instructive developmental program within the CD8 T cells that does not cease until memory CD8 T cell formation (47, 156-158). For instance, Kaech et al. showed that a brief antigen encounter (less than 24 hours) was sufficient in driving CD8 T cell activation, proliferation, and differentiation into effector and subsequently into memory CD8 T cells. Furthermore, they noted that once initiated, this developmental process continued in the absence of further antigenic stimulation (47). Although these studies seem to suggest that memory CD8 T cell differentiation, once initiated, goes into "autopilot mode" and occurs

independently of any other extraneous signals, various extrinsic factors can greatly influence the overall development of fully functional memory CD8 T cells.

Duration and level of antigenic stimulation

As described-above, initial antigen encounter triggers a cell-intrinsic instructive developmental program within the CD8 T cells that does not cease until memory CD8 T cell formation (47, 156-158). However, the overall duration and magnitude of the antigenic stimulation can greatly alter the overall quality of memory CD8 T cells that are generated (the "decreasing-potential" model) (4). For example, prolonged or persistent antigenic stimulation (e.g. chronic infection) results in the eventual exhaustion (loss of function) and/or deletion of the antigen-specific CD8 T cells (159, 160). Similarly, Sarkar et al. demonstrated that naïve P14 CD8 T cells that were adoptively transferred into LCMV-Arm infected animals at 4 days post-infection proceeded to become better memory CD8 T cells compared to those that were transferred on the day of infection (161). Usharauli et al. also found that limiting the duration of antigen stimulation resulted in the generation of effector CD8 T cells that produced greater amount of IL-2 and that subsequently differentiated into better memory cells, further suggesting that shorter the duration of antigenic stimulation, better the memory cells generated (162). However, overly brief antigenic stimulation (e.g. 2-6 hours) has been shown to result in modest proliferation of CD8 T cells, but these cells never gain effector function and are quickly deleted (163, 164). Therefore, there is a fine balance in the overall duration and level of antigenic stimulation that is required for the development of fully functional memory CD8 T cells – the Goldilocks model of effector and memory CD8 T cell

development (165).

Inflammation

Studies have also suggested that excessive inflammation early on in the immune response negatively affects memory CD8 T cell development by driving cells toward the terminal differentiation of effector CD8 T cells. For example, it was observed that reduced inflammation early on in infection resulted in not only decreased contraction of effector CD8 T cells, but also resulted in the more rapid acquisition of characteristics associated with memory CD8 T cells (166-168). Along these lines, Joshi et al. reported that the inflammatory cytokine IL-12 regulated the development of memory CD8 T cells by modulating T-bet expression in antigen-specific CD8 T cells (169). They showed that an increase in IL-12 correlated with higher expression of T-bet, which is known to impede memory CD8 T cell development by inducing the terminal differentiation of antigen-specific CD8 T cells toward KLRG-1^{hi} and $CD127^{lo}$ effector CD8 T cells (170-172).

Despite these experiments, the exact magnitude of the effect of inflammation on memory development remains controversial. In support, Sarkar et al. argued that much of these earlier experiments addressed the role of inflammation in the presence of continued antigenic stimulation (143). When they examined the effect of inflammation alone by directly comparing P14 (specific for the D^b GP33-41^+ epitope of LCMV) CD8 T cell response in mice infected with wild-type vaccinia virus with those infected with vaccinia virus expressing the D^b GP33-41^+ epitope of LCMV, Sarkar et al. observed that inflammation alone did not drive the terminal differentiation of effector CD8 T cells.

Instead, it was the prolonged exposure to inflammation in the context of continued antigenic stimulation that resulted in impaired memory CD8 T cell development. In fact, various studies have shown that different inflammatory signals early on in the immune response are actually important for maximal clonal expansion and memory formation. For instance, type I interferon (IFN-I) receptor deficient CD8 T cells exhibit impaired clonal expansion, resulting in defective memory development compared to WT CD8 T cells after primary infection with various pathogens (e.g. VV, VSV, LM, and LCMV) (42). This defect in CD8 T cell expansion is not due to impaired proliferation as IFN-I receptor deficient CD8 T cells actually divide much more rapidly compared to WT CD8 T cells. Instead, studies have shown that type I IFN signaling via STAT1 is essential for the survival of effector CD8 T cells in the context of an acute infection (41, 44, 173).

Similarly, IL-12 has also been described to play an important role for optimal proliferation and clonal expansion of activated antigen-specific CD8 T cells. IL-12 deficient animals (IL-12 -/-) display significant impairment in CD8 T cell expansion compared to WT mice; therefore, IL-12 -/- mice are highly susceptible to LM infection (174, 175). Experiments by Henry et al. demonstrated that IL-12 was important for maximal clonal expansion by increasing the production of the chemokines CCL1 and CCL17, which promoted the stable cognate interaction between CD8 T cells and DCs during initial T cell priming (176). IL-12 has also been described to promote the survival of effector CD8 T cells by inducing the expression of anti-apoptotic molecule Bcl-3 (177). Interestingly, despite the impairment in clonal expansion, IL-12 deficient animals have substantially more memory CD8 T cells and greater protective immunity against re-infection (174, 175). This is in agreement with work in which it was shown that the

contraction of effector CD8 T cells and memory CD8 T cell development were controlled by the magnitude of inflammation early on in the immune response (166, 167).

As a whole, the above-described studies highlight the dual effects that inflammatory cytokines have on memory CD8 T cell development, depending upon both the timing and the environment in which the cytokines are presented. Evolutionarily speaking, this makes sense as the inflammatory cytokines produced early on in the immune response play critical roles in promoting pathogen clearance. The negative effect of inflammation on memory development would ensure that the differentiation of memory CD8 T cells does not occur until after the infection is controlled and inflammation has subsided.

Precursor frequency

As described earlier, there are only about 100-200 CD8 T cells specific for a given antigen in a naïve mouse (31, 32). Therefore, many studies focusing on memory CD8 T cell development have often relied on TCR (T cell receptor) transgenic systems (e.g. P14, OT-I, etc.) to circumvent this issue. These systems allow for the transfer of large number of initial precursor cells, maximizing the total number of antigen-specific effector and memory CD8 T cells that are generated and making analysis easier (178).

Despite the increase in the frequency of initial precursors, the overall behavior of the transferred transgenic CD8 T cells after immunization/infection has often been assumed to accurately model the endogenous CD8 T cell immune response. However, various research groups have begun to question the validity of these earlier studies arguing that the results from these studies do not accurately depict immune responses

under physiological conditions. For instance, experiments with different transgenic systems have shown that upon their generation, memory CD8 T cells continue to linearly differentiate from "effector" memory ($CD62L^{lo}$ and $CCR7^{lo}$; less efficient at producing IL-2; less proliferative capacity; found mostly in non-lymphoid tissues; T_{EM}) to "central" memory ($CD62L^{hi}$ and $CCR7^{hi}$; efficiently produce IL-2; high proliferative capacity; T_{CM}) CD8 T cells (12, 179-182). However, Marzo et al. has argued that at low precursor frequency (comparable to that observed in normal animals), T_{EM} cells do not further differentiate into T_{CM} cells (183). They reported that they only observed the generation of T_{CM} when the initial antigen-specific CD8 T cell precursor frequency was significantly high, suggesting that the earlier assumption that immune responses generated with non-physiological numbers of transgenic CD8 T cells accurately mimic the endogenous response is incorrect. Similarly, Badovinac et al. observed that the initial precursor frequency of antigen-specific T cells dictates most facets of the CD8 T cell immune response (e.g. kinetics, proliferation, phenotypic expression, effector function, and memory development) (184). For example, at high or intermediate number of precursor frequency, they found that antigen-specific CD8 T cells underwent much reduced expansion (8-12 divisions compared to 16-19 divisions observed with low precursor frequency) and more closely resembled memory than effector CD8 T cells, both in phenotypic expression and in function at day 7-8 post LM infection.

Along these lines, Sarkar et al. also observed that the initial precursor frequency of antigen-specific CD8 T cells influenced memory CD8 T cell development, but this influence was largely kinetics (161). In agreement with the findings by Badovinac et al., Sarkar et al. noted that both the fold expansion of the antigen-specific CD8 T cells and

the intensity of the effector CD8 T cell differentiation (e.g. reduced granzyme B expression with higher precursor frequency at day 6 post LCMV-infection) were much reduced at higher precursor frequency (184). However, in contrast to the findings by Marzo et al., they observed that regardless of the initial precursor frequency, T_{EM} cells gradually differentiated into T_{CM} cells, albeit at different rates (161, 163). They explained that the contrast in their results from that observed by Marzo et al. could be that they did not monitor over a sufficient period of time to observe T_{EM} to T_{CM} conversion within the endogenous CD8 T cell population. In summary, the initial precursor frequency of antigen-specific CD8 T cells can alter the overall rate of memory CD8 T cell differentiation, but not the overall quality of these memory cells.

CD4 T cell help

The role of helper CD4 T cells in the development of fully functional memory CD8 T cells is an area of research that continues to baffle scientists today. Based on current understanding, it is generally believed that CD8 T cells and CD4 T cells intimately engage with one another via antigen presenting cells to initiate an effective immune response, but then carry out their effector function independent of one another. More specifically, antigen-specific CD4 T cells recognize peptide presented in the context of major histocompatibility complex (MHC) class II molecules on dendritic cells (DCs). This interaction allows helper CD4 T cells to "license" DCs via CD40-CD40L signaling resulting in the up-regulation of different co-stimulatory molecules (e.g. CD70, B7 family) and the production of various cytokines (e.g. type I IFN, IL-12, IL-15, IL-21)

by the DCs, which, collectively, aid in the activation of naïve antigen-specific CD8 T cells and the development of effector and memory CD8 T cells (43, 185-196) (Figure 4).

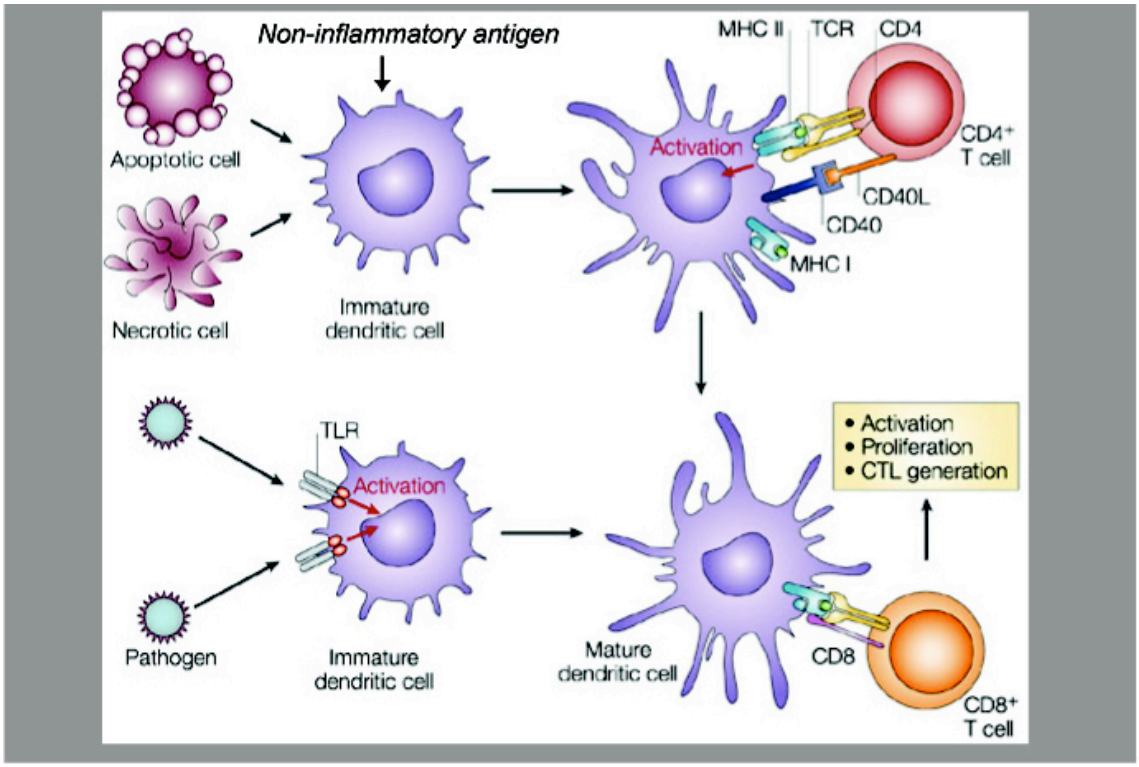


Figure 4. Role of CD4 T cells in the activation of antigen-specific CD8 T cells. For most non-inflammatory antigens and apoptotic/necrotic bodies, antigen-specific CD4 T cells are required to help activate the DCs via CD40-CD40L. Activated DCs can then induce the activation of antigen-specific CD8 T cells and their differentiation into effector and memory cells. By contrast, most inflammatory antigens can bypass the need for CD4 T cells by stimulating via the TLRs or directly infecting the DCs.

For most non-inflammatory antigens (e.g. minor H antigens, islet beta cell antigens, peptide-pulsed DCs, tumor antigens, and tissue grafts), the generation of both effector and memory CD8 T cells is closely dependent on this CD4 T cell help (197-201). Immunization of CD4 T cell deficient animals with different non-inflammatory antigens has been shown to result in the tolerance of antigen-specific CD8 T cells rather than in the generation of potent effector and memory cells. Studies have shown that in vivo

administration of agonistic anti-CD40 antibodies can rescue this defect, affirming the importance of CD40-CD40L signaling in the "licensing" of antigen-bearing DCs in the generation of both effector and memory CD8 T cells in the context of non-inflammatory antigens (202, 203).

Interestingly, the generation of cytotoxic CD8 T lymphocytes against many live pathogens (e.g. lymphocytic choriomeningitis virus, ectromelia virus, listeria monocytogenes, influenza virus, sendai virus, vaccinia virus, and vascular stomatitis virus) has been described to be independent of CD4 T cell help (39, 204-209). It is believed that these inflammatory pathogens may either directly infect or activate the DCs via the Toll-like receptors (TLRs) and thereby, bypass the need for CD4 T cell help. In support, Wu et al. showed that direct infection of splenic DCs with influenza virus resulted in the up-regulation of different co-stimulatory molecules. These DCs were able to induce CD8 T cell activation and efficient CTL activity (210). Similarly, Johnson et al. demonstrated that CD40L expression on the DCs was significantly up-regulated after brief stimulation with different TLR agonists (e.g. poly I:C and CpG) (211). Based on these observations, it was initially proposed that depending on the nature of the antigen (non-inflammatory vs. inflammatory), CD4 T cell help may or may not be required for the induction of an effective CD8 T cell immune response.

However, closer examination of these "unhelped" CD8 T cells has revealed that the subsequent stages of CD8 T cell differentiation are significantly impaired in the absence of CD4 T cell help. For example, although CD4 T cell deficient mice infected with either recombinant Listeria monocytogenes expressing chicken ovalbumin protein (rLm-OVA) or recombinant vaccinia virus expressing the LCMV-gp33 epitope (rVV-

gp33) initially clear antigen and exhibit normal numbers of antigen-specific CD8 T cells, these animals display severe defects in their recall capabilities upon secondary challenge (212, 213). This impairment in protection has been shown to be a direct consequence of the reduced proliferative capacity of these "unhelped" memory CD8 T cells upon secondary exposure. Along these lines, Janssen et al. reported that memory CD8 T cells generated in the absence of CD4 T cells were susceptible to TRAIL (TNF-related apoptosis-inducing ligand) mediated AICD upon re-stimulation (214). They observed that despite expressing similar level of TRAIL receptor DR5 (death receptor 5) compared to WT memory cells, unhelped memory CD8 T cells produced higher amount of TRAIL upon secondary activation, resulting in increased susceptibility to TRAIL mediated AICD. However, subsequent experiments by Badovinac et al. showed that TRAIL deficiency in memory CD8 T cells delayed, but did not prevent the defects associated with unhelped memory CD8 T cells; therefore, there may be both TRAIL-dependent and –independent help provided by CD4 T cells (215). Collectively, these studies have suggested that regardless of the nature of the antigen, CD4 T cells are absolutely required for the generation of fully functional memory CD8 T cells (Figure 5).

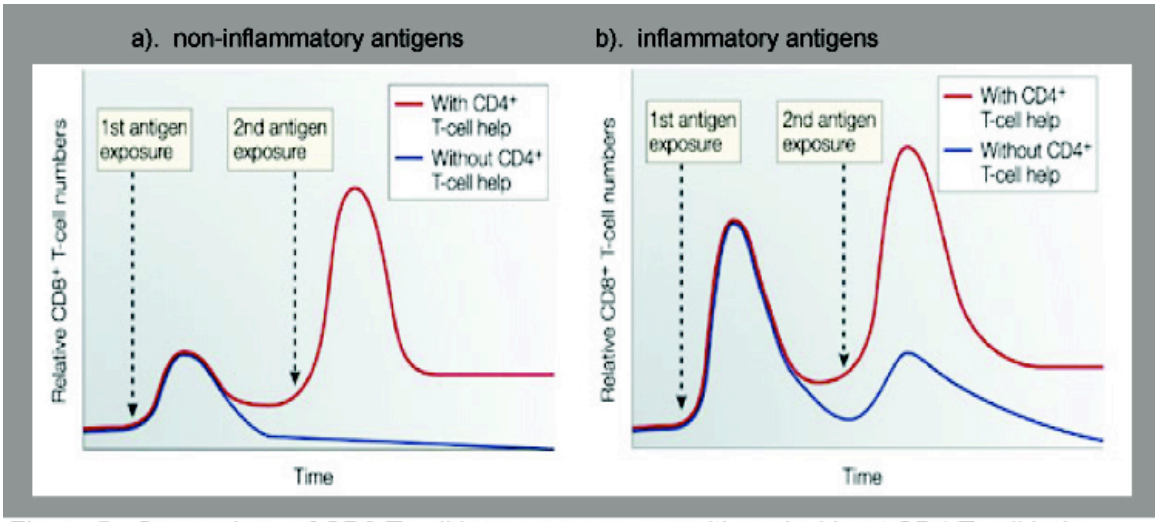


Figure 5. Comparison of CD8 T cell immune response with and without CD4 T cell help. a) Magnitude of primary response toward most non-inflammatory antigens is rather modest at the peak of the primary effector stage. The primary response in CD4 T cell deficient animals is similar. However, "unhelped" CD8 T cells quickly become anergic and fail to develop into fully functional effector and memory cells. b) Similarly, the primary response toward most inflammatory antigens is similar in wild-type and CD4 T cell deficient animals. However, functional effector CD8 T cells are generated in the absence of CD4 T cell help, but these "unhelped" cells fail to differentiate into healthy memory cells and exhibit defective secondary response.

Although the importance of CD4 T cell help in the generation of memory CD8 T cells has been well established, both the mechanism by which CD4 T cells provide this "help" and when during the immune response this "help" is required have been more difficult to elucidate. Currently, the general consensus in the field is that, as observed with non-inflammatory antigens, helper CD4 T cells are required during the initial priming of antigen-specific CD8 T cells to "program" them to differentiate into fully functional memory cells. Along these lines, Shedlock et al. observed that memory CD8 T cells generated in WT animals exhibited normal secondary response when adoptively transferred into CD4 -/- mice, but memory cells generated in CD4 -/- animals and transferred into WT mice failed to do so, demonstrating the importance of CD4 T cell help early on in the CD8 T cell immune response (212). Similarly, Williams et al.

reported that IL-2 provided by activated CD4 T cells during the initial CD8 T cell priming was crucial for the generation of fully functional memory CD8 T cells (153). They observed that despite the similarity in their primary response to infection with either LCMV or rLM-OVA, IL-2Rα (CD25) deficient CD8 T cells failed to mount a robust secondary response after rechallenge, compared to WT CD8 T cells. This defect was rescued when exogenous IL-2 was provided at the time of primary immunization, further highlighting the importance of CD4 T cell help during the initial priming of antigen-specific CD8 T cells to generate fully functional memory cells.

However, all this was further complicated by a study published by Sun et al. in which they argued that CD4 T cells are not required for the "programming" of memory CD8 T cells, but rather for their long-term maintenance (216). They demonstrated that the adoptive transfer of effector CD8 T cells generated in WT animals into uninfected MHC class II deficient animals resulted in the gradual loss of these transferred cells and in the subsequent loss of protection after secondary challenge. Conversely, when effector or memory CD8 T cells generated in MHC class II deficient animals were adoptively transferred into WT recipients, the transferred cells were stably maintained and retained their ability to mount a robust secondary response upon rechallenge. Accordingly, they argued that helper CD4 T cells promoted the stable maintenance of memory CD8 T cells by regulating the production of a yet unidentified element required for memory maintenance (216). Similarly, others have called into question the long held dogma that helper CD4 T cells "program" the development of memory CD8 T cells during the initial priming. Instead, it has been proposed that the primary role of CD4 T cell help in generating CD8 T cell memory after infection is to augment the proliferation and/or

survival of the antigen-specific CD8 T cells through co-stimulatory signaling (217). Hence, more work will be required to identify exactly when and how helper CD4 T cells regulate the generation of optimal memory CD8 T cells in the context of acute infections

This is obviously not an exhaustive list of all variables that can potentially influence the development of fully functional memory CD8 T cells. However, it does begin to highlight the complexity involved in the CD8 T cell immune response generated toward different antigens. Much more work is needed to better identify all the different elements that are involved in the generation of healthy memory CD8 T cells. Chapter 2 of this thesis will focus more on how and when CD4 T cells are required for the development of fully functional virus-specific memory CD8 T cells.

Regulation of the homeostatic turnover and long-term maintenance of memory CD8 T cells

As described earlier, a hallmark of memory CD8 T cells is their continued persistence long after the infection has been resolved, which enables memory cells to play an essential role in conferring lifelong protective immunity for the host (70). This long-term persistence has been shown to be associated with IL-7 and IL-15 mediated survival and homeostatic turnover of memory CD8 T cells (97-99, 107, 108, 132). Despite this understanding, there still remain questions that have yet to be fully addressed.

What are the rules that characterize the homeostatic turnover of memory CD8 T cells?

Initial studies addressing the mechanisms of long-term maintenance of memory CD8 T cells had suggested that memory CD8 T cells were non-dividing and that the continued persistence of these cells long after the resolution of infection was primarily due to their long lifespan (4, 218). However, subsequent BrdU (5-bromo-2'-deoxyuridine) incorporation experiments have demonstrated that this population of memory cells actively undergo cellular division (130). As described earlier, this turnover of memory CD8 T cells has been shown to be dependent on IL-15 mediated signaling (99, 107, 132).

Despite this understanding, the rules that characterize this homeostatic turnover of memory CD8 T cells remain less well elucidated. For instance, do all memory CD8 T cells undergo homeostatic turnover or is there a subpopulation of cells that do not homeostatically divide? Furthermore, do memory cells divide after a fixed time or is this turnover stochastic (probability that a cell divides does not depend on its previous history)? Lastly, what is the precise rate at which this homeostatic division of memory CD8 T cells occurs? As described above, many of the past studies have relied primarily on BrdU incorporation to address the turnover of CD8 T cells at various stages of chronic or acute infections (130, 219-222). Although these studies have been of tremendous value in ascertaining a qualitative picture of CD8 T cell turnover, the limitations in the data that can be generated with BrdU incorporation have made it difficult to attain a more rigorous quantitative analysis of the homeostatic turnover of memory CD8 T cells. For instance, BrdU is a synthetic thymidine analog that gets incorporated into the DNA of cells as they divide; therefore, it is effective in identifying bulk cells that have undergone cellular division (223). However, it is not possible with BrdU alone to identify memory

cells that have undergone different rounds of divisions – information that is critical in providing a more quantitative analysis. Furthermore, there are other problems associated with interpreting the results from BrdU incorporation experiments that the accuracy of these results remains an issue (224, 225).

Is the homeostatic turnover of memory CD8 T cells dependent on epitope specificity?

As discussed earlier, clonal competition and the initial strength of the TCR stimulus can greatly alter the overall rate of memory CD8 T cell differentiation. For example, Sarkar et al. demonstrated through in vivo experiments that the rate of conversion from T_{EM} to T_{CM} for LCMV-specific memory CD8 T cells varied depending on their epitope specificity (161). They reported that LCMV nuclear protein (NP) was produced earlier and at a higher level during infection than glycoprotein (GP) and this pattern of protein expression corresponded well to the higher level of NP396 epitope available for T cell stimulation compared with the glycoprotein epitopes (e.g. GP33 and GP276). Hence, the conversion rate from T_{EM} to T_{CM} was the slowest for $NP396^+$ memory CD8 T cells compared to either $GP33^+$ or $GP276^+$ memory cells.

Based on these observations, the rate of memory differentiation may also vary depending on the epitope specificity. Currently, it is unknown whether the rate at which memory CD8 T cells turnover is contingent on the specificity of the cells. Many of the earlier studies addressing the homeostatic turnover of memory CD8 T cells relied on CD44 expression to identify the memory CD8 T cell population as memory cells express high level of CD44 (130, 226). However, it should be noted that recently activated and effector CD8 T cells also express comparable levels of CD44; therefore, there is a clear

need to utilize some of the more recent technologies (e.g. tetramer and TCR transgenic systems) to better address this question.

What role do helper CD4 T cells play in the homeostatic turnover and the long-term maintenance of memory CD8 T cells, in both their quantity and their quality?

CD4 T cell help has been described to be important in the generation and maintenance of CD8 T cell immune response in the context of both acute and chronic infections. For instance, CD4 T cell help is important in preventing the exhaustion and deletion of antigen-specific CD8 T cells associated with many chronic infections (e.g. Clone-13) (227-230). As described earlier, for most acute infections, helper CD4 T cell had been traditionally thought to play an important role in the development of fully functional memory CD8 T cells (212, 213, 231). However, more recent data provided by Sun et al. have suggested that helper CD4 T cells play a necessary role in the long-term maintenance of memory CD8 T cells (216). Despite this understanding, how helper CD4 T cells aid in promoting the continued persistence of memory CD8 T cells long after the resolution of infection has yet to be clearly identified. For example, is CD4 T cell help required for the homeostatic turnover of memory CD8 T cells or for their long-term survival? In addition, do memory CD8 T cells continue to require helper CD4 T cells to also maintain their overall quality (phenotypic profile and recall capabilities)?

In Chapter 3 of this thesis, we use both CFSE labeling experiments and mathematical modeling to better address some of the above-described questions that pertain to the homeostatic turnover of memory CD8 T cells.

Utilizing the immunosuppressant drug FTY720 to study effector and memory CD8 T cell trafficking through different peripheral tissues

As discussed earlier, naïve CD8 T cells continuously recirculate between the peripheral blood and the secondary lymphoid tissues (e.g. lymph nodes and spleen) in search of DCs presenting their cognate antigen (232, 233). Upon antigen recognition activated CD8 T cells undergo extensive proliferation and differentiate into effector CD8 T cells that emigrate from the lymphoid tissues into the periphery, where they carry out their cytolytic activity. In the absence of antigen recognition, naïve CD8 T cells migrate out back into the peripheral blood and go on to survey other lymphoid organs. This trafficking of CD8 T cells in and out of different lymphoid tissues is critical for the induction of an effective cellular immune response. Hence, much research has been done to identify the various elements that regulate CD8 T cell migration.

Naïve CD8 T cell trafficking into the secondary lymphoid tissues

Entry of CD8 T cells into the LNs occurs primarily via the high endothelial venules (HEVs) and involves a three-step process (rolling, tight adhesion, and transmigration) that is tightly regulated by a family of selectins, integrins, and chemokines (234). More specifically, this process begins with CD62L (L-selectin) expressed on naïve CD8 T cells binding its ligand (peripheral node addressins or PNADs – highly glycosylated and sulfated sialomucins) on the HEVs; this interaction results in the slowing down and rolling of the lymphocytes along the endothelial cells of the HEVs (235-237). Next, the chemokines CCL21/CCL19 (produced by the HEVs) interact with their receptor, resulting in the activation of LFA-1 (β2-integrin; lymphocyte function

associated antigen 1) expressed on CD8 T cells (238, 239). Activated LFA-1, then, promotes the tight adhesion of the CD8 T cells to the HEVs by binding ICAM-1 and ICAM-2 (intracellular adhesion molecule 1 and 2) expressed on the surface of the high endothelial venules (240, 241). This allows the lymphocytes to transmigrate between adjacent endothelial cells, gaining entry into parenchyma of the LNs.

The importance of this three-step process in the trafficking of naïve T cells into the LNs becomes much more apparent when we examine mice deficient in any of these cell surface proteins involved in migration. For instance, the LNs of CD62L deficient or CCL21/CCL19 deficient animals display significantly reduced cellularity due to the impaired migration of lymphocytes across the HEVs (242, 243). Similarly, Kadono et al. observed a significant reduction (>50%) in the migration of cells to peripheral LNs in ICAM-1 deficient animals (244). Collectively, these studies highlight the importance of the different family of selectins, integrins, and chemokines in CD8 T cell trafficking into the LNs.

Trafficking of CD8 T cells into the spleen, on the other hand, does not involve the high endothelial venules, but integrins and chemokines, nonetheless, play important roles. CD8 T cells are initially released into marginal zone of the spleen from terminal arterioles that open into the red pulp or the marginal sinus (232). From there, the lymphocytes gain entry into the T (periarteriolar lymphoid sheath; PALS) and the B cell zones of the spleen through a process that is dependent on both integrins and chemokines. In support, Lo et al. observed that blocking LFA-1 and $\alpha 4\beta 1$ integrin signaling resulted in a significant defect in T cell migration into the white pulp of the spleen (245). Furthermore, Cyster and Goodnow highlighted the importance of chemokines in CD8 T

cell migration into the white pulp by demonstrating that the treatment of cells with pertussis toxin, a known inhibitor of G protein-coupled chemokine receptor signaling, had no effect on the overall accumulation of lymphocytes in the spleen, but had impaired cells from gaining into the white pulp of the spleen (246). Hence, although the precise mechanisms may differ, family of selectins, integrins, and chemokines play important roles in CD8 T cell migration to the various secondary lymphoid tissues.

Egress of CD8 T cells from the peripheral LNs

As shown above, the requirements for entry into the secondary lymphoid tissues have been well characterized for quite some time. However, the mechanism by which CD8 T cells migrate back out into peripheral blood from these tissues has only now begun to be more carefully elucidated. Traditionally, it has been described that once naïve CD8 T cells successfully cross the HEV of the LNs, these cells randomly crawl along the surface of the FRC (fibroblastic reticular cells) network toward the paracortex region (T cell area) of the LNs in search of cognate antigen (247) (Figure 6). In the absence of antigen recognition, these lymphocytes leave the T cell regions of the LNs and drain into the medulllary sinuses either via the cortical sinusoids or directly from the paracortex. From there, CD8 T cells flow freely into efferent lymph and circulate back into the blood via the thoracic duct (232) (Figure 6). Experiments with the immunosuppressant drug FTY720 have implicated sphingosine-1-phosphate signaling as being crucial for the egress of CD8 T cells from the peripheral LNs.

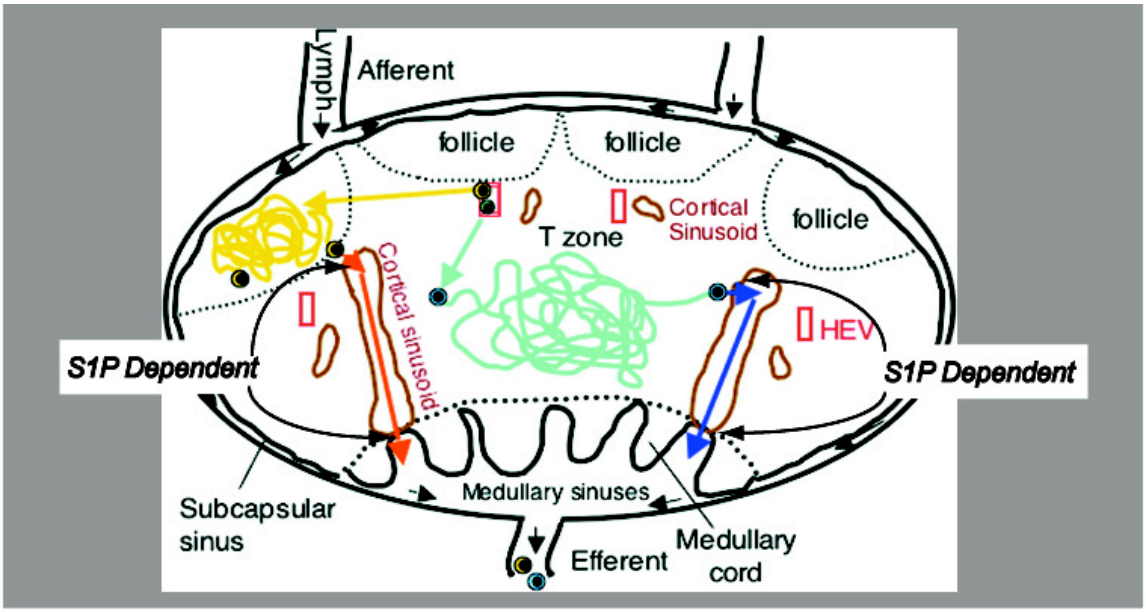


Figure 6. Lymphocyte recirculation through a lymph node. Diagram represents a lymph node cross-section and depicts possible paths taken by a B cell (yellow) or T cell (blue) after entry via the HEVs. After extensive migration in the follical or T cell zone, respectively, the cells exit via the efferent lymphatics. Cortical sinusoids (brown), which are lymphatic-related structures present in the outer T cell zone, connect with the medullary sinuses. Alternatively, lymphocytes may exit directly into the medullary or subcapsular sinuses. Entry into either the cortical sinusoids or medullary sinuses is linked to S1P signaling.

FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride)

FTY720 is an immunosuppressant chemically derived from a fungal metabolite (myriocin/ISP-1 – sphingosine-related molecule that binds and inhibits serine palmitoyl-transferase, an enzyme involved in sphingolipid biosynthesis) extracted from an herb (Iscaria sinclarii) commonly used by the Chinese for its anti-aging effects (232, 248, 249). It has been shown to be effective in both reducing allograft transplant rejections and onset of many autoimmune diseases in both animals and humans (250). Unlike cyclosporin A and tacrolimus, FTY720 induces its immunosuppressive effects not by interfering with T cell activation, but by decreasing the number of circulating lymphocytes in the peripheral blood and lymph fluid (232, 251-253).

Originally, this decrease in circulating lymphocytes was believed to be the direct result of FTY720 mediated apoptosis of these cells. In support, Suzuki et al. observed that human mononuclear cells treated with 5 uM FTY720 in vitro resulted in a dose-dependent reduction in cell viability with a corresponding decrease in Bcl-2 expression and increase in Bax protein levels (254). Similarly, Isoyama et al. demonstrated that when rats were intramuscularly injected with 10 mg/kg/day FTY720 for 7 days, there were reduced numbers of cells within the thymus of these animals. They argued that this was due to FTY720 mediated apoptosis as they noted an increase in apoptosis in the cortical region of the thymus by TUNEL analysis (255).

However, the concentration of FTY720 used in vivo for therapeutic purposes (0.1 to 1 mg/kg), is significantly less than that used in these studies (250, 256). Therefore, it is highly unlikely that the mechanism in which FTY720 treatment results in peripheral lymphopenia is linked to apoptosis of the circulating lymphocytes. Instead, Chiba et al. proposed an alternative mechanism in which FTY720 treatment induced the sequestration of lymphocytes within the peripheral LNs by accelerating lymphocyte migration from peripheral blood to the LNs (257). They argued that by sequestering cells within the LNs and away from the peripheral tissues, FTY720 was able to mediate its immunosuppressive activities and reduce the onset of transplant rejections and autoimmune diseases in various model systems. Subsequent studies have provided further insight into the mechanism by demonstrating that members of the sphingosine kinase family phosphorylate FTY720, both in vitro and in vivo (258-261). Upon phosphorylation, FTY720 efficiently binds 4 out of the 5 S1P receptors ($S1P_1$, $S1P_3$,

$S1P_4$, and $S1P_5$); this interaction with the S1P receptors (primarily $S1P_1$) is crucial for the FTY720 mediated immunosuppressive activities (262, 263).

Sphingosine-1-phosphate (S1P) metabolism and its source in vivo

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite that is generated through phosphorylation of sphingosine with sphingosine kinases (264). Upon phosphorylation, S1P can either function as an intracellular second messenger or be secreted into extracellular fluid. From there, it binds, both in an autocrine and paracrine manner, one of the 5 pertussis-toxin sensitive G protein-coupled receptors (GPCRs) – $S1P_{1-5}$ – to induce various biological processes (e.g. angiogenesis, cell migration, survival, and immune regulation) (265-271).

Although all cells produce S1P during routine sphingolipid degradation, in majority of these cells, most cells do not extracellularly secrete S1P. Instead, in these cells, S1P is rapidly and irreversibly degraded by S1P lyase and dephosphorylated by S1P phosphatase (272). Accordingly, in most tissues, S1P level is extremely low (1-100 nM) compared to both the blood and lymph (0.3 to 3 uM) (249). This increased concentration of S1P observed in the peripheral blood was initially associated with platelets as they were found to both accumulate high level of S1P and secrete S1P into the blood upon activation (273, 274). However, subsequent work by Pappu et al. determined that erthyrocytes, not the platelets, were the major source of S1P in plasma as the transfusion of wild-type erythrocytes restored plasma S1P concentration in conditional sphingosine kinase deficient animals (275). Similarly, Hanel et al. observed that erythrocytes were capable of secreting abundant amount of S1P when cultured in vitro with human plasma

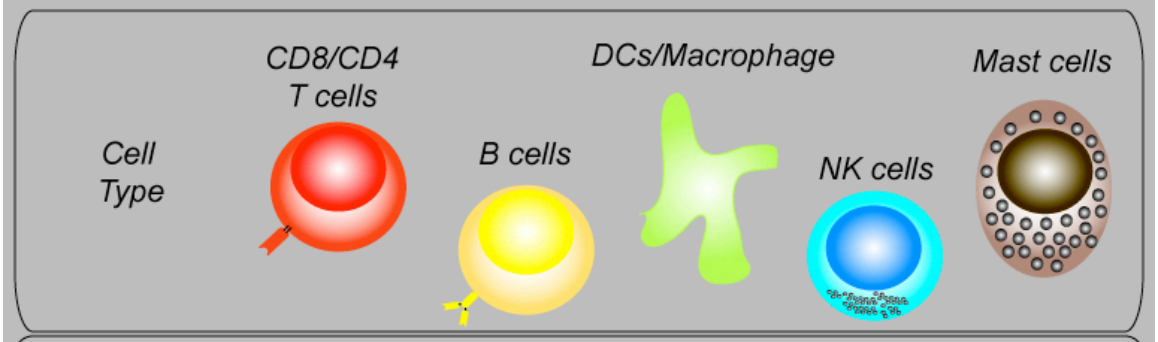
(276). Interestingly, however, they observed that although erythrocytes released S1P into the blood, they did not synthesize the sphingolipid metabolite themselves, but instead, incorporated it from the surrounding medium. Hence, it has been hypothesized that sphingosine was continuously released from different tissues into the peripheral blood, where it was subsequently incorporated, phosphorylated, and secreted by the erythrocytes; this was important to stably maintain the S1P concentration in the blood under normal steady state conditions (276).

Alternatively, erythrocytes, themselves, may not phosphorylate sphingosine, but only incorporate already phosphorylated sphingosine secreted other tissues to prevent S1P degradation. In support, Venkataraman et al. suggested that the vascular endothelium, in addition to the hematopoietic system, was a major source of plasma S1P as they observed that the reconstitution of irradiated wild type animals with bone marrow from animals deficient in sphingosine kinases did not result in reduced S1P concentration in the peripheral blood (277). In contrast, Pappus et al. noted a 10-fold reduction in plasma S1P level in their study when wild-type animals were reconstituted with bone marrow from sphingosine kinase deficient animals (275). Although they failed to provide an explanation for the discrepancy between the two studies, Pham et al. did acknowledge that the endothelial cells were a significant source of S1P in vivo, especially within the lymphatic tissues (278). Along these lines, various studies have shown that growth factors and cytokines (e.g. PDGF, epidermal growth factor, TNF-α, and nerve growth factor) do activate the sphingosine kinases within different cell types (e.g. mast cells, macrophages, dendritic cells, astrocytes, etc.) and thereby, increase cellular S1P level in these cells (249, 279, 280). However, the contribution of these cells to the

overall level of S1P in the peripheral blood and in the lymph has not been thoroughly investigated. In all, this tight regulation of S1P secretion in vivo creates a gradient in S1P concentration (blood > lymph > tissues), which is crucial for the trafficking of cells through different lymphoid tissues.

S1P receptor expression and the function of S1P on immune cell trafficking

Table 1. S1P receptor expression and their effect on immune cells



S1P Receptor Expression	$S1P_{1,3,4}$	$S1P_{1,3,4}$	$S1P_{1,2,3,4}$	$S1P_{1,4,5}$	$S1P_{1,2}$
Chemotaxis	Stimulated at low conc.; decreased at high conc.	Stimulated at low conc.; decreased at high conc.	Stimulated	Stimulated	Stimulated
Cytokine Production	Decreased IFN-γ; Increased IL-10		Decreased IL-12 & TNF-α; Increased IL-10		Increased degranulation

Different cells of the immune system express distinct S1P receptor expression profiles, enabling these cells to uniquely respond to S1P (Table 1). Human dendritic cells (DCs), for example, express $S1P_{1-4}$ and exhibit varying responses to S1P depending on the maturation state of the DCs (281). In vitro, immature DCs exposed to S1P display a chemotactic response to S1P, whereas DCs activated with LPS fail to exhibit any chemotactic activity. Instead, Idzko et al. observed that S1P-treated mature DCs produced more IL-10, promoting a bias toward Th_2 immune responses (281). Others

have made similar observations with murine DCs, but S1P appears to induce chemotaxis and actin polymerization in these cells regardless of their maturation status (282, 283). Human and murine CD4 and CD8 T cells also express S1P receptors (CD4: $S1P_1$ and $S1P_4$; CD8: $S1P_1$, $S1P_4$, and $S1P_5$) and exhibit varying responses depending mainly on the concentration of the S1P present in the system (284). For instance, at sub-plasma concentration of S1P (10-100 nM), there is enhanced CD4 T cell chemotactic response toward CCL21 and CCL5 in a transwell migration assay, but at higher concentrations, S1P dramatically inhibits this response (285-288). This inhibition is not due to cytotoxicity as washing the cells before conducting the chemotaxis assay fully reverses the inhibition.

In addition to their role on lymphocyte chemotaxis, S1P has also been shown to be important for the optimal function of different cell types. For example, $CD4^+CD25^+$ regulatory T cells also express $S1P_1$ and $S1P_4$, but at much lower levels compared to non-regulatory CD4 T cells; therefore, they display reduced chemotaxis to S1P in vitro (289). Wang et al. observed that the regulatory T cell mediated suppression of both anti-CD3 + anti-CD28 induced proliferation and IL-2 production by T cells were greatly enhanced with S1P treatment at normal blood and lymph concentrations. They concluded that this increase in suppression was mediated by IL-10 as these cells produced greater amount of IL-10 upon S1P treatment, highlighting the importance of S1P on regulatory T cell function (290).

The true biological significance of the role of S1P in chemotaxis was not truly appreciated until the development of animals deficient in S1P receptor expression. Not surprisingly, mice deficient in $S1P_1$ expression (the most widely expressed of the S1P

receptors) are embryonically lethal at day 13.5 due to defects in vascular development and die from severe hemorrhage (291, 292). Hence, Allende et al. utilized the Lck-Cre conditional knockout technology to generate a T cell specific $S1P_1$ deficient mouse model in order to better assess the role of S1P on T cell migration in vivo (293). They observed a significant defect in the number of mature CD4 and CD8 T cells in the peripheral blood with a corresponding accumulation of cells within the thymus in these mice, suggesting that the egress of cells from the thymus and into the peripheral blood was dependent on S1P signaling. Matloubian et al. made similar observations in their study utilizing fetal liver chimeras (transplanted lethally irradiated wild-type mice with day 12.5 fetal liver cells from $S1P_1$ deficient animals) (294). In addition to the defect in thymic egress, they noted that although $S1P_1$ deficient thymocytes were readily able to enter secondary lymphoid organs when adoptively transferred into wild-type recipients, these cells failed to migrate back out into the lymph/peripheral blood. They concluded from these observations that the egress of lymphocytes from both the thymus and peripheral lymphoid organs was tightly regulated by S1P signaling exclusively through $S1P_1$. In support, Lo et al. showed, by regulating the expression level of $S1P_1$ on lymphocytes using retroviral transduction techniques, that the rate at which lymphocytes emigrated from the secondary lymphoid tissues was directly proportional to the overall expression level of $S1P_1$ on the lymphocytes, further highlighting the importance of S1P signaling in the trafficking of immune cells through different peripheral tissues (295).

Mechanisms of S1P/FTY720-mediated regulation of lymphocyte trafficking through the peripheral LNs

As described earlier, phosphorylated FTY720 binds $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ with great affinity. Initial studies with in vitro experiments have shown that the ligation of FTY720 to its receptors results in the activation of different downstream signaling components with similar or slightly higher affinity as S1P itself, suggesting that FTY720 functions as an agonist for the different S1P receptors (262, 263). However, subsequent studies have found that FTY720 treatment only resulted in nonresponsiveness of the cells to subsequent S1P exposure, but also resulted in the internalization of the S1P receptors (294, 296). This internalization of the S1P receptors was not unique to FTY720 alone, as Liu et al. had previously shown that S1P binding to the receptors resulted in receptor internalization (297). However, with S1P the receptors are recycled and eventually brought back to the plasma membrane, whereas with FTY720, the internalized receptors are not recycled, but instead targeted to the ubiquitinylation and proteasomal degradation pathway (298, 299).

Based on these latter studies, it has been proposed that FTY720 impedes immune cell trafficking by acting as a functional antagonist, inducing the down-modulation of the surface expression of $S1P_1$ and the subsequent non-responsiveness of the cells to further S1P exposure (256). In support, Grigorova et al. proposed a multistep model of T cell egress from the peripheral LNs to highlight the importance of S1P signaling in immune cell trafficking (300). According to this model (Figure 6), naïve CD8 T cells encounter the cortical sinusoids during their "random" crawl along the FRC network of the paracortex of the LNs, and begin probing the walls of these sinuses as they decide whether to migrate back to the T cell zone or to traverse into the cortical sinuses. This decision to enter the cortical sinuses requires S1P signaling to overcome the CCR7-

mediated LN retention signals. Accordingly, Pham et al. explained that the primary cause for the lymphopenia observed in FTY720 treated animals was that the lymphocytes in the peripheral LNs of these animals failed to overcome this LN retention signal. This was due to the FTY720-induced down-regulation of $S1P_1$ expression and thus, sequestering the cells within the T- and B-cell zones of the LNs (301). Along these lines, Mandala et al. observed that after FTY720 administration, there was an absence of lymphocytes in both the subcapsular and medullary sinuses of the LNs with most of the lymphocytes "log-jammed" on the abluminal side of the sinus endothelium (263). Under normal steady state conditions (i.e. no FTY720 treatment), $S1P_1$ expressing lymphocytes successfully gain entry into a cortical sinus branch in response to the S1P produced by the lymphatic endothelial cells that line these "egress compartments" of the LNs (278). From there, the cells continue to migrate until they are eventually transported by the lymph flow to the medullary sinuses, where they exit via the efferent lymphatic vessels. Once the cells ultimately circulate back into the peripheral blood via the thoracic duct, there is a transient down-regulation of $S1P_1$ expression due to the concentration level of S1P in the peripheral blood. This down-regulation of the receptors allows cells to continue their journey in search of cognate antigen in other peripheral lymphoid tissues. Along these lines, Lo et al. proposed that this cyclical ligand-induced modulation of $S1P_1$ expression is a crucial regulator of the migratory properties of lymphocytes in vivo and that FTY720 mediates its immunosuppressive activities by targeting this mechanism of lymphocyte trafficking (295).

The biological relevance of the above-proposed model has been well visualized through different in vivo studies. For example, Schwab et al. reported that the

lymphopenia observed in animals after treatment with 2-acetyl-4-tetrahydroxybutylimidazole (THI; a component of caramel color) was directly linked to the surface expression of $S1P_1$ on the lymphocytes. They observed that animals treated with THI had significantly higher amount of S1P within the peripheral LNs (comparable to that observed in blood and in lymph) due to disruption of S1P lyase activity. This increase in the LNs resulted in decreased surface expression of $S1P_1$ on both T- and B-lymphocytes (similar to what is seen in FTY720 treated animals), resulting in impaired egress (302). Similarly, Shiow et al. determined that the transient shutdown of egress from peripheral LNs observed soon after infection was directly linked to the expression pattern of $S1P_1$ on the lymphocytes (303). They found that treating cells with type I IFN, through either poly(I:C) treatment or LCMV infection, resulted in the induction of CD69 expression, which negatively regulated the surface expression of $S1P_1$ on the lymphocytes, rendering these cells unable to exit the peripheral LNs. Approximately 3 days after initial infection when activated CD8 T cells began appearing in the periphery, they noted reduced expression of CD69 on the activated cells with a corresponding increase in both the transcription of the genes that regulate $S1P_1$ expression (e.g. KLF2 – kruppel-like factor 2) and the S1P responsiveness of these cells (294, 304, 305). These studies, collectively, emphasize the importance of S1P/FTY720 signaling in regulating the migratory properties of these cells in vivo. However, there exists an alternative model to explain for the role of S1P on the egress of lymphocytes from peripheral LNs that does not require S1P acting directly on the lymphocytes through $S1P_1$. Instead, this model proposes that S1P regulates lymphocyte trafficking indirectly by signaling through the $S1P_1$ expressed on the lymphatic endothelium (Figure 7).

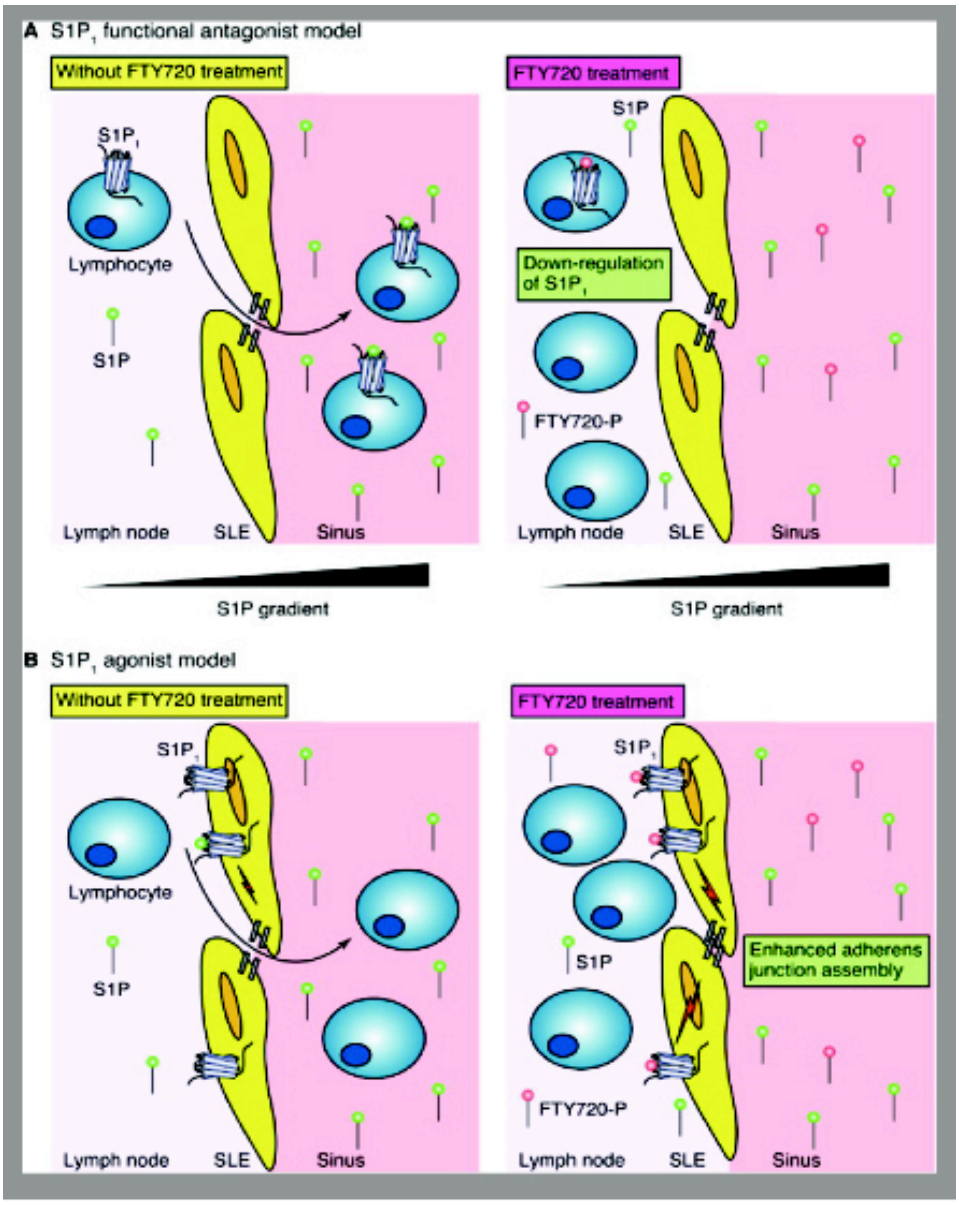


Figure 7. Two models for the molecular mechanism of FTY720-induced immunosuppression. A) In the S1P1 functional antagonist model, S1P1 on lymphocytes figures prominently. Under normal conditions, lymphocytes exit the lymph nodes via the sinus-lining endothelium (SLE) in response to S1P gradient. FTY720 treatment results in the down-regulation of S1P1 on the lymphocytes, leading to a loss of the migratory response. B) In the S1P1 agonist model, S1P1 on the SEL is important. Under steady state conditions, lymphocytes can readily squeeze through the SLE. However, in the presence of FTY720, FTY720 activates S1P1 on SLE and stimulates adherence junction assembly, resulting in reduced permeability.

As briefly described earlier, the importance of S1P and $S1P_1$ on the vascular endothelium can be clearly observed with animals deficient in either $S1P_1$ or deficient in both members of the sphingosine kinase family, as these mice are embryonically lethal due to defects in vascular development and die from severe hemorrhage (291, 292, 306). In fact, $S1P_1$ was initially isolated as an inducible gene from human umbilical vein endothelial cells (HUVEC) treated with PMA (phorbol 12-myristate 13-acetate) and was originally called EDG-1 (endothelial differentiation G-protein coupled receptor 1) (307). Lee et al. subsequently demonstrated that the primary ligand for EDG-1 was S1P and the family of EDG receptors was accordingly renamed to S1P receptors (308). Various groups have since demonstrated that S1P signaling through these receptors (specifically $S1P_1$ expressed on the endothelial cells) regulates various aspects of normal vascular endothelium development (309). More specifically, Garcia et al. reported that S1P promoted endothelial cell barrier integrity by increasing extracellular matrix tethering through rearrangement of the actin cytoskeleton (310). Similarly, Lee et al. demonstrated that the treatment of HUVEC with S1P resulted in increased localization of VE-cadherin, α-, β-, and γ-catenin along discontinuous structures at cell–cell contact regions, suggesting that S1P promoted the formation of adherence junctions (311). These studies suggest that, under normal conditions, the copious level of S1P in the peripheral blood interacts with $S1P_1$ expressed on vascular endothelial cells and thereby, prevents excessive vascular permeability.

Based on the above-described observations, various groups have proposed an alternative model in which S1P indirectly regulates lymphocyte trafficking by signaling through the $S1P_1$ expressed on the lymphatic endothelium to control vascular

permeability. Under normal steady-state conditions, S1P level in the parenchyma of the LNs is negligible and therefore, lymphocytes can readily traverse across the permeable lymphatic endothelium and migrate into the peripheral blood. Accordingly, this model suggests that FTY720 induces sequestration of lymphocytes within the peripheral LNs by acting not as an antagonist, but instead as an agonist and signaling through the $S1P_1$ on the endothelial cells. This results in the promotion of lymphatic endothelium barrier integrity by "tightening" the cell-cell junctions between LN sinus cells (312, 313). In support, Wei et al. utilized two-photon microscopy to study the effects of a known pharmacological $S1P_1$ agonist SEW2871 on lymphocyte trafficking within explanted LNs (314). They observed that the application of SEW2871 had no significant effect on lymphocyte migration across the cortical regions of the LNs, but it had prevented these cells from gaining entry into the medullary sinuses. In contrast, W123 or VPC23019, known $S1P_1$ antagonists, had no significant effect on the egress of lymphocytes from the peripheral LNs. Based on these observations, several groups have concluded that FTY720-mediated $S1P_1$-dependent nodal sequestration involved obligate agonism, not functional antagonism as proposed earlier (314, 315).

These experiments have clearly highlighted the importance of $S1P_1$ signaling for the egress of CD8 T cells from the peripheral LNs. Further work will be required to determine which of the two prevailing models dominates under physiological conditions in vivo. In addition, whether virus-specific effector CD8 T cells also utilize this mechanism for their egress from the LNs requires further investigation. In Chapter 4 of

this thesis, we will address the role of $S1P_1$ signaling in the egress of LCMV-specific effector CD8 T cells from the peripheral LNs.

"Programming" of memory CD8 T cell trafficking

One of the consequences of CD8 T cell activation is the change in the expression of tissue homing receptors as these cells differentiate from naïve to effector to memory CD8 T cells. This change allows activated CD8 T cells to migrate to various lymphoid and non-lymphoid tissues in search of foreign antigen (15, 316). As discussed earlier, this ability to migrate to different peripheral tissues (both lymphoid and non-lymphoid) provides memory CD8 T cells with a selective advantage over naïve cells in providing protection against secondary infections. Although this idea that memory CD8 T cells are widely disseminated throughout the body has been known for some quite time, how these cells "know" where to migrate has only recently begun to be addressed. Studies in various animal models have suggested that both the site of infection and the type of tissues that is infected have an immense influence on the overall pattern of distribution of the resulting memory CD8 T cells. For example, CD8 T cells primed in the mesenteric LNs preferentially up-regulate the expression of gut homing molecules $\alpha 4\beta 7$ and CCR9 compared to those cells primed in either the peripheral LNs or the spleen (317-319). Furthermore, DCs isolated from Peyer's patches (PPs – a secondary lymphoid tissue within the intestinal mucosa) are more efficient in the induction of a4b7 expression on CD8 T cells in vitro (320). Collectively, these observations suggest that the environment within which CD8 T cells are primed "instructs" cells with unique homing potential. However, recent experiments by Masopust et al. suggest that this may not always be the

case, as they observed that cells primed in the spleen both up-regulated α4β7 expression and migrated to the intestinal mucosa just as effectively as those primed in the mesenteric LNs (321). Similarly, Kaufman et al. reported that intramuscular immunization with recombinant adenovirus (rAd) vector-based vaccines expressing SIV Gag resulted in highly effective CD8 T cell responses at multiple mucosal effector sites, suggesting that the initial site of infection did not always dictate the final destination of the cells (322). Further work will be required to better elucidate how memory CD8 T cells are able to "know" where to migrate upon their development.

Resident vs. Non-resident memory CD8 T cells

Another issue of great interest in the study of memory CD8 T cell trafficking is whether memory cells continuously re-circulate through the different peripheral tissues or whether memory cells become resident once they migrate to a particular tissue. Many of the earlier studies have argued for the former (33). When the vasculature of two mice was joined via parabiosis, memory CD8 T cells from both mice were isolated from a single lung, liver, or peritoneal cavity, suggesting that memory cells from these peripheral sites were not sessile, but could freely migrate via the blood (323). In another study, memory CD8 T cells within the lung were described to be short-lived and a continuous recruitment of the systemic population was necessary to maintain numbers (324). These studies collectively suggest that memory CD8 T cells can freely migrate in and out of various peripheral tissues and this may be critical in providing improved protection against secondary challenges. However, it is still unclear whether this re-circulation paradigm applies to all memory CD8 T cells. In the above mentioned

parabiosis experiment, it was observed that there was minimal mixing of memory CD8 T cells within the gut epithelium (intraepithelial lymphocytes, IELs) between the conjoined mice (323). Furthermore, memory CD8 T cells within the IELs differ drastically in terms of both their phenotypic expression and function compared to memory cells in other tissues, suggesting that these cells do not freely circulate through different tissues (325). We will better address this in Chapter 4 through the use of FTY720 to study memory CD8 T cell migration.

Chapter 2: Role of CD4 T cell help in the development of fully functional LCMV-specific memory CD8 T cells

Abstract

An important question that continues to linger in the field of immunological memory pertains to the role of CD4 T cells in the development of memory CD8 T cells. Previous studies have shown that the "help" provided by CD4 T cells are essential in memory development as CD8 T cell activation in the absence of CD4 T cells results in defective memory CD8 T cells that fail to provide efficient protection against secondary challenges. However, there still lacks a general consensus on when during an immune response these CD4 T cells are required for the development of healthy memory cells. Furthermore, there is also still much debate on the mechanism by which CD4 T cells "help" CD8 T cells. In this study, we utilized a series of adoptive transfer experiments with wild-type (WT) and CD4 T cell deficient (CD4 -/-) animals to control when, during an immune response, LCMV-specific CD8 T cells were provided this "help" from CD4 T cells – CD4 T cells were present during either (i) the entire immune response, (ii) days 0-7 post-infection when naïve to effector transition occurs, (iii) days 7-60 post-infection when effector to memory transition (and memory maintenance) occurs, or (iv) none of the different stages of immune response. To our surprise, we noted that LCMV-specific CD8 T cells did not require CD4 T cells to differentiate into healthy memory cells as CD8 T cells primed in CD4 -/- animals and subsequently transferred into uninfected CD4 -/- animals developed into normal memory CD8 T cells. Instead, we found that the absence of CD4 T cells results in impaired antigen clearance and this persistence of low

amount of antigen is directly responsible for the observed defect in memory development in LCMV-infected CD4 -/- animals. We conclude from our observations that in the context of an acute infection, CD4 T cells are not required for either the "programming" or "maintenance" of memory CD8 T cells. Instead, we propose that CD4 T cells are essential for the complete clearance of antigen and thereby, creating an ideal environment for memory CD8 T cell development to occur.

Introduction

The gradual differentiation of naïve CD8 T cells into potent cytotoxic T lymphocytes (CTLs) and subsequently into rapidly responding memory CD8 T cells that are maintained for the life of an individual is the fundamental basis of an effective adaptive immune response (4). The requirements for this differentiation of CD8 T cells, especially the involvement of helper CD4 T cells (T_H) in the generation of memory CD8 T cells, have been the topic of much intense discussion in recent years (197, 212, 213). It is generally believed that CD8 T cells and CD4 T cells intimately engage with one another via antigen presenting cells (APCs) to initiate an effective immune response, but then carry out their effector functions primarily independent of one another. For example, the primary CD8 T cell response towards many non-inflammatory antigens (e.g. minor H Ags, tumor Ags, tissue grafts, and peptide-pulsed dendritic cells) has been shown to require this cognate help (326-328). Antigen-specific CD4 T cells recognize peptide presented in the context of major histocompatibility complex (MHC) class II molecules on dendritic cells (DCs). This interaction allows T_H cells to "license" DCs through CD40-CD40L signaling resulting in the up-regulation of various co-stimulatory molecules (e.g. CD70, B7 family) and the production of various cytokines (e.g. IFN-γ and IL-12) by the DCs. The now "licensed" DCs, then, induces the activation and differentiation of Ag-specific naïve CD8 T cells into potent CTLs, which subsequently further differentiate into fully functional memory CD8 T cells. In contrast, the generation of CTLs against many acute infectious pathogens (e.g. lymphocytic choriomeningitis virus, ectromelia virus, listeria monocytogenes, influenza, and vascular stomatitis virus) has previously been described to be independent of CD4 T cell help. It is believed that

these inflammatory pathogens may either directly infect or activate the DCs via Toll-like receptors (TLRs) and thereby, bypass the need for T_H cells (193, 329). However, closer examination of these "unhelped" CD8 T cells revealed that the subsequent stages of memory CD8 T cell development are significantly impaired in the absence of CD4 T cells as the ability to undergo secondary expansion and to protect against re-infection were severely compromised (212, 213). Thus, it has been proposed that CD4 T cells play a critical role in the generation of an effective adaptive immune response against all types of antigens by delivering the necessary signals for the generation of fully functional memory CD8 T cell pool. Despite much progress, it remains an enigma as to when, during a CD8 T cell response to an acute infectious pathogen, T_H cells are required for the generation of fully functional memory CD8 T cells. As observed with many non-inflammatory antigens, T_H cells were traditionally thought to play an important role during the initial priming and help "program" CD8 T cells to gradually differentiate into fully functional memory CD8 T cells (328). However, recent work by Sun et al. has questioned this traditional dogma by suggesting that CD4 T cells are not required for the programming of memory CD8 T cells, but rather for their long-term maintenance (216).

In this study, we aimed to more clearly identify when T_H cells were required to induce the development of functional memory CD8 T cells during acute LCMV infection in mice. Through a series of adoptive transfer experiments utilizing a combination of wild type (WT) and CD4 T cell deficient animals (CD4 -/-), we controlled when during the immune response LCMV-specific CD8 T cells were exposed to T_H cells. Surprisingly, we found that the development of healthy memory CD8 T cells was not directly dependent on CD4 T cell help at all. Fully functional memory CD8 T cells were

generated in the complete absence of T_H cells. Instead, we found that the impaired memory CD8 T cells observed in infected CD4 -/- animals were due to the impairment in antigen clearance in these animals. These results argue that T_H cells are not directly required for either the "programming" or "maintenance" of memory CD8 T cells. Instead, CD4 T cells play a crucial role in the complete clearance of LCMV antigen and thereby, creating a suitable environment for the gradual differentiation and development of memory CD8 T cells to occur.

Results

Memory CD8 T cells generated in CD4 -/- mice infected with acute LCMV are functionally and phenotypically impaired

Within normal immunocompetent individuals, ~95% of effector CD8 T cells undergo massive contraction with the resulting fraction of cells progressively differentiating into fully functional memory CD8 T cells over a course of several weeks (5). This model was based on the observation that "hallmark" qualities of memory CD8 T cells – high proliferative capacity to both antigen and homeostatic signals, increased production of IL-2 upon in vitro stimulation, higher expression of Bcl-2, IL-7Rα (CD127), L-selectin (CD62L), and CD27, decreased expression of KLRG-1 and Granzyme B – were gradually acquired by the antigen-specific CD8 T cell populations several weeks following infection (179). However, in the absence of CD4 T cells, it has been shown that although the generation of antigen-specific effector CD8 T cells appears normal, the differentiation into functional memory CD8 T cells is greatly impaired (212, 213). As seen in Fig 1a, infection of both WT and CD4 -/- resulted in comparable generation of effector CD8 T cells both in terms of their number and phenotypic expression (e.g. CD62L) at day 8 post-infection. Interestingly, however, LCMV-specific CD8 T cells generated in the absence of CD4 T cells failed to up-regulate CD62L expression at later time points post-infection. For example, at day 100 post-LCMV infection, approximately 50% of D^b NP396-404$^+$ CD8 T cells expressed CD62L in WT animals whereas only about 10% of cells were CD62L$^+$ in CD4 -/- mice. This was also true for CD127 expression as only about 13% of D^b NP396-404$^+$ CD8 T cells were CD127$^+$ in CD4 -/- compared to 73% in WT animals (Fig 1b). LCMV-specific memory

CD8 T cells generated in CD4 -/- animals also failed to produce IL-2 upon in vitro stimulation compared to those generated in CD4 T cell competent animals. Only about 3-5% of LCMV-specific CD8 T cells produced IL-2 upon in vitro stimulation with either D^b GP33-41, GP276-286, or NP396-404 peptides. However, in WT animals, approximately 25-50% of LCMV-specific effector CD8 T cells produced IL-2 when stimulated in vitro with any of the three-immunodominant LCMV peptides (Fig 1c). All in all, these data support other previous findings that memory CD8 T cells generated in the absence of T_H cells are functionally and phenotypically impaired.

Absence of CD4 T cells during effector to memory transition, but not during naïve to effector differentiation results in impaired memory CD8 T cell development

As suggested in Fig 1 and further supported by other previous findings (197, 330), T_H cells are thought to play an important role in generating fully functional memory CD8 T cells. However, exactly when CD4 T cells are required for the development of optimal memory CD8 T cells has yet to be clearly demonstrated. CD4 T cell help may perhaps be required during the entire immune response or only during the different phases of CD8 T cell response to acute viral or bacterial infections. For example, T_H cells may play an important role during the first phase of the response (Phase 1; days 0-8 post infection) when naïve CD8 T cells become activated and differentiate into effector CD8 T cells. In this scenario, T_H cells would provide various signals (e.g. CD40L, IL-2, etc.) and "program" Ag-specific CD8 T cells to differentiate eventually into functional memory CD8 T cells. Alternatively, CD4 T cells may be required during the later stages of CD8 T cell response (Phase 2; days 8 and onward post infection) when antigen has been

cleared from the system and the differentiation of a stable memory CD8 T cell population occurs. A third possibility is that CD4 T cell help is required at all stages of CD8 T cell immune response. Lastly, a fourth and remote possibility is that T_H cells are not actually directly required for the development of functional memory CD8 T cells, but rather something unique about an infected CD4 -/- animal impedes the differentiation of effector CD8 T cells into healthy memory CD8 T cells.

To identify when exactly T_H cells are required, we designed a set of adoptive transfer experiments that controlled when CD8 T cells were exposed to T_H cells during the immune response – CD4 T cell help was provided to CD8 T cells during either the entire immune response (group i), phase 1 when naïve to effector transition occurs (group ii), phase 2 when effector to memory transition takes place (group iii), or none of the different stages of immune response (group iv) (Fig 2a). Specifically, naïve Thy1.1$^+$ P14 CD8 T cells were adoptively transferred into WT or into CD4 -/- mice and subsequently infected with LCMV. On day 7 post-infection, effector P14 CD8 T cells from either WT or CD4 -/- animals were isolated, purified, and adoptively transferred into either infection matched WT or CD4 -/- mice. Mice were then bled at various time points post-transfer and the changes both in the number and in the phenotypic expression were assessed. As seen in Fig 2b, there were no significant differences in terms of the percentage of donor P14 CD8 T cells observed in the peripheral blood of any of the groups at all time points post-transfer. Effector P14 CD8 T cells isolated from either WT or CD4 -/- animals and subsequently transferred into either infection matched WT or CD4 -/- mice all underwent comparable contraction resulting in a similar number of memory CD8 T cells that were

maintained stably in all tissues examined (e.g. peripheral blood, spleen, lymph nodes, and liver; Fig 2c) for at least the duration of the experiments.

In terms of phenotypic expression, however, the absence of T_H cells during phase 2 (effector to memory differentiation), but not during phase 1 (naïve to effector differentiation) resulted in reduced CD127 and CD62L expression by 60 days post-transfer (Fig 2d). Initially, at one day post-transfer, donor P14 CD8 T cells in all four groups expressed similar levels of CD127 (~10%) and CD62L (~1%). However, as early as 10 days post-transfer and definitely by 60 days post-transfer, differences in CD127 and CD62L expression were clearly apparent both in the peripheral blood and in other tissues (e.g. spleen – Fig 2e; liver and LNs – data not shown). Donor P14 CD8 T cells that were exposed to T_H cells throughout the entire immune response (group i) gradually expressed higher levels of both CD127 and CD62L as shown in Fig 2d. By day 60 post-transfer, when recipient mice were sacrificed, these cells expressed high levels of CD127 (~75%), CD62L (~28%), CD27 (~90%), and Bcl-2 (~97%), while expressing low levels of KLRG-1 (~10%) and PD-1 (~3%) – profile characteristic of healthy memory CD8 T cells – whereas those that had never been exposed to T_H cells (group iv) failed to express high levels of CD127 and CD62L (~20% and ~12%, respectively) even after 60 days post-transfer. Surprisingly, the absence of T_H cells only during the naïve to effector differentiation phase (group iii) did not result in impaired CD127 and CD62L expression (~75% and ~40%, respectively). In fact, these CD8 T cells closely resembled those memory cells that had been provided T_H cells throughout the entire immune response (group i) for all phenotypic markers examined. This suggests that the presence of T_H cells during phase 2 (effector to memory differentiation) of CD8 T cell immune response

is critical for the development of fully functional memory CD8 T cells and not necessarily during the initial activation/expansion (phase 1) of the cells. This observation was further confirmed as providing CD4 T cells only during phase 1 (naïve to effector differentiation; group ii) resulted in reduced CD127 (~12%), CD62L (~7%), CD27 (~13%), and Bcl-2 (~57%) expression – all at levels that were comparable to those donor P14 CD8 T cells that had never been provided CD4 T cell help at all (group iv) (Fig 2e).

A hallmark of memory CD8 T cells is its ability to mount a much more rapid and potent CD8 T cell response upon secondary challenges. Hence, next we examined whether the absence of T_H cells during the different phases of CD8 T cell immune response affects the overall function of memory CD8 T cells. Approximately 60 days post-transfer, donor P14 CD8 T cells from all groups were isolated from recipient mice and stimulated in vitro with D^b GP33-41 peptide to measure the production of IFN-γ, TNF-α, and IL-2 (Fig 2f). In agreement with the phenotypic data, providing T_H cells only during phase 1 (naïve to effector differentiation) was not sufficient in generating fully functional memory CD8 T cells as donor P14 CD8 T cells isolated from group ii produced TNF-α and IL-2 at levels (~65% and ~4%, respectively) that were comparable to levels produced by those memory cells that had never seen T_H cells at all (~70% and ~2%; group iv). Donor P14 CD8 T cells that were provided T_H cells only during phase 2 (effector to memory differentiation) of CD8 T cell immune response (group iii) produced TNF-α and IL-2 (~90% and ~20%, respectively) at levels that were comparable to that produced by WT control (group i). Next, to determine whether the differences observed in cytokine production correlated with differences in protection against secondary challenges in vivo, naïve Thy1.1$^+$ P14 CD8 T cells were adoptively transferred into WT

or into CD4 -/- mice and subsequently infected with LCMV-Armstrong. On day 7 post-infection, effector P14 CD8 T cells from either WT or CD4 -/- animals were isolated, purified, and adoptively transferred into either infection matched WT or CD4 -/- mice. Approximately 60 days post-transfer, donor P14 CD8 T cells were purified from infected WT and CD4 -/- mice and equal number of cells was adoptively transferred into naïve WT mice. Recipient mice were then subsequently infected with VV-gp33 and bled at various time points post secondary challenge to assess the recall response of the transferred P14 CD8 T cells (Fig 2g). As shown in Fig 2h, regardless of whether memory P14 CD8 T cells were initially primed in the presence or absence of T_H cells, absence of CD4 T cells during the effector to memory transition phase results in memory cells that display poor recall response upon secondary challenge. Even by 15 days post secondary challenge, responding P14 CD8 T cells from groups ii and iv constituted only about 5-10% of total peripheral blood, whereas in groups where T_H cells were present either during the entire immune response (group i) or only during the effector to memory differentiation phase (group iii), responding P14 CD8 T cells constituted approximately 15-20% of the total peripheral blood. Altogether, these results suggest that the absence of CD4 T cells during the effector to memory transition, but not during the naïve to effector differentiation results in impaired memory CD8 T cell development.

Fully functional LCMV-specific memory CD8 T cells do develop in the absence of T_H cells

Traditionally, the defect in memory CD8 T cell development observed in CD4 -/- animals has been associated with the absence of T_H cells. However, it is now well

understood that various other factors (e.g. antigen load, cytokines, co-stimulation, and inflammation) are intimately involved in the development of fully functional memory CD8 T cells (161, 166). Therefore, it may be possible that the defect in memory development observed in CD4 -/- animals is due to some other factors rather than the absence of T_H cells. To address this, naïve Thy1.1$^+$ P14 CD8 T cells were adoptively transferred into WT or into CD4 -/- mice and subsequently infected with LCMV. On day 7 post-infection, effector P14 CD8 T cells from either WT or CD4 -/- animals were isolated, purified, and adoptively transferred into either WT or CD4 -/- mice as done previously. However, this time cells were transferred into naive recipients rather than infection matched animals as done previously. Recipient mice were then bled at various time points post-transfer and the changes in both number and phenotypic expression was assessed (Fig 3a). As shown in Fig 3b, there were no significant differences in terms of percentage of donor P14 CD8 T cells observed in the peripheral blood of any of the groups at all time points post-transfer. Donor P14 CD8 T cells isolated from either WT or CD4 -/- animals and subsequently transferred into naive WT or CD4 -/- mice underwent comparable contraction resulting in a similar number of memory CD8 T cells that were maintained stably in all tissues examined (e.g. peripheral blood, spleen, lymph nodes, and liver; data not shown) for at least the duration of the experiments.

In terms of phenotypic expression, interestingly, the absence of T_H cells during the effector to memory transition phase (phase 2) did not result in impaired CD127 and CD62L expression when P14 CD8 T cells were transferred into naïve recipients (Fig 3c). Approximately one day post-transfer, donor P14 CD8 T cells in all four groups expressed similar levels of CD127 (~15%) and CD62L (~5%). Whereas the defect in memory

development quickly became apparent when effector P14 CD8 T cells were adoptively transferred into infection-matched CD4 -/- animals, transfer of cells into naïve CD4 -/- (group ii) animals did not result in defective CD127 and CD62L expression even as far out as 60 days post-transfer. Furthermore, these donor CD8 T cells closely resembled their WT counter-parts (group i) in that they all expressed high levels of CD127 (~85%), CD62L (~60%), CD27 (~90%), and Bcl-2 (~99%), while expressing low levels of KLRG-1 (~15%) suggesting that T_H cells are not critical during the effector to memory transition phase for the development of healthy memory CD8 T cells (Fig 3d). In fact, CD4 T cells did not appear to be critical during any phases of CD8 T cell immune response as donor P14 CD8 T cells primed in CD4 -/- mice and subsequently transferred into naïve CD4 -/- mice (group iv) developed into memory cells that closely resembled WT control (group i) in terms of phenotypic expression ($CD127^{hi}$, $CD62L^{hi}$, $CD27^{hi}$, $Bcl\text{-}2^{hi}$, $KLRG\text{-}1^{lo}$, $PD\text{-}1^{lo}$).

In agreement with the observed phenotypic data, donor P14 CD8 T cells transferred into naïve CD4 -/- recipients also developed into fully functional memory CD8 T cells. As seen in Fig 3e, P14 CD8 T primed in WT animals and subsequently transferred into naïve CD4 -/- recipients (group ii) produced TNF-α and IL-2 at levels (97% and 44%, respectively) that were comparable to that produced by CD8 T cells that had been provided T_H cells throughout the entire immune response, when stimulated in vitro with D^b GP33-41 peptide (group i; TNF-α: 98% and IL-2: 41%). This observation further suggests that CD4 T cells may not be as essential as originally believed for the development of fully functional memory CD8 T cells. In support, P14 CD8 T cells primed in CD4 -/- recipients and transferred into naïve CD4 -/- animals (group iv) also

produced high levels of TNF-α and IL-2 (98% and 48%, respectively) when stimulated with peptide. Next, to determine whether the data on cytokine production correlated with protection against secondary challenges in vivo, naïve Thy1.1^{+} P14 CD8 T cells were adoptively transferred into WT or into CD4 -/- mice and subsequently infected with LCMV-Armstrong. On day 7 post-infection, effector P14 CD8 T cells from either WT or CD4 -/- animals were isolated, purified, and adoptively transferred into naive WT or CD4 -/- mice. Approximately 60 days post-transfer, donor P14 CD8 T cells were purified from recipient WT and CD4 -/- mice and adoptively transferred into new naïve WT mice. These mice were then subsequently infected with VV-gp33. Animals were sacrificed 5 days post secondary challenge and the recall response of the transferred memory CD8 T cells was assessed (Fig 3f). As observed in Fig 3g, donor P14 CD8 T cells from all groups responded comparably upon secondary challenge with VV-gp33. Approximately 5 days post re-challenge, similar number of responding donor P14 CD8 T cells were observed in the spleen of mice from the different groups (Fig 3g). This observation suggests that regardless of whether donor P14 CD8 T cells were primed in the presence or absence of CD4 T cells, transfer of effector CD8 T cells into uninfected recipients (WT or CD4 -/-) resulted in the differentiation and development of healthy memory CD8 T cells. All in all, these results suggests that fully functional LCMV-specific memory CD8 T cells can and do develop in the absence of T_H cells. Therefore, the defect in memory CD8 T cell development observed in LCMV infected CD4 -/- recipients is not the result of the absence of CD4 T cells per se, but more due to some environmental factors present only in infected CD4 -/- animals.

CD4 T cells are not required for the differentiation of all LCMV-specific CD8 T cells into fully functional memory cells

As described elsewhere, there are many inherent advantages to the P14 transgenic system (331, 332). However, we next wanted to examine whether the above-described observations are true for all LCMV-specific memory CD8 T cells or whether it is particular to the transgenic memory P14 CD8 T cells. To address this, naïve WT mice were infected with LCMV. On day 8 post-infection, endogenous effector CD8 T cells from LCMV-infected WT mice were isolated, purified, and equal numbers were adoptively transferred into: a) WT naïve; b) WT day 8 post-LCMV infection; c) CD4 -/- naïve; and d) CD4 -/- day 8 post-LCMV infection. Approximately 75 days post-transfer, animals were sacrificed and both the number and the changes in the overall quality of the donor CD8 T cells specific for the three major immunodominant epitopes of LCMV (D^b GP33-41, D^b NP396-404, D^b GP276-286) were assessed (Fig 4a). As shown in Fig 4b, the overall percentage of total donor CD8 T cells in the spleen of recipient mice did not differ drastically among the different groups. However, a closer examination of the donor cells specific for the three major immunodominant epitopes of LCMV individually revealed that there were some significant differences between LCMV-infected CD4 -/- mice compared to the other three groups. For example, although there were no significant differences in the percentage of D^b GP33-41$^+$ donor CD8 T cells (as observed earlier with P14 CD8 T cells), the percentage of D^b NP396-404$^+$ donor cells differed significantly between LCMV-infected CD4 -/- mice compared to the other groups. In LCMV-infected CD4 -/- animals, approximately 21% of the transferred cells in the spleen consisted of NP396$^+$ CD8 T cells, whereas in both LCMV-infected and naïve WT

animals, NP396^{+} CD8 T cells made up only about 6-7% of the donor cell pool. This difference was also noticed when the absolute number of NP396^{+} donor CD8 T cells was calculated (Fig 4c). It is important to note that this increase in number of both D^{b} NP396-404^{+} and D^{b} GP276-286^{+} donor CD8 T cells in LCMV-infected CD4 -/- is not strictly due to the absence of CD4 T cells as both the percentage and absolute number of cells in naïve CD4 -/- mice did not significantly differ from WT control. This suggests that the increase in frequency observed in LCMV-infected CD4 -/- recipients is more due to the infection rather than due to the absence of T_H cells.

In terms of phenotypic expression, as observed previously with the P14 transgenic system, the absence of T_H cells during the effector to memory differentiation (phase 2) did not result in impaired LCMV-specific memory CD8 T cell development. Although the transfer of donor effector CD8 T cells into LCMV-infected CD4 -/- mice did result in memory CD8 T cells that were defective in their phenotypic expression (CD127^{lo}, CD62L^{lo}, Bcl-2^{lo}, and Ly6-c^{lo}), this, however, was not due to the absence of T_H cells. This is so as the transfer of D^{b} GP33-41^{+} donor CD8 T cells into naïve CD4 -/- recipients resulted in memory CD8 T cells that expressed high levels of CD127, CD62L, Bcl-2, and Ly6C – profile characteristic of healthy memory CD8 T cells (Fig 4d). This observation was not unique to the D^{b} GP33-41^{+} donor CD8 T cells, but was also true for D^{b} NP396-404^{+} and D^{b} GP276-286^{+} donor CD8 T cells (data not shown).

In regards to their overall function, donor CD8 T cells transferred into LCMV-infected CD4 -/- mice did produce reduced levels of TNF-α (~92%) and IL-2 (~11%) when stimulated in vitro with D^{b} GP33-41 peptide compared to WT control (TNF-α: ~99%; IL-2: ~26%). Furthermore, this impairment in cytokine production was much

more dramatic for CD8 T cells specific for the D^b NP396-404 epitope of LCMV. Whereas approximately 95% and 23% of $NP396^+$ donor CD8 T cells from either LCMV-infected WT or naïve WT recipient mice produced TNF-α and IL-2 upon in vitro stimulation with D^b NP396-404 peptide, only ~70% and ~11% of D^b $NP396\text{-}404^+$ donor CD8 T cells from LCMV-infected CD4 -/- produced TNF-α and IL-2, respectively. However, as observed previously with the P14 transgenic system, this impairment in cytokine production was not due to the absence of CD4 T cells as donor D^b $NP396\text{-}404^+$ CD8 T transferred into naïve CD4 -/- animals produced TNF-α and IL-2 (~99% and ~30%, respectively) at levels comparable to that produced by donor cells from WT recipient animals (also true for D^b $GP33\text{-}41^+$ and $GP276\text{-}286^+$ CD8 T cells). Collectively, these results suggest that all LCMV-specific CD8 T cells (endogenous and transgenic) do not require T_H cells to develop into fully functional memory CD8 T cells. Instead, the defect in memory development observed in LCMV-infected CD4 -/- mice is a direct consequence of the unique environment created by the infection itself.

Absence of CD4 T cells during LCMV infection results in impaired antigen clearance

Traditionally, CD8 T cells have been described to play an essential role in the immune response in mice against LCMV and these cells were thought to be sufficient in the complete clearance of LCMV antigen (333, 334). However, in certain strains of mice (e.g. CD40 -/-, MHC class II -/-, and B cell -/-) although no virus can be detected at the peak of the CD8 T cell immune response by traditional plaque assay method, virus recrudescence has been shown to occur at later time points post-infection suggesting that CD8 T cells alone may not be sufficient in the complete clearance of virus in certain

scenarios (335, 336). Therefore, we next wanted to examine whether CD4 -/- animals can completely clear antigen upon infection with acute strain of LCMV. It has been shown previously that CD4 -/- mice generate a CTL response of sufficient magnitude to control LCMV infection as no virus can be observed as measured by plaque (204). However, it is not known whether this is due to complete viral clearance or due to limitations in the sensitivity of the plaque assay method as was the case with CD40 -/-, MHCII -/- and B cell -/- animals. Therefore, we utilized memory P14 CD8 T cells as a more sensitive approach to measuring antigen in LCMV-infected CD4 -/- animals. Specifically, memory P14 CD8 T cells, isolated and purified from WT LCMV-immune mice, were CFSE labeled in vitro and adoptively transferred into WT or CD4 -/- mice infected with LCMV 8 days previously. Recipient mice were then bled at different time points post-transfer and both the frequency and CFSE dilution of the donor memory P14 CD8 T cells in the peripheral blood was assessed (Fig 5a). As shown in Fig 5b, donor memory P14 CD8 T cells adoptively transferred into WT animals were stably maintained for at least 21 days post-transfer. At 7 days post-transfer, approximately 0.052% of the peripheral blood consisted of the transferred memory P14 CD8 T cells. By 21 days post-transfer, the frequency of donor cells in WT animals remained at approximately 0.058% of the total peripheral blood. In terms of CFSE dilution, although there was some occurrence of basal level of IL-15 mediated homeostatic turnover, majority of the donor memory P14 CD8 T cells retained their CFSE expression at least for the duration of the experiments. Interestingly, in LCMV-infected CD4 -/- recipients, the frequency of donor memory P14 CD8 T cells in the peripheral blood increased significantly from day 7 (0.043%) to day 21 post-transfer (0.22%). This increase in frequency correlated with

increase in proliferation as donor memory P14 CD8 T cells adoptively transferred into LCMV-infected CD4 -/- animals underwent massive proliferation that by 21 days post-transfer, approximately 85% of the transferred cells in the peripheral blood had completely diluted out their CFSE expression.

Next, to confirm that the observations made in the peripheral blood was also true in other peripheral tissues, recipient mice were sacrificed at 28 days post-transfer and the percentage, CFSE profile, and phenotypic expression of the donor memory P14 CD8 T cells in different tissues (spleen, lymph nodes, and liver) was analyzed. As observed in the peripheral blood, there was a significant increase in the percentage of donor memory P14 CD8 T cells in the spleen and liver (0.13% and 0.19%, respectively) of LCMV-infected CD4 -/- mice compared to WT recipients (spleen: 0.04%; liver: 0.068%). This increase in percentage correlated with increase in absolute number of donor CD8 T cells in different tissues of LCMV-infected CD4 -/- animals compared to WT control (data not shown). Furthermore, >80% of donor memory cells in the spleen and liver of CD4 -/- recipient mice had completely diluted out their CFSE expression, whereas in WT animals, majority of the donor cells (>90%) still retained their CFSE expression. Interestingly, this was not the case when donor cells in the peripheral LNs of WT and CD4 -/- recipient mice were analyzed. The percentage of donor memory cells in the LNs of CD4 -/- mice was actually less compared to WT control (0.027% vs. 0.049%, respectively). In addition, a larger fraction (~80%) of donor memory P14 CD8 T cells in the LNs of LCMV-infected CD4 -/- mice retained their CFSE expression compared to those in the spleen and liver of the same mouse. However, it should be noted that the donor memory P14 CD8 T cells in the LNs of CD4 -/- animals had proliferated to a

greater extent compared to those memory cells in the LNs of WT mice (Fig 5c). Lastly, memory P14 CD8 T cells transferred into WT recipients continued to express high levels of CD127, CD62L and CD27, while expressing low levels of PD-1 – profile characteristic of healthy memory CD8 T cells. On the other hand, donor cells transferred into LCMV-infected CD4 -/- animals displayed more of an activated phenotype as they expressed significantly lower levels of CD127, CD62L and CD27 (Fig 5d). In all, these results suggest that in the absence of CD4 T cells, there is an impairment in the complete clearance of virus resulting in the persistence of LCMV-Armstrong antigen at levels that can not be observed by traditional plaque assay method, but capable of driving the proliferation and activation of memory P14 CD8 T cells.

Not the absence of CD4 T cells, but rather the persistence of low level of viral antigen impedes normal memory CD8 T cell development in LCMV-Armstrong infected CD4 -/- animals

The role of antigen in the differentiation of CD8 T cells from naïve to effector to memory cells has now been well characterized (47). The initial activation and the subsequent differentiation from naïve to effector CD8 T cells are strictly dependent on the presence of antigen. However, the gradual transition from effector to memory CD8 T cells occurs in the complete absence of antigen (78). In fact, prolonged antigenic stimulation (e.g. chronic infections) can impede memory CD8 T cell development and can even result in the eventual exhaustion and/or deletion of the CD8 T cells. Now that we have established that CD4 -/- animals fail to completely clear antigen upon LCMV infection, we next wanted to address whether the defective memory CD8 T cell

development observed in LCMV-infected CD4 -/- mice was a direct consequence of the impaired antigen clearance or due to some other unique environmental factors present in an infected CD4 -/- animal. To address this, naïve P14 CD8 T cells were adoptively transferred into WT mice and subsequently infected with LCMV. At day 8 post-infection, effector P14 CD8 T cells were purified and equal numbers of cells were adoptively transferred into the following groups: i) WT day 8 post LCMV infection; ii) naïve CD4 -/-; iii) CD4 -/- day 8 post LCMV infection; and iv) CD4 -/- day 8 VV infection. Recipient mice were then bled at various time points post-transfer and the expression of both CD127 and CD62L on the donor CD8 T cells was assessed (Fig 6a). In agreement with earlier data (Fig 3), donor P14 CD8 T cells transferred into naïve CD4 -/- animals gradually expressed both CD127 and CD62L at levels that were comparable to those cells transferred into LCMV-infected WT control mice (Fig 6b). In addition, donor CD8 T cells transferred into LCMV-infected CD4 -/- animals expressed significantly lower levels of CD127 and CD62L as expected. Interestingly, however, the adoptive transfer of effector P14 CD8 T cells into VV-infected CD4 -/- animals did not result in impaired CD127 and CD62L expression on the donor CD8 T cells even as far out as 48 days post-transfer. In fact, the overall kinetics of both CD127 and CD62L expression on CD8 T cells transferred into VV-infected CD4 -/- animals closely resembled that of those cells transferred into LCMV-infected WT control mice (Fig 6b). Furthermore, at 60 days post-transfer when recipient mice were sacrificed, donor P14 CD8 T cells in the spleen of VV-infected CD4 -/- animals continued to express high levels of both CD127 (~97%) and CD62L (~74%) comparable to those cells transferred into WT control. They also expressed high levels of CD27 (~90%) and Bcl-2 (~100%)

while expressing low levels of KLRG-1 (~11%) – profile characteristic of healthy memory CD8 T cells (Fig 6c). In addition, these donor CD8 T cells were fully functional as they rapidly produced IFN-γ, TNF-α, and IL-2 upon in vitro stimulation with D^b GP33-41 peptide. For example, whereas donor cells transferred into LCMV-infected CD4 -/- animals produced impaired levels of TNF-α (~85%) and IL-2 (~4%), those cells transferred into VV-infected CD4 -/- produced significantly higher levels of both TNF-α (~92%) and IL-2 (~35%) – levels that were comparable to WT control (~90% and ~40%, respectively). Altogether, these results suggest that the development of fully functional LCMV-specific memory CD8 T cells is not dependent on CD4 T cells as traditionally believed. Instead, the defect in memory development observed in LCMV-infected CD4 -/- animals is a direct consequence of the impaired antigen clearance that occurs in the absence of CD4 T cells.

Discussion

The importance of T_H cells in the development of fully functional memory CD8 T cells towards many various antigens has been now well documented. For most non-inflammatory antigens (e.g. minor H Ags, tumor Ags, tissue grafts, and peptide-pulsed DCs), these cells are thought to play a critical role during the initial activation and priming of CD8 T cells and thereby, "program" antigen-specific CD8 T cells to gradually differentiate into long-lived healthy memory cells (328). However, for most inflammatory antigens (e.g. lymphocytic choriomeningitis virus, ectromelia virus, listeria monocytogenes, influenza virus, and vascular stomatitis virus), the role that T_H cells play in mediating this formation of healthy and protective memory CD8 T cells remains an enigma (330). Although the effector CD8 T cells generated in the absence of CD4 T cells are highly functional and display potent cytolytic activity, the development of healthy memory CD8 T cells has been shown to be greatly compromised in CD4 T cell deficient animals (Fig 1) (212, 213). Despite this knowledge, the precise mechanism by which T_H cells mediate this formation of healthy and protective memory CD8 T cells has yet to be clearly elucidated. Furthermore, it is currently unknown when exactly this "help" provided by CD4 T cells is required during the CD8 T cell immune response towards various inflammatory pathogens.

To begin addressing these questions, we utilized a series of adoptive transfer experiments involving both WT and CD4 -/- animals to control the presence or absence of T_H cells during CD8 T cell immune response to LCMV. The initial findings from these experiments suggested that T_H cells are not required during the initial naïve to effector CD8 T cell transition phase. Instead, the presence of CD4 T cells appeared to be

more critical as effector CD8 T cells differentiate into long-lived memory CD8 T cells. Effector P14 CD8 T cells isolated from WT animals and subsequently transferred into LCMV-infection matched CD4 -/- animals failed to differentiate into fully functional memory CD8 T cells. These cells failed to express high levels of CD127, CD62L, CD27, and Bcl-2; produced suboptimal levels of IL-2 upon in vitro stimulation; and responded poorly upon secondary challenge. This observation is in direct contrast to the current belief that T_H cells are important during the initial activation of antigen-specific CD8 T cells. It is currently postulated that the signals provided by CD4 T cells during these early stages of immune response help "program" CD8 T cells to gradually differentiate into long-lived healthy memory cells (326, 327). This belief has been based primarily on past studies that demonstrated that the early events (e.g. antigenic stimulation, inflammatory cytokines, co-stimulatory signaling, etc.) of CD8 T cell immune response towards different inflammatory antigens instruct naïve CD8 T cells to commit to clonal expansion and differentiation into cytotoxic effector cells that subsequently develop into long-lived protective memory CD8 T cells (47, 161). However, our initial observations suggest that T_H cells play a negligible role during the initial priming and activation of CD8 T cells and instead, appear to be more critical during the effector to memory transition phase of CD8 T cell immune response. In support, Sun et al. had demonstrated that CD4 T cells are required for the long-term maintenance, not programming, of memory CD8 T cells after acute infection (216). They observed that the transfer of effector P14 CD8 T cells into naïve MHC class II -/- animals resulted in impaired memory CD8 T cell development. Furthermore, these LCMV-specific memory CD8 T cells were not stably maintained and declined in number over the course of the

experiments. Based on these observations, they suggested that CD4 T cells are critical not during the initial activation of cells, but rather during the long-term maintenance by perhaps producing an unknown trophic factor that is necessary to maintain both the function and survival of memory CD8 T cells.

Interestingly, however, we observed in our studies that effector P14 CD8 T cells transferred into naïve CD4 -/- animals differentiated into fully functional memory CD8 T cells. These cells expressed high levels of CD127, CD62L, CD27, and Bcl-2; produced optimal levels of IL-2 upon in vitro stimulation; and responded rapidly upon secondary challenge – all at levels that were comparable to WT controls. Furthermore, this observation was not unique to the P14 transgenic system as memory development for all epitopes of LCMV examined (e.g. D^b GP33-41, NP396-404, GP276-286) was not impaired when effector cells were adoptively transferred into naïve CD4 -/- animals. These observations clearly argue against T_H cells being necessary either during the effector to memory differentiation phase or during the long-term maintenance phase for normal development and function of memory CD8 T cells. It should be noted though that when effector P14 CD8 T cells were adoptively transferred into naïve MHC class II -/- animals, we did observe a defect in memory CD8 T cell development (Supp Fig 1). These cells expressed lower levels of CD127, CD62L, CD27, and Bcl-2 compared to those cells transferred into either naïve WT or naïve CD4 -/- animals. They also produced lower levels of IL-2 upon in vitro stimulation with D^b GP33-41 peptide. However, when compared to cells that were transferred into LCMV-infection matched MHC class II -/- mice, the observed defect in memory development was much more modest in naïve MHC class II -/- animals. Effector P14 CD8 T cells that were adoptively

transferred into LCMV-infected MHC class II -/- mice failed to differentiate into healthy memory CD8 T cells as they expressed significantly lower levels of phenotypic markers characteristic of healthy memory CD8 T cells (e.g. CD127, CD62L, CD27, and Bcl-2) and failed to produce IL-2 upon in vitro stimulation. In all, our observation that memory CD8 T cell development is not impaired (CD4 -/-) or greatly improved (MHCII -/-) when effector P14 CD8 T cells are transferred into naïve CD4 T cell deficient animals suggests that T_H cells play a negligible role either during the effector to memory differentiation phase or during the long-term maintenance phase for normal development and function of memory CD8 T cells.

It is interesting to speculate on the cause for the discrepancy observed in memory development between naïve CD4 -/- and naïve MHC class II -/- animals. It has been suggested previously that CD4 -/- mice are not ideal models to study CD4 T cell deficiency as the $CD8^+$ population in CD4 -/- mice is heavily contaminated with MHC class II-restricted T cells that may compensate for the absence of T_H cells (337). Based on these studies, it may be assumed that the lack of defect in memory CD8 T cell development observed in naïve CD4 -/- animals is due to the "help" provided by these MHC class II-restricted $CD8^+$ T cells. However, these "T_H-like" cells are not capable of compensating for the CD4 T cell deficiency as CD4 -/- mice infected with different pathogens results in defective memory CD8 T cell development (Fig. 1) (212, 338). Furthermore, as described previously, memory CD8 T cell development was greatly improved in naïve compared to LCMV-infected MHC class II -/- mice, although admittedly not to the same extent as observed in naïve CD4 -/- animals. Instead, we hypothesize that the moderate defect in memory development observed in naïve MHC

class II -/- mice is more due to the intrinsic quality of the animals rather than the absence of CD4 T cells. Uninfected MHC class II -/- mice have been shown to be more susceptible to various disorders (e.g. pancreatitis; inflammatory bowel disease) (339, 340) and a greater percentage of the endogenous CD8 T cells appear to express PD-1 (data not shown). How these observations relate to the subtle defect in memory development observed in naïve MHC class II -/- animals appears to be seen, but experiments are currently underway that may hopefully better explain the discrepancy in memory CD8 T cell development observed between naïve CD4 -/- and naïve MHC class II -/- animals. Regardless of the reason for this discrepancy, our data suggests that T_H cells are not required during any stages of the CD8 T cell immune response for the development of fully functional memory P14 CD8 T cells.

As described earlier, CD4 -/- mice are traditionally thought to be able to generate a CTL response of sufficient magnitude to control LCMV-Armstrong infection as no virus can be detected by plaque assay (204). However, in certain strains of mice (e.g. CD40 -/-, MHC class II -/-, and B cell -/-) it has been shown that although no virus can be detected by plaque assay at the peak of the CD8 T cell immune response, virus recrudescence can occur at later times post-infection suggesting that CD8 T cells alone may not be sufficient in the complete clearance of virus in certain scenarios (335, 336). To address whether the defect in memory CD8 T cell development in LCMV-infected CD4 -/- animals is due to impairment in antigen clearance, we utilized memory P14 CD8 T cells as sensor cells by labeling them with CFSE in vitro and adoptively transferring equal number of cells into LCMV-infected WT and CD4 -/- animals. As expected, memory cells transferred into WT animals had undergone minimal rounds of division and

retained their overall phenotypic expression ($CD127^{hi}$, $CD62L^{hi}$, and $CD27^{hi}$) and function (optimal IL-2 production upon in vitro stimulation; data not shown) as far out as 28 days post-transfer. However, when transferred into LCMV-infected CD4 -/- animals, memory P14 CD8 T cells underwent rapid proliferation and reverted back to a more effector-like CD8 T cells in terms of both their phenotypic expression ($CD127^{lo}$, $CD62L^{lo}$, and $CD27^{lo}$) and function (suboptimal IL-2 production; data not shown) suggesting that the antigen clearance in CD4 -/- animals is impaired resulting in the persistence of LCMV antigen at levels that can not be detected by plaque assay, but sufficient to drive memory P14 CD8 T cell proliferation and activation. It should be noted, though, that this persistence of antigen differed in magnitude compared to that observed in other chronic infections (e.g. Clone-13) as PD-1, an inhibitory molecule shown to correlate with antigen load, was never expressed on the CD8 T cells in our studies. Lastly, our observation that fully functional memory CD8 T cells ($CD127^{hi}$, $CD62L^{hi}$, $CD27^{hi}$, KLRG-1^{lo}, Bcl-2^{hi}, and optimal IL-2 production) are generated when effector P14 CD8 T cells were transferred into VV-infected CD4 -/- animals provide as further evidence that the impairment in memory development observed in CD4 -/- animals is a direct consequence of the impaired antigen clearance and not due to the absence of CD4 T cells.

Interestingly, though, we unexpectedly observed in our sensor experiment that the donor memory cells found in the lymph nodes of LCMV-infected CD4 -/- animals greatly differed from those found in other tissues (e.g. spleen and liver) in terms of their phenotypic expression and CFSE profile. These cells expressed significantly higher levels of both CD127 and CD62L and had undergone less rounds of division compared to

memory cells in other tissues, albeit greater than WT controls. The exact reason for this discrepancy observed between the lymph nodes and other peripheral tissues (e.g. blood, spleen, and liver) in LCMV-infected CD4 -/- animals is currently unknown, but we postulate that it may simply be that there is no residual LCMV antigen in the lymph nodes of these mice. Alternatively, the difference may be due to the CD62L expression on the donor memory CD8 T cells. It has now well been documented that the main mechanism by which CD8 T cells enter the lymph nodes from the periphery is via the high endothelial venules and CD62L expression is a necessary requirement for this entry (341, 342). Therefore, the donor memory cells that had seen antigen in the periphery and undergone rapid proliferation lack the necessary CD62L expression to migrate into the lymph nodes and are instead concentrated within the peripheral tissues. Regardless of the reason for this discrepancy between the lymph nodes and peripheral tissues, our data demonstrate that in the absence of CD4 T cells, there is an obvious impairment in viral clearance that results in the persistence of LCMV antigen at levels that can not be detected by plaque assay method, but sufficient to drive memory P14 CD8 T cell proliferation and activation. It is this persistence of low level of antigen that impedes memory CD8 T cell development in CD4 T cell deficient animals.

The role of antigen persistence and its negative effects on memory CD8 T cell development has been now well documented for various systems (78). Hence, our observation that the defect in memory development in LCMV-infected CD4 -/- animals is caused by impaired viral clearance is not unique on its own merits. What is novel in our findings is that the development of fully functional memory CD8 T cells can occur in the complete absence of T_H cells. Contrary to current belief (328, 330), our results argue that

T_H cells are not directly required for either the programming or the long-term maintenance of memory CD8 T cells. However, we are not suggesting that CD4 T cells play no role in the generation of fully functional memory cells, as it is obvious that in LCMV-infected CD4 -/- animals, there is a clear defect in memory development. We postulate that CD4 T cells play a critical role in the complete clearance of antigen after a viral or bacterial infection and thereby, creating a suitable environment in which memory CD8 T cell development can occur. Currently, it is not exactly known how T_H cells contribute to the clearance of LCMV antigen, but it is interesting to speculate on the potential mechanisms. CD4 T cells are not traditionally known for their cytotoxic activity as majority of these cells are not known to express molecules often associated with CTL function (e.g. perforin and granzyme B). However, at least in humans, it has been shown that a small subpopulation of CD4 T cells can directly function as effector cells by executing cytotoxicity in a peptide-specific and MHC class II-restricted manner (343). Therefore, it may be that LCMV-specific CD4 T cells work in conjunction with effector CD8 T cells in carrying out their cytotoxic activity resulting in the complete clearance of virus from the animals. Alternatively, T_H cells are known to play an essential role in the generation of an effective humoral immune response (344). The help provided by CD4 T cells (via CD40-CD40L signaling) to antigen-specific B cells early on in the immune response is critical for the development of high affinity and isotype switched antibodies. Along this line, it has been previously demonstrated that the therapeutic injections of LCMV-specific antibodies into LCMV-infected CD40 -/- animals results in the complete clearance of viral antigen and the rescue of the defect in memory CD8 T cell development otherwise observed in these animals (345, 346).

Therefore, CD4 T cells may play a critical role in the clearance of antigen through their interaction with antigen-specific B cells and the production of antibodies, which appear to be necessary for the removal any residual antigens that effector CD8 T cells may have failed to clear from the animals.

To date several studies have addressed the importance of T_H cells in the development of memory CD8 T cells towards many various antigens (206, 212, 213, 230, 347-350). However, there has yet to be a clear agreement on the exact role that CD4 T cells play in the development of healthy memory CD8 T cells. Contrary to popular belief, our data clearly demonstrates that T_H cells are not directly required for the development of fully functional LCMV-specific memory CD8 T cells. Instead, CD4 T cells appear to be critical in the complete clearance of LCMV-antigen and thereby, creating a suitable environment in which memory CD8 T cell development can occur. This may potentially explain for all the variations and discrepancies among the various groups that have addressed this so far. Depending on the nature of the infection and the experimental system used to represent CD4 T cell deficiency, effector CD8 T cells alone may or may not have been sufficient in the complete clearance of antigen. Regardless, we conclude from our findings that memory CD8 T cell development occurs independently of T_H cells in the context of most acute infections. Our results have important implications for the better understanding of memory CD8 T cell development during various "chronic" infections and for the design of vaccines and other therapies that may be used to treat such diseases.

Materials and Methods

Virus

LCMV Armstrong and recombinant vaccinia virus (VV-gp33) expressing the D^b GP33-41 epitope of LCMV were propagated, titered, and used as previously described (351, 352).

Mice

C57BL/6 (B6), CD4 -/-, and MHC class II -/- mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Taconic Farms (Germantown, NY). Thy1.1$^+$ P14 transgenic mice with CD8 T cells expressing the TCR specific for the LCMV epitope D^b GP33-41 were obtained from The Jackson Laboratory and backcrossed to B6 mice in our colony (47). Immune and effector P14 CD8 T cells were made by transferring 10^5 naïve Thy1.1$^+$ P14 CD8 T cells into B6 mice i.v. injection and subsequently infected with $2x10^5$ pfu of LCMV-Armstrong. For secondary challenge experiments, ~$3x10^4$ immune Thy1.1$^+$ P14 CD8 T cells were adoptively transferred into B6 mice and subsequently infected with $5x10^6$ pfu VV-gp33. All mice were used in accordance with NIH and the Emory University Institutional Animal Care and Use Committee guidelines.

Magnetic bead separation and adoptive transfer

At day 7 or 8 post acute LCMV infection, CD8 T cells from the spleens of B6 or CD4 -/- mice containing P14 effector CD8 T cells were isolated using anti-CD8 MACS magnetic beads and columns (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of MACS-purified samples was >90%. The number of P14 cells

in each purified CD8 T cell population was calculated and equal numbers (~5-10x10^6) were adoptively transferred i.v. into B6, CD4 -/-, or MHC class II -/- mice that were either infection matched (day 7 or 8 post acute LCMV infection) or uninfected (naïve).

Lymphocyte isolation, cell surface and intracellular staining

Lymphocytes were prepared from the blood, spleen, liver, and bracial and inguinal lymph nodes as described (143). All antibodies were purchased from BD Biosciences (San Diego, CA), except for anti-mouse IL-7Ra antibodies from eBiosciences (San Diego, CA) and KLRG-1 antibodies from Southern Biotech (Birmingham, AL). Cells were stained for surface or intracellular proteins and cytokines as described previously (46). For intracellular cytokine staining, splenocytes were stimulated with D^b GP33-41 peptide for 5 hours, fixed, permeabilized, and stained as described previously (161).

CFSE labeling and adoptive transfer

Immune or naïve P14 CD8 T cells were isolated and subsequently labeled with CFSE from Molecular Probes (Eugene, OR) by incubating at 5 mM in PBS, quenching with FCS, and washing as described previously (353). Approximately 10^6 immune or naïve P14 CD8 T cells were adoptively transferred into acute LCMV infected WT or CD4 -/- mice. Mice were then bled and/or sacrificed at various time points post-transfer and CFSE division was assessed.

Figure Legends

Figure 1. Memory CD8 T cells generated in CD4 -/- mice infected with acute LCMV are functionally and phenotypically impaired. (A) Longitudinal analysis of D^b NP396-404$^+$ CD8 T cell frequency and CD62L expression in WT and CD4 -/- animals after LCMV infection. C57BL/6 (WT) and CD4 -/- animals were infected with LCMV-Armstrong and at days 8, 15, 35, 80, 100 post-infection, the splenocytes were stained with D^b NP396-404 MHC class I tetramer and antibodies to CD8α and L-selectin (CD62L). For each mouse strain, the left hand panel shows CD8α and D^b NP396-404 staining (the percent of D^b NP396-404$^+$ CD8 T cells are shown) and the right hand panel is gated on tetramer-positive cells and show the expression of L-selectin at different times post-infection (the percent of CD62Lhi cells are indicated). (B) Comparison of CD127 expression on D^b NP396-404$^+$ memory CD8 T cells from WT and CD4 -/- mice at 80 days post LCMV infection. The percent of CD127^{hi} D^b NP396-404$^+$ CD8 T cells is indicated. (C) Production of IFN-γ and IL-2 by memory CD8 T cells from WT and CD4 -/- mice. Approximately 80 days post-infection splenocytes from WT and CD4 -/- animals were incubated with three different LCMV peptides (D^b GP33-41, NP396-404, and GP276-286) and BFA for 5 h in vitro. The cells were then stained for extracellular CD8 and intracellular IFN-γ and IL-2 expression. The FACS plots are gated on IFN-γ$^+$ CD8 T cells. The percent of IFN-γ$^+$ CD8 T cells producing IL-2 is indicated.

Figure 2. Absence of CD4 T cells during effector to memory transition, but not during naïve to effector differentiation results in impaired memory CD8 T cell development. (A) Experimental setup. Naïve Thy1.1$^+$ P14 CD8 T cells were adoptively transferred into

WT or CD4 -/- mice. Animals were then infected with LCMV-Armstrong. Approximately 7 days post-infection, effector P14 CD8 T cells were isolated, purified, and adoptively transferred into either infection matched (day 8 post LCMV-Armstrong infection) WT or CD4 -/- animals. Mice were then bled at various time points post-transfer and changes in number and in overall quality of donor cells were assessed. (B) Kinetics of donor effector CD8 T cell contraction and memory CD8 T cell development. At days 1, 10, 22, and 60 post-transfer, recipient mice were bled and the percentage of Thy1.1^{+} donor P14 CD8 T cells in the peripheral blood was assessed (n=4). (C) Comparison of absolute number of donor CD8 T cells in different peripheral tissues (spleen, lymph nodes, and liver) of recipient animals approximately 60 days post adoptive transfer (n=4). (D) Longitudinal analysis of cell-surface expression of CD127 and CD62L on donor P14 CD8 T cells after adoptive transfer into LCMV-infected WT or CD4 -/- mice. Data shown are gated on Thy1.1^{+} donor CD8 T cells and the percent of CD127hi (left panel) or CD62Lhi (right panel) P14 CD8 T cells is indicated. (E) Phenotypic properties of donor CD8 T cells in the spleen of LCMV-infected recipient mice approximately 60 days post adoptive transfer. Data shown are gated on Thy1.1^{+} donor CD8 T cells. The percent of CD127^{hi}, CD62L^{hi}, KLRG-1^{hi}, CD27^{hi}, PD-1^{hi}, or Bcl-2^{hi} P14 CD8 T cells is indicated. (F) Production of IFN-γ, TNF-α, and IL-2 by donor P14 CD8 T cells at 60 days post-transfer into recipient animals. Splenocytes from LCMV-infected WT and CD4 -/- recipient mice were incubated with LCMV peptide (D^{b} GP33-41) and BFA for 5 h in vitro. Cells were then stained for extracellular CD8 and intracellular IFN-γ, TNF-α, and IL-2 expression. Data shown are gated on Thy1.1^{+} donor P14 CD8 T cells. The percent of IFN-γ^{+} CD8 T cells producing TNF-α or IL-2 is

indicated. (G) Experimental setup for recall analysis. Approximately 60 days post initial adoptive transfer, donor P14 CD8 T cells were re-isolated from recipient WT or CD4 -/- mice and equal numbers of cells (~75,000/mouse) were transferred into naïve WT animals. These animals were then challenged with VV-gp33 (5×10^6 PFU i.v.) and the percentage of responding donor cells were assessed in the peripheral blood at various time points post-infection. (H) Comparison of the recall response after secondary challenge (n=4). All data shown are representative of 4 different experiments.

Figure 3. Fully functional LCMV-specific memory CD8 T cells do develop in the absence of TH cells. (A) Experimental setup. Naïve Thy1.1$^+$ P14 CD8 T cells were adoptively transferred into WT or CD4 -/- mice. Animals were then infected with LCMV-Armstrong. Approximately 7 days post-infection, effector P14 CD8 T cells were isolated, purified, and adoptively transferred into uninfected WT or CD4 -/- animals. Mice were then bled at various time points post-transfer and changes in number and in overall quality of donor cells were assessed. (B) Kinetics of donor effector CD8 T cell contraction and memory CD8 T cell development. At days 1, 10, 22, and 60 post-transfer, recipient mice were bled and the percentage of Thy1.1$^+$ donor P14 CD8 T cells in the peripheral blood was assessed (n=4). (C) Kinetics of CD127 and CD62L expression on donor P14 CD8 T cells after adoptive transfer. Data shown are gated on Thy1.1$^+$ donor CD8 T cells. (n=4). (D) Phenotypic properties of donor CD8 T cells in the spleen of uninfected WT or CD4 -/- recipient animals approximately 60 days post adoptive transfer. Data shown are gated on Thy1.1$^+$ donor CD8 T cells. The percent of CD127^{hi}, CD62L^{hi}, KLRG-1^{hi}, CD27^{hi}, or Bcl-2^{hi} P14 CD8 T cells is indicated. (E)

Production of IFN-γ, TNF-α, and IL-2 by donor P14 CD8 T cells at 60 days post-transfer into recipient animals. Splenocytes from naive WT and CD4 -/- recipient mice were incubated with LCMV peptide (D^b GP33-41) and BFA for 5 h in vitro. Cells were then stained for extracellular CD8 and intracellular IFN-γ, TNF-α, and IL-2 expression. Data shown are gated on Thy1.1$^+$ donor P14 CD8 T cells. The percent of IFN-γ$^+$ CD8 T cells producing TNF-α or IL-2 is indicated. (F) Experimental setup for recall analysis. Approximately 60 days post initial adoptive transfer, donor P14 CD8 T cells were re-isolated from recipient WT or CD4 -/- mice and equal numbers of cells (~40,000/mouse) were transferred into naïve WT animals. These animals were then challenged with VV-gp33 (5 x 10^6 PFU i.v.). Animals were then sacrificed at 5 days post VV-gp33 infection and the total number of responding donor cells was calculated in the spleen and depicted in (G). All data shown are representative of 4 different experiments.

Figure 4. CD4 T cells are not required for the differentiation of all LCMV-specific CD8 T cells into fully functional memory cells. (A) Experimental setup. Naïve WT animals were infected with LCMV-Armstrong. At 8 days post-infection, LCMV-specific effector CD8 T cells were isolated, purified, and adoptively transferred into either uninfected or LCMV-infection matched WT and CD4 -/- animals. Recipient mice were then sacrificed at approximately 75 days post-transfer and both the number and overall quality of these cells were assessed. (B) Percentage of donor LCMV-specific effector CD8 T cells in the spleen of recipient mice at day 75 post adoptive transfer. Splenocytes from recipient mice were stained with D^b GP33-41, D^b GP276-286, or D^b NP396-404 MHC class I tetramer and antibodies to Thy1.1 and CD8α. The percentage of total donor CD8 T cells

(most left hand panel) and the percentage of donor CD8 T cells specific for the different epitopes of LCMV (remaining three panels) are indicated. (C) Comparison of the total number of donor D^b GP33-41$^+$, D^b GP276-286$^+$, or D^b NP396-404$^+$ CD8 T cells in the spleen of recipient mice 75 days post-transfer. (n=3). (D) Phenotypic expression of donor CD8 T cells in the spleen of recipient animals at day 75 post adoptive transfer. Histograms depict the MFI of different phenotypic markers analyzed. Data shown are gated on D^b GP33-41$^+$ donor CD8 T cells. (E) Production of IFN-γ, TNF-α, and IL-2 by donor LCMV-specific CD8 T cells approximately 75 days post-transfer into recipient animals. Splenocytes from recipient animals were incubated with three different LCMV peptides (D^b NP396-404, GP33-41, or GP276-286) and BFA for 5 h in vitro. The cells were then stained for extracellular Thy1.1 and CD8 and intracellular IFN-γ, TNF-α, and IL-2 expression. The percent of donor CD8 T cells producing IFN-γ (most left hand panel) and percent of IFN-γ$^+$ donor CD8 T cells also producing TNF-α (middle panel) or IL-2 (most right hand panel) are indicated. All data shown are representative of 3 different experiments.

Figure 5. Absence of CD4 T cells during LCMV infection results in impaired antigen clearance. (A) Experimental setup. Memory P14 CD8 T cells isolated from WT immune mice (>90 days post LCMV-Armstrong infection) were purified and labeled in vitro with CFSE. Equal numbers of these memory cells were adoptively transferred into LCMV-infected WT or CD4 -/- animals (8 days post-infection). Recipient mice were then bled at various time points post-transfer and both the percentage and CFSE profile of the donor cells were assessed. (B) Comparison of the frequency and CFSE profile of donor

memory P14 CD8 T cells in the peripheral blood of recipient animals at days 7, 14, and 21 post-transfer. The upper panel shows Thy1.1 and CD8 staining (the percent of donor cells is indicated) and the lower panel is gated on donor memory P14 CD8 T cells and shows the CFSE profile at different times post-infection (the percent of cells having completely diluted out their CFSE expression is indicated). (C) Comparison of the percentage and CFSE profile of donor memory cells in different tissues of LCMV-infected WT or CD4 -/- animals approximately 28 days post-transfer. The top panel shows Thy1.1 and CD8 staining (the percent of donor cells is indicated) and the lower panel is gated on donor memory P14 CD8 T cells and shows the CFSE profile (the percent of cells having completely diluted out their CFSE expression is indicated). (D) Phenotypic profile of donor memory CD8 T cells in the spleen of recipient animals at day 28 post-transfer. Data shown are gated on Thy1.1^{+} donor memory P14 CD8 T cells. All data shown are representative of 4 different experiments.

Figure 6. Not the absence of CD4 T cells, but rather the persistence of low level of LCMV antigen impedes normal memory CD8 T cell development in LCMV-Armstrong infected CD4 -/- animals. (A) Experimental setup. Naïve Thy1.1^{+} P14 CD8 T cells were adoptively transferred into WT mice and animals were subsequently infected with LCMV-Armstrong. Approximately 8 days post-infection, effector P14 CD8 T cells were isolated, purified, and adoptively transferred into different recipient animals (day 8 LCMV-infected WT, naïve CD4 -/-, day 8 LCMV-infected CD4 -/-, and day 8 VV-infected CD4 -/-). Recipient mice were then bled at various time points post-transfer and the overall quality of donor cells was assessed. (B) Kinetics of CD127 and CD62L

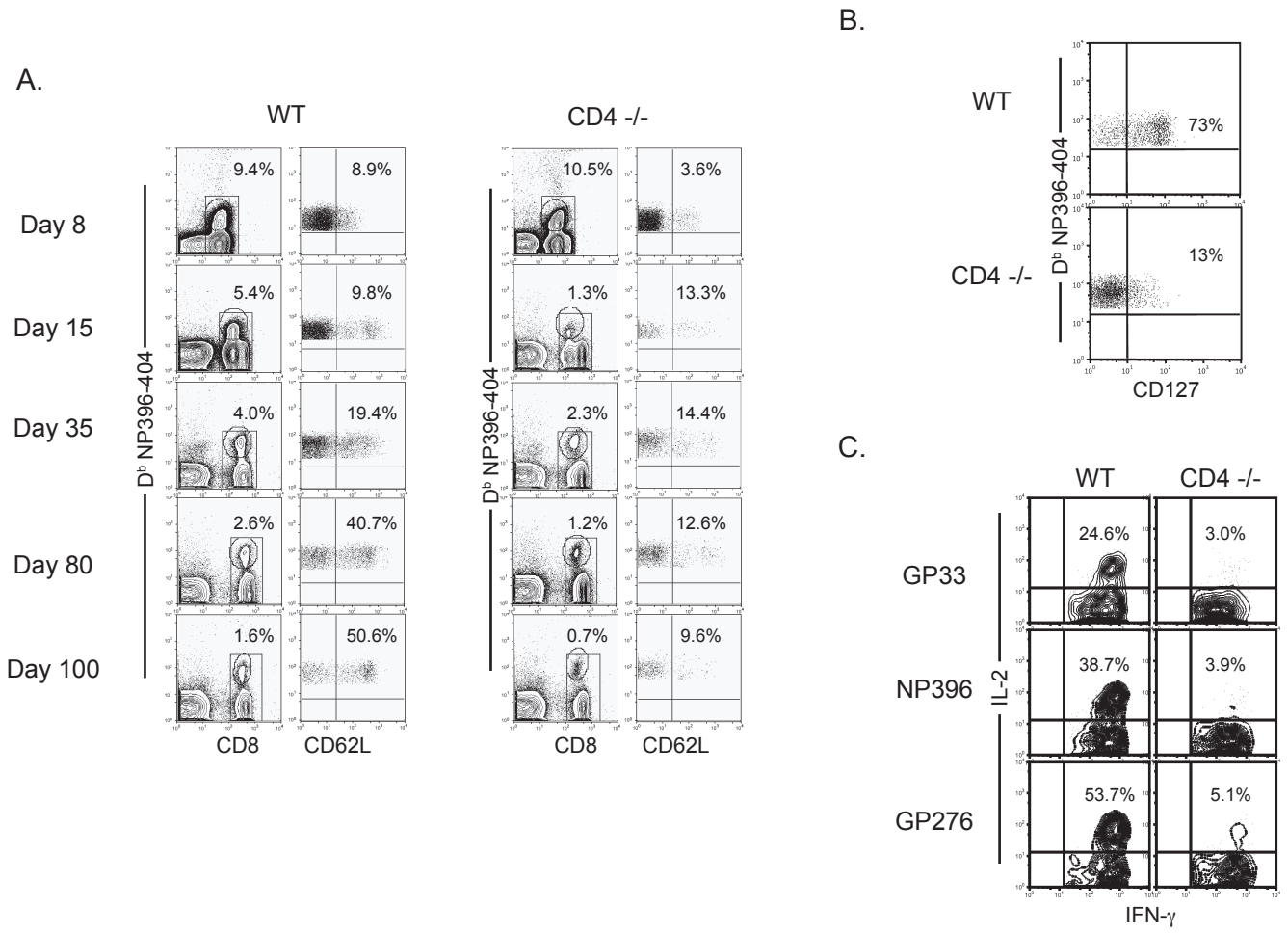
expression on donor P14 CD8 T cells after adoptive transfer into recipient mice. Data shown are gated on Thy1.1^{+} donor CD8 T cells. (n=4). (C) Phenotypic properties of donor CD8 T cells in the spleen of different recipient animals approximately 60 days post adoptive transfer. Data shown are gated on Thy1.1^{+} donor CD8 T cells. The percent of CD127^{hi}, CD62L^{hi}, KLRG-1^{hi}, CD27^{hi}, or Bcl-2^{hi} donor P14 CD8 T cells is indicated. (D) Production of IFN-γ, TNF-α, and IL-2 by donor P14 CD8 T cells at 60 days post-transfer into recipient animals. Splenocytes from different recipient groups were incubated with LCMV peptide (D^{b} GP33-41) and BFA for 5 h in vitro. Cells were then stained for extracellular Thy1.1 and CD8 and intracellular IFN-γ, TNF-α, and IL-2 expression. Data shown are gated on Thy1.1^{+} donor P14 CD8 T cells. The percent of IFN-γ^{+} CD8 T cells producing TNF-α or IL-2 is indicated. All data shown are representative of 3 different experiments.

Supplementary Figure Legends

Figure S1. Effector P14 CD8 T cells adoptively transferred into naïve MHC class II -/- animals failed to develop into fully functional memory CD8 T cells. (A) Experimental setup. Naïve Thy1.1^{+} P14 CD8 T cells were adoptively transferred into WT mice and animals were subsequently infected with LCMV-Armstrong. Approximately 8 days post-infection, effector P14 CD8 T cells were isolated, purified, and adoptively transferred into either uninfected or LCMV-infection matched WT, CD4 -/-, or MHC class II -/- animals. Recipient mice were then bled at various time points post-transfer and the overall quality of donor cells was assessed. (B) Longitudinal analysis of cell-surface expression of CD127 and CD62L on donor P14 CD8 T cells after adoptive transfer into

different recipient mice. Data shown are gated on Thy1.1^{+} donor CD8 T cells and the percent of CD127^{hi} (left panel) or CD62L^{hi} (right panel) P14 CD8 T cells is indicated. (C) Comparison of absolute number of donor CD8 T cells in different peripheral tissues (spleen and liver) of recipient animals approximately 60 days post adoptive transfer (n=4). (D) Phenotypic properties of donor CD8 T cells in the spleen of LCMV-infected recipient mice approximately 60 days post adoptive transfer. Data shown are gated on Thy1.1^{+} donor CD8 T cells. Histograms depict the MFI of different phenotypic markers analyzed. (E) Production of IFN-γ, TNF-α, and IL-2 by donor P14 CD8 T cells at 60 days post-transfer into recipient animals. Splenocytes from different recipient groups were incubated with LCMV peptide (D^{b} GP33-41) and BFA for 5 h in vitro. Cells were then stained for extracellular Thy1.1 and CD8 and intracellular IFN-γ, TNF-α, and IL-2 expression. Data shown are gated on Thy1.1^{+} donor P14 CD8 T cells. The percent of donor CD8 T cells producing IFN-γ (most left hand panel) and percent of IFN-γ^{+} donor CD8 T cells also producing TNF-α (middle panel) or IL-2 (most right hand panel) are indicated. All data shown are representative of 3 different experiments.

Figure 1.


A.
WT
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B.
WT
CD4 -/-
Dᵇ NP396-404
CD127
73%
13%
C.
WT
CD4 -/-
GP33
NP396
GP276
IL-2
IFN-γ
24.6%
3.0%
38.7%
3.9%
53.7%
5.1%

Figure 2.

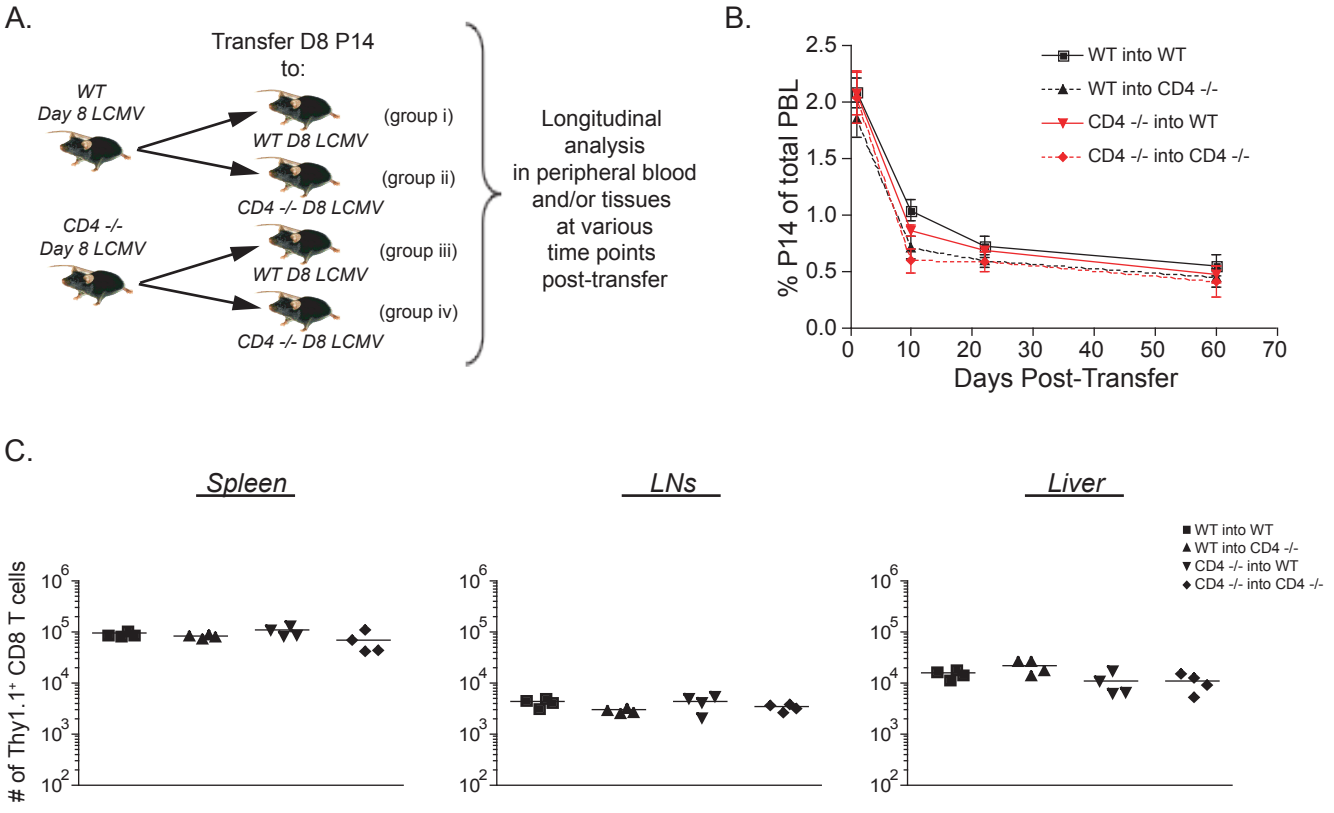

A.
Transfer D8 P14
to:
WT
Day 8 LCMV
WT D8 LCMV
(group i)
CD4 -/- D8 LCMV
(group ii)
CD4 -/-
Day 8 LCMV
WT D8 LCMV
(group iii)
CD4 -/- D8 LCMV
(group iv)
Longitudinal
analysis
in peripheral blood
and/or tissues
at various
time points
post-transfer
B.
% P14 of total PBL
Days Post-Transfer
WT into WT
WT into CD4 -/-
CD4 -/- into WT
CD4 -/- into CD4 -/-
C.
Spleen
LNs
Liver
# of Thy1.1+ CD8 T cells
WT into WT
WT into CD4 -/-
CD4 -/- into WT
CD4 -/- into CD4 -/-

Figure 2

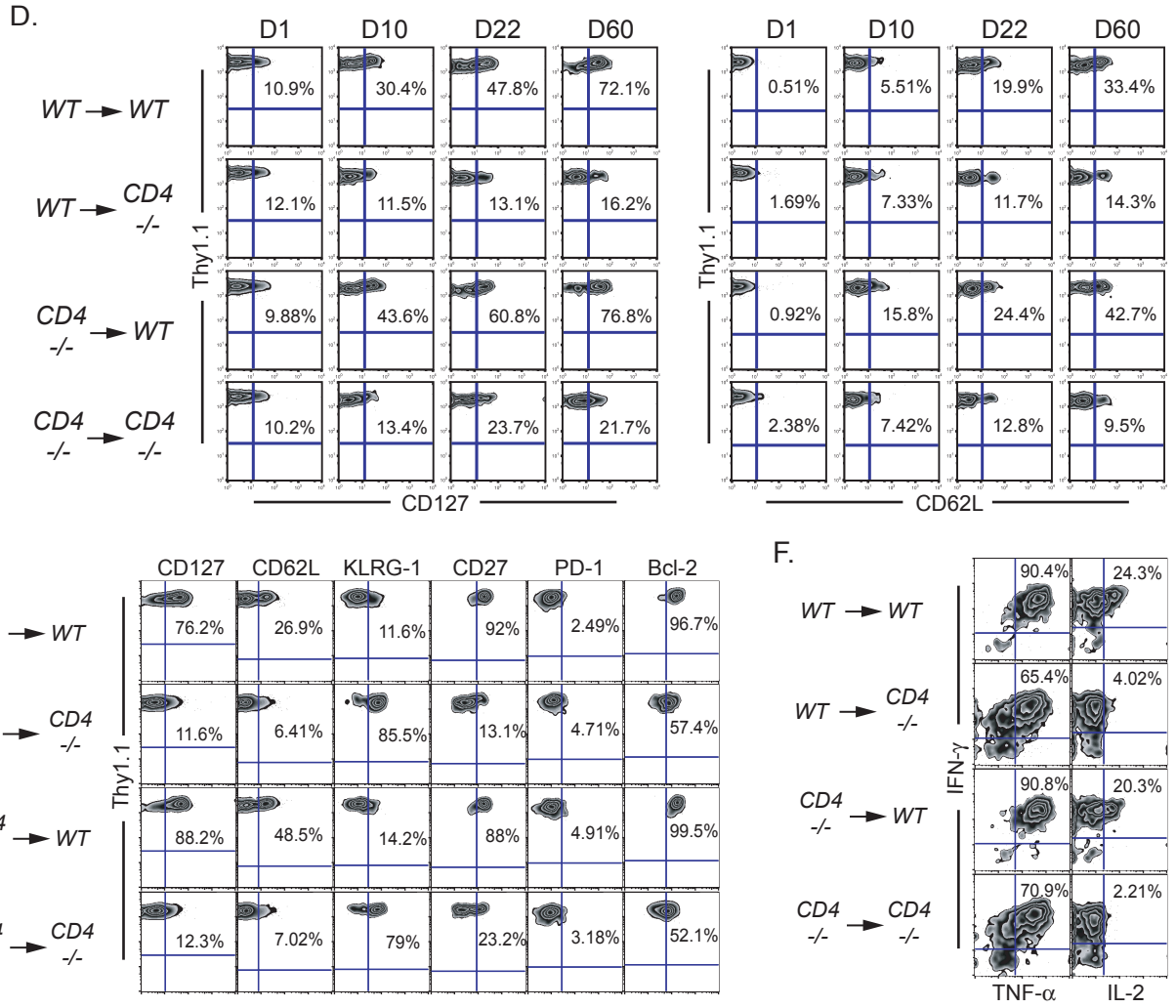
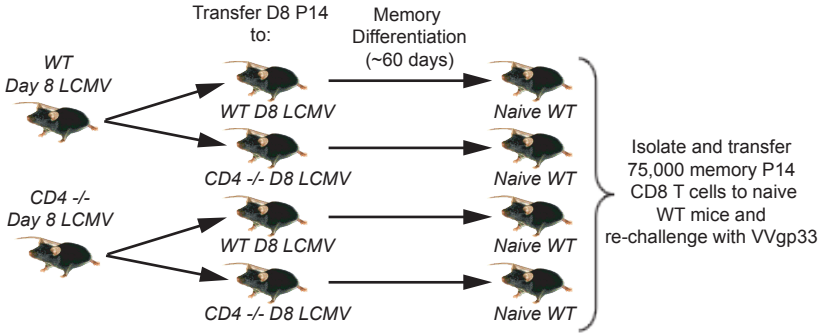

D.
D1
D10
D22
D60
WT → WT
WT → CD4 -/-
CD4 -/- → WT
CD4 -/- → CD4 -/-
10.9%
30.4%
47.8%
72.1%
12.1%
11.5%
13.1%
16.2%
9.88%
43.6%
60.8%
76.8%
10.2%
13.4%
23.7%
21.7%
Thy1.1
CD127
0.51%
5.51%
19.9%
33.4%
1.69%
7.33%
11.7%
14.3%
0.92%
15.8%
24.4%
42.7%
2.38%
7.42%
12.8%
9.5%
CD62L
E.
CD127
CD62L
KLRG-1
CD27
PD-1
Bcl-2
76.2%
26.9%
11.6%
92%
2.49%
96.7%
11.6%
6.41%
85.5%
13.1%
4.71%
57.4%
88.2%
48.5%
14.2%
88%
4.91%
99.5%
12.3%
7.02%
79%
23.2%
3.18%
52.1%
F.
90.4%
24.3%
65.4%
4.02%
90.8%
20.3%
70.9%
2.21%
IFN-γ
TNF-α
IL-2

Figure 2.

G.


Transfer D8 P14
to:
Memory
Differentiation
(~60 days)
WT
Day 8 LCMV
WT D8 LCMV
Naive WT
CD4 -/- D8 LCMV
Naive WT
CD4 -/-
Day 8 LCMV
WT D8 LCMV
Naive WT
CD4 -/- D8 LCMV
Naive WT
Isolate and transfer
75,000 memory P14
CD8 T cells to naive
WT mice and
re-challenge with VVgp33

H.

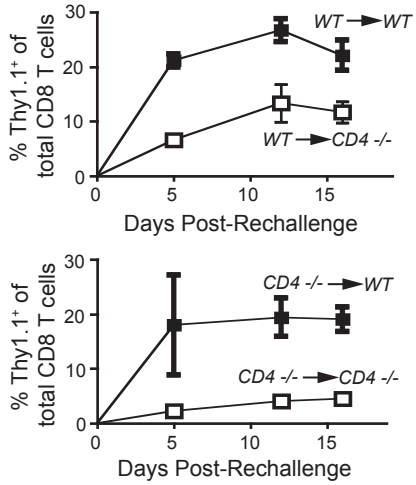

% Thy1.1+ of
total CD8 T cells
30
20
10
0
0
5
10
15
WT → WT
WT → CD4 -/-
Days Post-Rechallenge
% Thy1.1+ of
total CD8 T cells
30
20
10
0
0
5
10
15
CD4 -/- → WT
CD4 -/- → CD4 -/-
Days Post-Rechallenge

Figure 3.

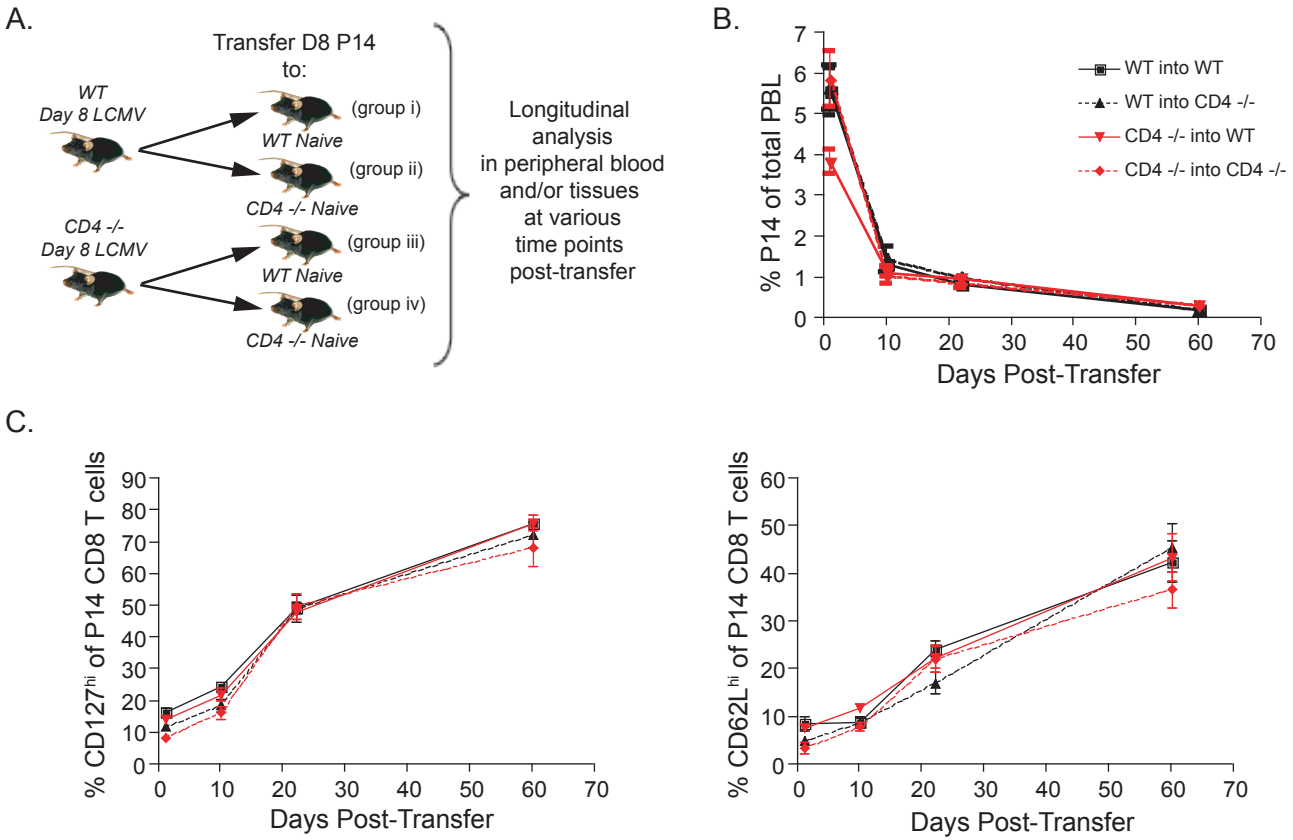

A.
Transfer D8 P14
to:
WT
Day 8 LCMV
(group i)
WT Naive
(group ii)
CD4 -/- Naive
CD4 -/-
Day 8 LCMV
(group iii)
WT Naive
(group iv)
CD4 -/- Naive
Longitudinal
analysis
in peripheral blood
and/or tissues
at various
time points
post-transfer
B.
% P14 of total PBL
Days Post-Transfer
WT into WT
WT into CD4 -/-
CD4 -/- into WT
CD4 -/- into CD4 -/-
C.
% CD127hi of P14 CD8 T cells
Days Post-Transfer
% CD62Lhi of P14 CD8 T cells
Days Post-Transfer

Figure 3.

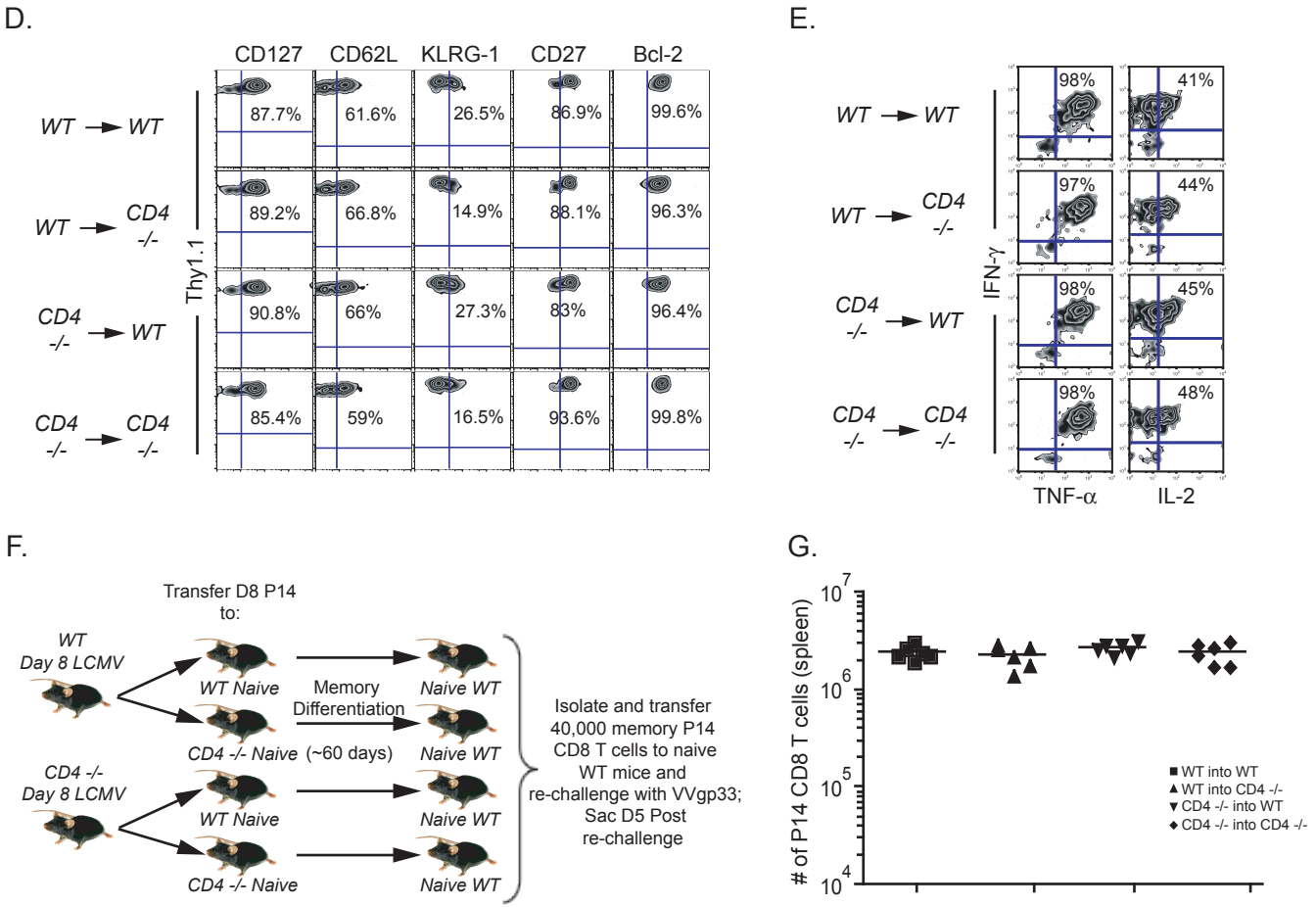

D.
CD127
CD62L
KLRG-1
CD27
Bcl-2
WT → WT
WT → CD4 -/-
CD4 -/- → WT
CD4 -/- → CD4 -/-
Thy1.1
87.7%
61.6%
26.5%
86.9%
99.6%
89.2%
66.8%
14.9%
88.1%
96.3%
90.8%
66%
27.3%
83%
96.4%
85.4%
59%
16.5%
93.6%
99.8%
E.
98%
41%
97%
44%
98%
45%
98%
48%
IFN-γ
TNF-α
IL-2
F.
Transfer D8 P14 to:
WT Day 8 LCMV
CD4 -/- Day 8 LCMV
WT Naive
CD4 -/- Naive
Memory Differentiation
(~60 days)
Naive WT
Isolate and transfer 40,000 memory P14 CD8 T cells to naive WT mice and re-challenge with VVgp33; Sac D5 Post re-challenge
G.
# of P14 CD8 T cells (spleen)
WT into WT
WT into CD4 -/-
CD4 -/- into WT
CD4 -/- into CD4 -/-

Figure 4.

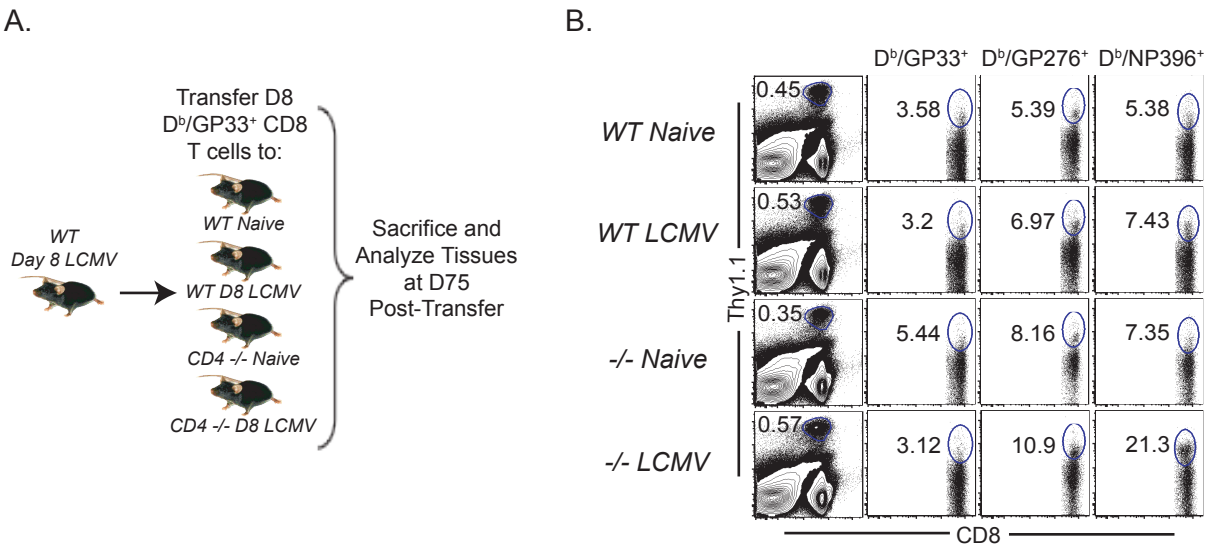

A.
Transfer D8 Db/GP33+ CD8 T cells to:
WT Naive
WT D8 LCMV
CD4 -/- Naive
CD4 -/- D8 LCMV
WT Day 8 LCMV
Sacrifice and Analyze Tissues at D75 Post-Transfer
B.
Db/GP33+
Db/GP276+
Db/NP396+
WT Naive
WT LCMV
-/- Naive
-/- LCMV
Thy1.1
CD8
0.45
3.58
5.39
5.38
0.53
3.2
6.97
7.43
0.35
5.44
8.16
7.35
0.57
3.12
10.9
21.3
C.
# of Db/GP33+ CD8 T Cells
# of Db/GP276+ CD8 T Cells
# of Db/NP396+ CD8 T Cells
WT Naive
WT Arm
KO Naive
KO Arm

Figure 4.

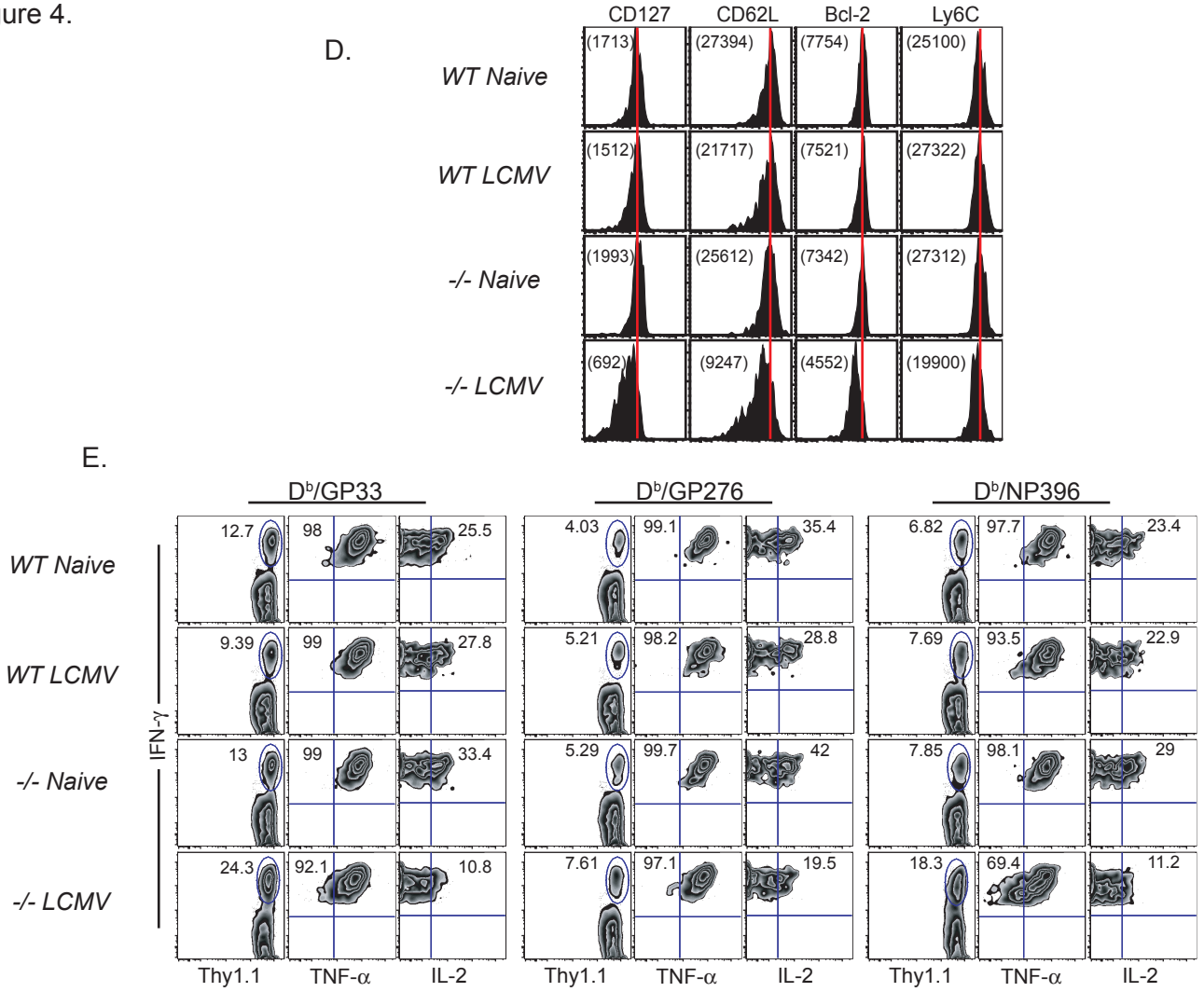

D.
CD127
CD62L
Bcl-2
Ly6C
WT Naive
WT LCMV
-/- Naive
-/- LCMV
(1713)
(27394)
(7754)
(25100)
(1512)
(21717)
(7521)
(27322)
(1993)
(25612)
(7342)
(27312)
(692)
(9247)
(4552)
(19900)
E.
Db/GP33
Db/GP276
Db/NP396
IFN-γ
Thy1.1
TNF-α
IL-2
12.7
98
25.5
4.03
99.1
35.4
6.82
97.7
23.4
9.39
99
27.8
5.21
98.2
28.8
7.69
93.5
22.9
13
99
33.4
5.29
99.7
42
7.85
98.1
29
24.3
92.1
10.8
7.61
97.1
19.5
18.3
69.4
11.2

Figure 5.

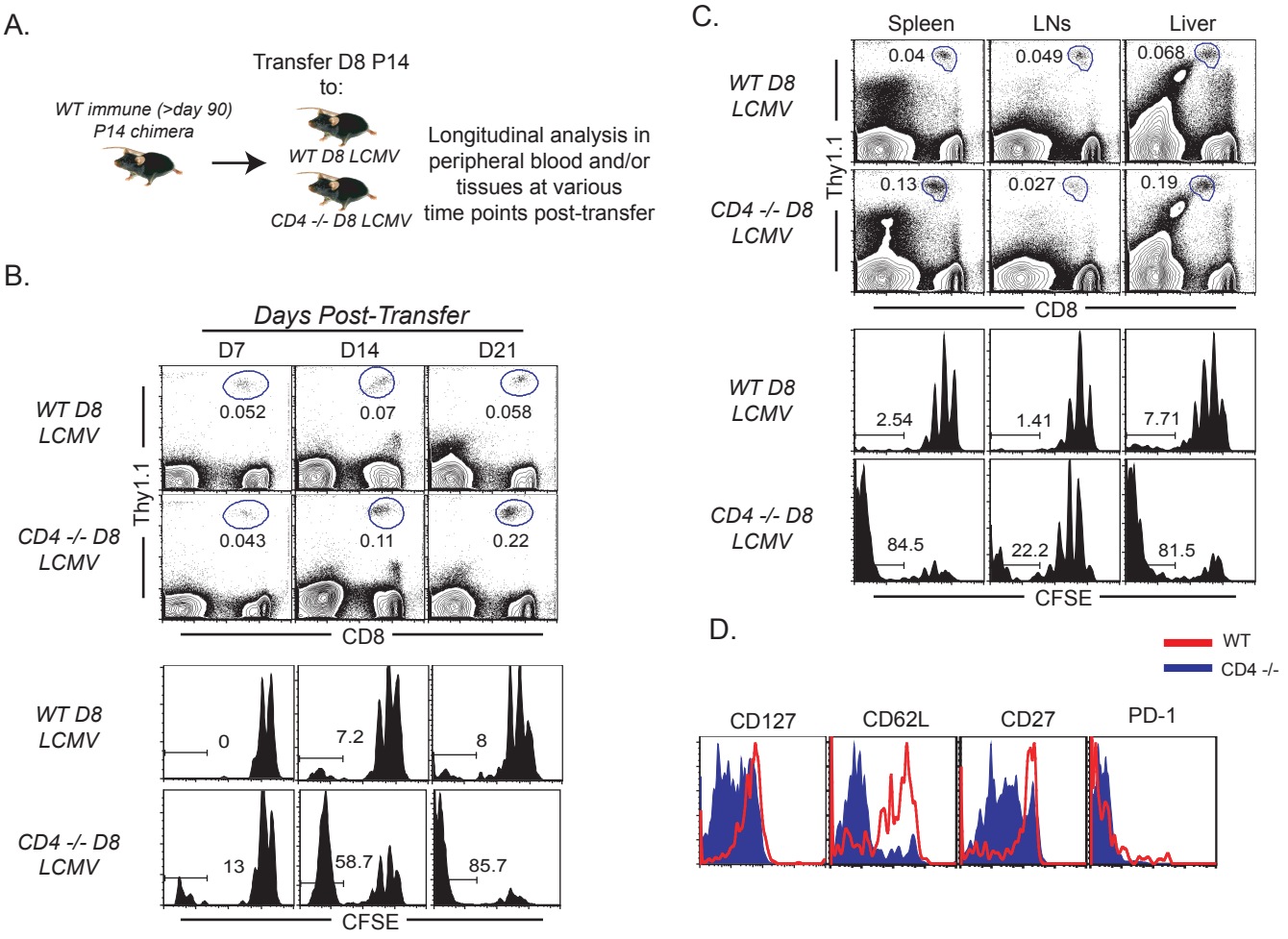
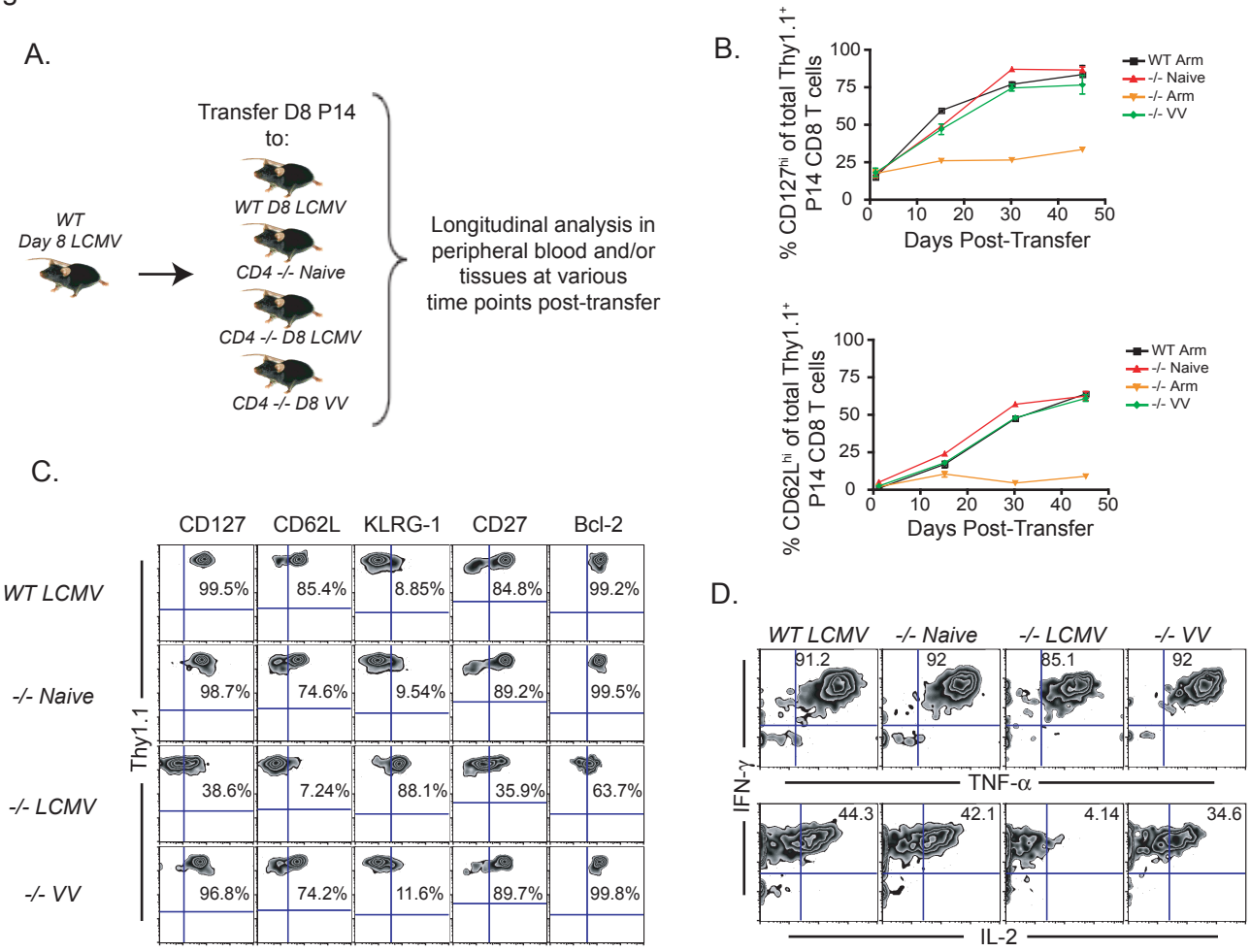
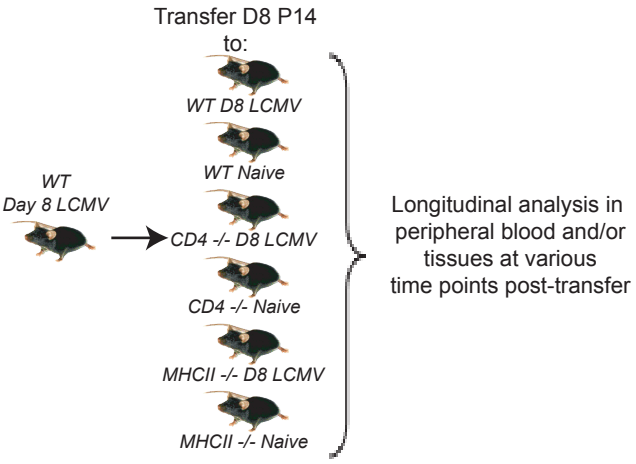

A.
Transfer D8 P14 to:
WT immune (>day 90) P14 chimera
WT D8 LCMV
CD4 -/- D8 LCMV
Longitudinal analysis in peripheral blood and/or tissues at various time points post-transfer
B.
Days Post-Transfer
D7
D14
D21
WT D8 LCMV
CD4 -/- D8 LCMV
Thy1.1
0.052
0.07
0.058
0.043
0.11
0.22
CD8
0
7.2
8
13
58.7
85.7
CFSE
C.
Spleen
LNs
Liver
0.04
0.049
0.068
0.13
0.027
0.19
2.54
1.41
7.71
84.5
22.2
81.5
D.
WT
CD4 -/-
CD127
CD62L
CD27
PD-1

Figure 6.

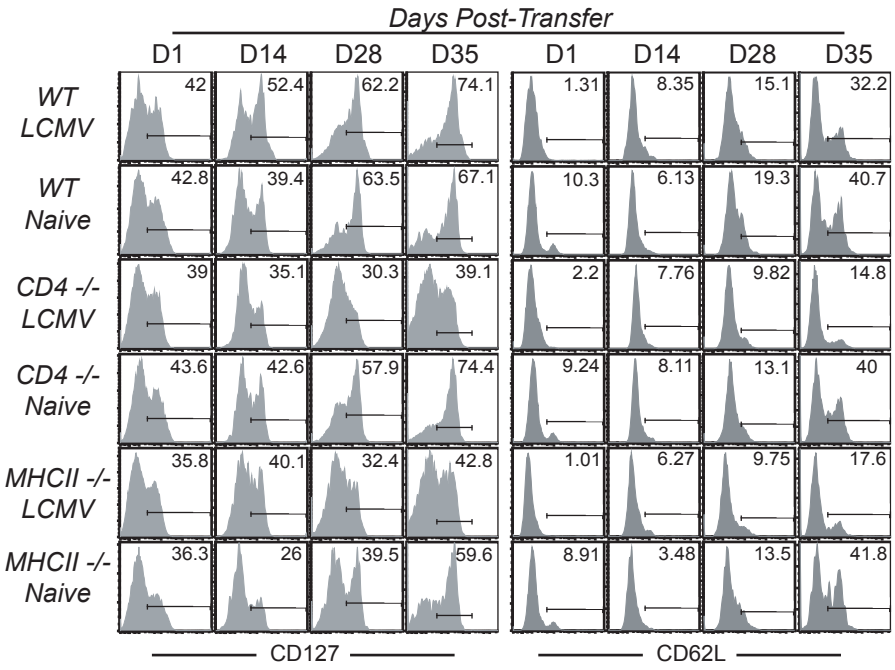

A.
Transfer D8 P14 to:
WT Day 8 LCMV
WT D8 LCMV
CD4 -/- Naive
CD4 -/- D8 LCMV
CD4 -/- D8 VV
Longitudinal analysis in peripheral blood and/or tissues at various time points post-transfer
B.
% CD127hi of total Thy1.1+ P14 CD8 T cells
Days Post-Transfer
WT Arm
-/- Naive
-/- Arm
-/- VV
% CD62Lhi of total Thy1.1+ P14 CD8 T cells
Days Post-Transfer
C.
CD127
CD62L
KLRG-1
CD27
Bcl-2
WT LCMV
-/- Naive
-/- LCMV
-/- VV
Thy1.1
99.5%
85.4%
8.85%
84.8%
99.2%
98.7%
74.6%
9.54%
89.2%
99.5%
38.6%
7.24%
88.1%
35.9%
63.7%
96.8%
74.2%
11.6%
89.7%
99.8%
D.
WT LCMV
-/- Naive
-/- LCMV
-/- VV
IFN-γ
TNF-α
IL-2
91.2
92
85.1
92
44.3
42.1
4.14
34.6

Supplementary Figure 1.

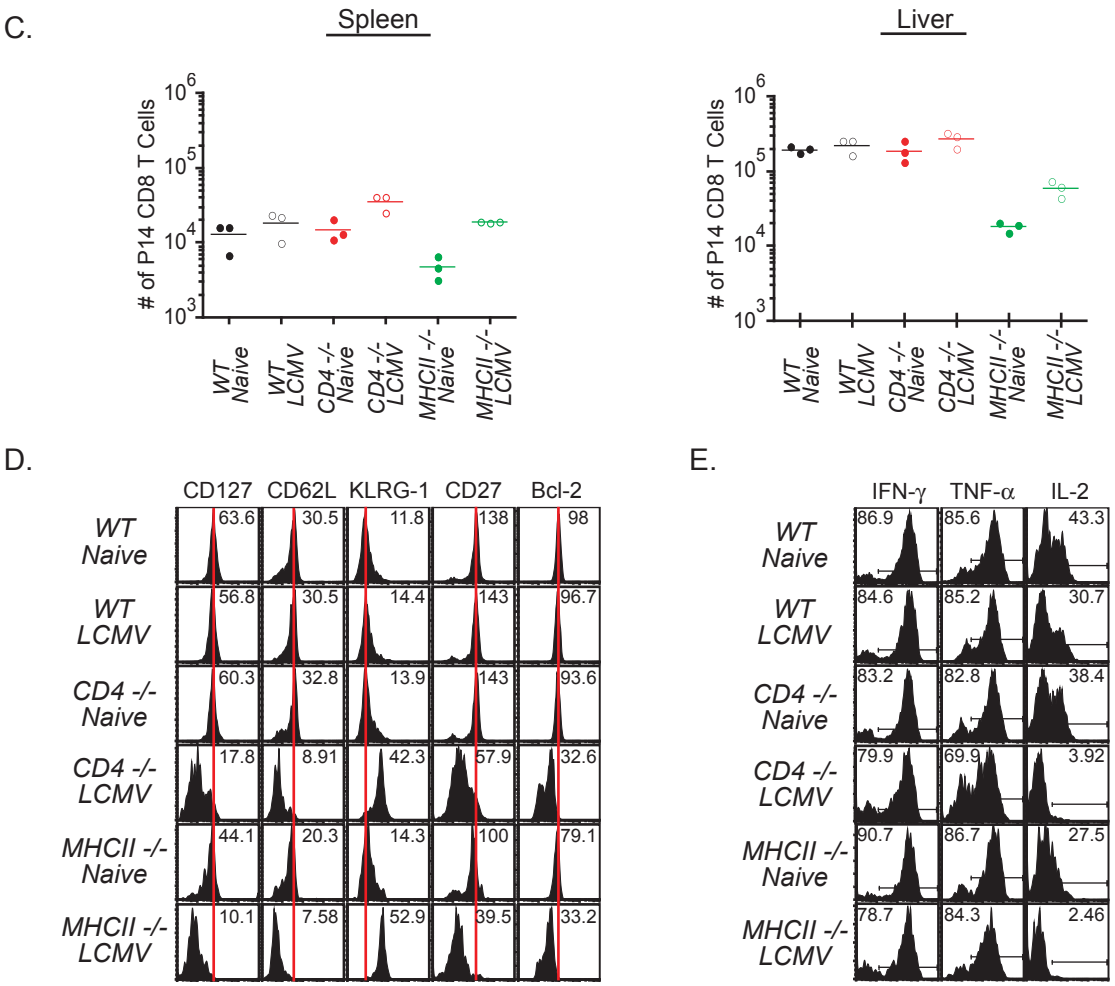
A.



B.



Supplementary Figure 1.



Chapter 3: Homeostatic turnover of virus-specific memory CD8 T cells occurs stochastically and is independent of CD4 T cell help

Abstract

The continued persistence of memory CD8 T cells long after the resolution of infection, which enables these cells to play an important role in conferring lifelong protective immunity, is associated with the increased survival and the homeostatic turnover of memory CD8 T cells. Despite this understanding, the rules and elements that regulate this long-term maintenance of memory cells require further characterization. In this study, we utilized a series of adoptive transfer experiments along with mathematical modeling to both rigorously quantify the homeostatic turnover and address the role of CD4 T cell help in the long-term maintenance of the quality of memory CD8 T cells. We made the following observations in this study: 1) the mean and the variance in the number of divisions both increased linearly at similar rates and the CFSE distribution of the divisions followed a Poisson distribution, which collectively suggested that the recruitment of memory cells into division was stochastic, 2) the homeostatic turnover was comparable among memory CD8 T cells specific for different epitopes of LCMV, and 3) memory CD8 T cells maintained in the absence of CD4 T cell help underwent normal homeostatic turnover and retained both their phenotypic expression and function. These results show that both the stochastic and TCR specificity independent homeostatic turnover and the long-term maintenance of the overall quality of virus-specific memory CD8 T cells are independent of CD4 T cell help.

Introduction

The hallmarks of memory CD8 T cells include both their ability to respond faster and more efficiently compared to naïve cells upon secondary challenge, and also their ability to persist long after the infection has been cleared (4, 5, 47, 354-356). This latter quality of memory cells is essential in providing lifelong protective immunity, both in mice and in humans (88, 357). Due to this importance in host immunity, there has been much interest over the years in understanding the mechanisms and regulations of the long-term maintenance of antigen-specific memory CD8 T cells. Initially, it was argued that this persistence of memory CD8 T cells was due to continual stimulation from small amounts of persisting antigen (358). However, subsequent studies have demonstrated that memory maintenance is independent of antigen, and is instead associated with IL-7 and IL-15 mediated survival and homeostatic turnover of memory CD8 T cells (93, 95-99, 107, 108, 132). More recent experiments have shown that helper CD4 T cells (T-helper cells) are also important for the continued persistence of memory CD8 T cells after acute *Listeria monocytogenes* (LM) or lymphocytic choriomeningitis virus (LCMV) infection (216). Although this role of T_H cells in the maintenance of memory CD8 T cells after an acute infection is a fairly recent discovery, for most chronic infections, it has long been established that CD4 T cell help is necessary to prevent the exhaustion and deletion of antigen-specific CD8 T cells (227-230).

Despite this substantial progress made in understanding the mechanisms and regulations of memory CD8 T cell maintenance, there still remain questions that have yet to be fully addressed. First, do all memory CD8 T cells undergo homeostatic turnover or is there a subpopulation of cells that do not homeostatically divide? Next, what

characterizes the turnover of memory cells – do cells divide after a fixed time or is this turnover stochastic? In addition, how do CD4 T cells aid (e.g. survival vs. homeostatic turnover) in promoting the persistence of memory CD8 T cells and is the antigen-specificity of these T_H cells important? Lastly, do memory CD8 T cells also continue to require CD4 T cell help to retain their overall quality (e.g. phenotypic profile and function)?

To more carefully address the above-described questions, we longitudinally analyzed, within individual mice, both the frequency and the homeostatic turnover of fully functional LCMV-specific memory CD8 T cells after adoptive transfer into naïve wild-type (WT) mice. This information allowed us to use mathematical modeling to more rigorously and more accurately quantify the homeostatic turnover and the dynamics of memory CD8 T cell maintenance. We also utilized naïve CD4 T cell deficient (CD4 -/-) and major histocompatibility complex class II deficient (MHCII -/-) mice as recipients to address the requirement for CD4 T cell help in the long-term maintenance of the overall quality of memory CD8 T cells. These animals are ideal models to study the role of CD4 T cells as they exhibit significant impairment in the generation and maintenance of memory CD8 T cells in the context of both acute and chronic infections and this impairment has been described to be due to the absence of CD4 T cells in these mice. This study provides a much needed longitudinal and rigorous quantitative analysis of the homeostatic turnover of memory CD8 T cells and helps investigate the maintenance of memory CD8 T cells in the absence of CD4 T cell help.

Results

Quantitative analysis of homeostatic turnover of LCMV-specific memory CD8 T cells

To quantitatively analyze the homeostatic turnover of fully functional memory CD8 T cells, approximately 1 x 10^5 naïve P14 CD8 T cells (specific for the D^b GP33-41 epitope of LCMV) were adoptively transferred into naïve congenic WT mice and then these P14 chimeric mice were infected with the Armstrong strain of LCMV. At >60 days post-infection the majority of the memory P14 CD8 T cells had further differentiated from effector memory ($CD62L^{lo}$, $CCR7^-$, $CD27^{lo/int}$, found mostly in non-lymphoid tissues, less efficient at IL-2 production, and reduced proliferative capacity) to central memory ($CD62L^{hi}$, $CCR7^+$, $CD27^{hi}$, efficiently produce IL-2, and great proliferative capacity) CD8 T cells. These (day >60) memory P14 CD8 T cells were purified, labeled in vitro with CFSE, and adoptively transferred into congenic naïve WT recipients (179). Recipient mice were then serially bled at various time points post-transfer and both the number and the CFSE profiles of the transferred memory cells were longitudinally assessed in individual mice. Consistent with earlier studies demonstrating long-term persistence of memory CD8 T cells, we observed that the transferred memory CD8 T cells were stably maintained in WT animals for the entire duration of the experiments (Fig 1a). At day 1 post-transfer, the percentage of donor memory cells observed in the peripheral blood ranged between 0.14 and 0.26%. While this percentage fluctuated slightly over time, overall, the number of transferred memory CD8 T cells remained constant as far out as 120 days post-transfer. More specifically, we calculated the mean rate of loss of donor memory cells to be 0.0005 per day, which was not statistically

different from 0 (p=0.78). This stability in the maintenance of the donor memory cells is shown in Fig 1c.

In regard to the homeostatic turnover of the transferred memory cells, the initial inspection of the CFSE profiles of these cells at different time points post-transfer suggested that the turnover of memory CD8 T cells occurred slowly, but continuously. In support, the percentage of cells that had undergone at least 1 round of division increased from about 60%, at day 21 post-transfer, to approximately 80-90%, by 60 days post-transfer (Fig 1b). Examination of the frequency of undivided cells at different time points revealed that the percentage of undivided cells decreased exponentially with time (Fig 2a), suggesting that there was a single homogeneous population of memory cells (no separate population of non-dividing memory CD8 T cells) and that the recruitment into division was stochastic. In order to confirm this, we subjected the CFSE profiles of donor memory CD8 T cells to further mathematical analysis (see Material and Methods). If all memory cells were capable of division and the recruitment into division were stochastic, then the frequency of cells having undergone different numbers of divisions at a given time would follow a Poisson distribution. A characteristic of the Poisson distribution is that the mean number of divisions and the variance in the number of divisions are the same and if the division rate does not change over time, they should both increase linearly with time. In Figure 2b, we observed that this indeed appeared to be the case. The mean number of divisions and the variance in the number of divisions both increased linearly with time at a rate of about 0.04 divisions/day, which translated into an average rate of division (λ) of approximately 0.02 divisions/day or an intermiotic time ($1/\lambda$) of approximately 50 days (see Materials and Methods). As might be

expected, the variance in the number of divisions was slightly less than the mean number of divisions because while the time for cells to undergo division was much smaller than the rate of recruitment into division as we had assumed it was not zero. In Figure 2c, we made maximum use of all the CFSE data by fitting the entire dataset for the CFSE distribution in the number of cells over time, in each mouse, to the model for stochastic division. We observed that the rate of division (λ) estimated from the fit to the entire CFSE distribution was indeed in agreement with that estimated from the rate of increase in the mean number of divisions described above. Collectively, these observations implied that the homeostatic turnover of memory CD8 T cells occurred stochastically, where the probability that a memory cell divided did not depend on its previous division history. This stochastic turnover resulted in the mean number of divisions of cells in the population increasing at a rate of about 0.04 divisions per day, which corresponds to an average rate of division (λ) of 0.02 divisions/day or an intermiotic time, which equals $1/\lambda$, of approximately 50 days.

Comparison of the homeostatic turnover of memory CD8 T cells specific for different epitopes of LCMV

Next, we wanted to examine whether the pattern of homeostatic turnover observed for transgenic Db GP33-41+ memory P14 CD8 T cells also held true for populations of endogenous memory CD8 T cells specific for different epitopes of LCMV in two distinct strains of mice. To do this, we infected both naïve WT C57BL/6 or naïve WT BALB/c mice with the Armstrong strain of LCMV. At >60 days post-infection, LCMV-infected animals were sacrificed, and the total splenocytes were labeled with

CFSE and adoptively transferred into congenic naïve C57BL/6 or BALB/c recipients. The recipient mice were then sacrificed at different time points post-transfer and the CFSE profiles of different populations of LCMV-specific memory CD8 T cells (D^b NP396-404$^+$ in C57BL/6 and L^d NP118-126$^+$in BALB/c) were assessed.

As observed in Fig 3a and b, the overall pattern of the homeostatic turnover of D^b NP396-404$^+$ and L^d NP118-126$^+$ memory CD8 T cells closely resembled that observed earlier with memory P14 CD8 T cells. For example, we observed that for both D^b NP396-404$^+$ and L^d NP118-126$^+$ memory CD8 T cells, the mean number of divisions and the variance in the number of divisions also increased linearly with time. In addition, the CFSE distribution of these memory CD8 T cells followed a Poisson distribution. These data indicated that the homeostatic turnover of memory cells of different specificities (D^b NP396-404$^+$ vs. L^d NP118-126$^+$) in different strains of mice (C57BL/6 vs. BALB/c) was consistent with a stochastic model of cell division. We also observed that the CFSE profile of the total CD44^{hi} memory CD8 T cells was virtually identical to that of D^b NP396-404$^+$ memory CD8 T cells at day 21 post-transfer, suggesting that all memory CD8 T cells, irrespective of their specificity and mouse strain exhibit similar homeostatic turnover (slow, continuous, and stochastic recruitment into division).

Role of CD4 T cell help in the homeostatic turnover and persistence of memory CD8 T cells

For most acute infections, CD4 T cell help has been traditionally thought to be important in the development of fully functional memory CD8 T cells (231). However, more recently, it has been proposed that CD4 T cell help is necessary for the long-term

persistence of memory CD8 T cells (216). Therefore, we next wanted to more rigorously determine the role of helper CD4 T cells for maintaining the quantity and quality of memory CD8 T cells. Briefly, fully functional memory P14 CD8 T cells were purified from WT LCMV immune mice (>60 days post-infection) as described earlier. Cells were then labeled with CFSE in vitro and adoptively transferred into either uninfected WT, CD4 -/-, or MHCII -/- mice. These recipient animals were then serially bled at various time points post-transfer and both the frequency and the CFSE profiles of the transferred memory cells were longitudinally assessed in individual mice (Fig 4a).

As shown in Fig 4b, we observed that the donor memory cells were stably maintained when adoptively transferred into either WT or CD4 -/- recipients, but slowly declined in number when transferred into MHCII -/- animals. At day 1 post-transfer, the percentages of donor memory cells in the peripheral blood of WT, CD4 -/-, and MHCII -/- mice were approximately the same (0.1 – 0.4%). By 70 days post-transfer when the animals were sacrificed, the transferred memory cells made up less than 0.1% of the peripheral blood in MHCII -/- animals, whereas in WT and CD4 -/- recipient mice, this percentage remained at or around the initial frequency (0.1 – 0.4%). In support, when the mean rate of the loss of donor cells was calculated for the different recipient groups, we found that this rate was not significantly different from 0 in both WT (0.0005 cells/day, 95% CI: -0.004 – 0.003) and CD4 -/- animals (-0.002 per day, 95% CI: -0.0027 – 0.007), indicating stable maintenance of the transferred cells in these recipient groups. However, in MHCII -/- animals, the donor memory CD8 T cells decayed at a mean rate of 0.015 per day (95% CI: -0.01 – 0.02; $p<0.0001$), which is equivalent to a half life of about 67 days. This observed trend in memory persistence among the different recipient animals was not

unique to the peripheral blood, but was also true for all tissues examined (e.g. spleen, lymph nodes, liver, and bone marrow). As shown in Fig 4c and d, we consistently observed a 5- to 10-fold decrease both in the percentage and in the total number of donor memory CD8 T cells in the tissues of MHCII -/- animals compared to WT and CD4 -/- mice.

In contrast with the observed loss of memory CD8 T cells in MHCII -/- recipients, we observed no substantial defect in the homeostatic turnover of the transferred memory CD8 T cells in any of the recipient mice (Fig 4e). Analysis of the CFSE profiles revealed that the mean number of divisions (Fig 4f) and the variance in the number of divisions (not shown) increased linearly with time in WT (mean: 0.04 divisions/day), CD4 -/- (mean: 0.048 divisions/day), and MHCII -/- (mean: 0.054 divisions/day) mice. In addition, the distribution of the number of donor cells having undergone different rounds of divisions followed a Poisson distribution in all recipient animals (Figure 4g). This indicated that the homeostatic turnover of donor memory cells from WT, CD4 -/-, and MHCII -/- recipients occurred slowly with the recruitment into division being stochastic.

Assessment of the role of CD4 T cell help in maintaining the overall quality of memory CD8 T cells

The hallmark of memory CD8 T cells is their ability to confer protective immunity by responding more rapidly and more efficiently upon secondary challenge. This intrinsic quality of memory cells is greatly dependent on CD4 T cell help (231). Along these lines, we next examined whether memory CD8 T cells continue to require CD4 T cell help to retain their overall quality. To address this, we adoptively transferred

fully functional and CFSE labeled memory P14 CD8 T cells, purified from LCMV immune WT P14 chimeric mice (>60 days post-infection), into either uninfected WT, CD4 -/-, or MHCII -/- animals as done previously (Fig 4a). At least 60 days post-transfer, the recipient mice were sacrificed and both the phenotypic profile and the functional quality of the donor memory CD8 T cells were assessed.

In regard to phenotypic expression, we observed no noticeable discrepancy among donor memory CD8 T cells from WT, CD4 -/-, and MHCII -/- animals (Fig 5a). For instance, donor memory cells isolated from the spleens of all three groups expressed high levels of CD62L, CD127, CD27, CD44, and CD122 (IL-2/IL-15Rβ) – profile characteristic of healthy memory CD8 T cells. In addition, these memory cells expressed negligible levels of markers associated with activation and effector activity (KLRG-1, CD25/IL-2Rα, CD69, PD-1, and Granzyme B). This lack of difference in phenotypic profile was also true among donor cells isolated from other peripheral tissues (e.g. liver, lung, bone marrow, peripheral blood, and lymph nodes; data not shown), suggesting that CD4 T cell help was not required for maintaining the phenotypic profile of memory CD8 T cells.

In agreement with the phenotypic data, we also did not observe any significant discrepancy in the functional quality of the transferred cells in all the recipient animals. When donor CD8 T cells maintained in WT, CD4 -/-, or MHCII -/- were isolated and re-stimulated in vitro with LCMV GP33 peptide, they all rapidly produced IFN-γ with approximately 94% and 20% of the cells also producing TNF-α and IL-2, respectively (Fig 5b). Similarly, we observed that the recall capability of the donor memory cells was unaffected by the absence of CD4 T cell help during memory maintenance. This was

demonstrated by adoptively transferring equal number of donor memory cells, that had been maintained in WT, CD4 -/-, or MHCII -/- animals for at least 60 days, into normal C57BL/6 mice and then challenging these mice with vaccinia virus expressing the GP33 epitope of LCMV (VV-GP33). Approximately 5 days post-challenge, the animals were sacrificed and the recall response of the donor memory CD8 T cells was analyzed (Fig 5c). As shown in Fig 5d, the donor memory cells maintained in WT, CD4 -/-, or MHCII -/- animals all responded comparably after VV-GP33 challenge. The responding donor cells constituted approximately 1 – 2% of the total peripheral blood in all the groups. In addition, we observed similar total number of donor cells both in the spleen (Fig 5e) and in the liver (data not shown). Altogether, these results suggested that although the long-term persistence of memory CD8 T cells may depend on CD4 T cell help (at least in MHCII -/- animals), the homeostatic turnover and the overall quality (both in phenotypic expression and in recall ability) were unaffected by the absence of helper CD4 T cells during the maintenance phase of CD8 T cell immune response.

Discussion

There has been much interest over the years in understanding the maintenance of memory CD8 T cells, as these cells are required to mount a much more rapid and efficient immune response upon secondary exposure to a given pathogen (4, 355, 359, 360). In this study, we longitudinally assessed, in individual mice, both the numbers and the homeostatic turnover of antigen-specific memory CD8 T cells. We did so after adoptive transfer of memory CD8 cells into either WT, CD4 -/-, or MHCII -/- recipients. This allowed us to use mathematical modeling to rigorously quantify the homeostatic turnover of memory CD8 T cells, and also address the role of CD4 T cell help both in this turnover and in the long-term maintenance of the overall quality of memory CD8 T cells.

Our initial analysis of memory CD8 T cells adoptively transferred into WT recipients revealed that these cells were stably maintained and underwent slow homeostatic turnover that was independent of epitope specificity (total $CD44^{hi}$, D^b GP33-41^+ P14, D^b NP396-404^+, and L^d NP118-126^+) and mouse strain (C57BL/6 and BALB/c). These results are in agreement earlier qualitative observations that demonstrated that memory CD8 T cells actively undergo homeostatic turnover and this turnover is important in the long-term maintenance of memory CD8 T cells (95, 130, 132, 360).

However, our study is distinct from these more qualitative studies in that it provides a much-needed rigorous quantitative analysis of the homeostatic turnover of antigen-specific memory CD8 T cells. Accordingly, we determined that the recruitment of memory CD8 T cells into division was stochastic rather than deterministic. Had division followed a deterministic model, in which all cells spend the same time between successive divisions, then, at any given time, the number of divisions that all the memory

cells had undergone would have differed by at most one division. In contrast, we observed that the variance in the number of divisions increased linearly with time at a rate similar to the increase in the mean number of divisions, which suggested a stochastic model for cell division, in which the probability that a cell divides does not depend on its previous history. We further validated the stochastic nature of memory CD8 T cell turnover by demonstrating that the numbers of divisions followed a Poisson distribution, as predicted by a stochastic model for cell turnover. Lastly, like other past studies (226, 361, 362), we assumed that death like division occurred stochastically (random birth-death or RBD model); this allowed us to infer that the mean number of divisions increased at twice the rate of division (λ), as each division would result in the production of two cells having one additional division, while death would kill cells of all divisions equally (361). Based on this assumption, we calculated that the turnover of memory CD8 T cells occurs at a rate of 0.02 divisions/day or an intermiotic time of about 50 days ($1/\lambda$). Our estimation is comparable to that proposed by Di Rosa and colleagues (intermiotic time of 63 days), who used BrdU to calculate the rate of turnover of memory-phenotype ($CD44^{hi}$) CD8 T cells in thymectomized naïve mice (226). This modest difference between the two estimations could be due to the different cell labeling techniques or the type of memory cells studied.

In addition to this rigorous quantitative analysis, our study differs from past studies by also analyzing the requirement for CD4 T cell help during the long-term maintenance of memory CD8 T cells, both in their quantity and in their quality. We first observed that antigen-specific helper CD4 T cells were not required for memory CD8 T cell maintenance, as memory cells adoptively transferred into naïve WT recipients –

uninfected and therefore, lack antigen-specific T cells – were stably maintained and retained both their phenotypic profile and functions. While this demonstrated that antigen-specific CD4 T cells were not required, it did not rule out a non-specific role for CD4 T cell help. Therefore, we utilized naïve CD4-/- mice as recipients for our adoptive transfer experiments, since these animals lack CD4 T cells due to the deletion of the CD4 gene (363). Analysis of the CFSE profiles of memory CD8 T cells adoptively transferred into naïve CD4 -/- recipients showed that the homeostatic turnover of memory cells was unaffected by the absence of CD4 T cell help. Furthermore as observed in WT recipient mice, the transferred memory CD8 T cells in CD4 -/- animals were stably maintained in their total number and retained both their phenotypic expression and functions for the duration of the experiments, suggesting that CD4 T cell help is not required for the long-term maintenance of memory CD8 T cells, both in their quantity and in their quality.

A potential problem with the use of CD4-/- mice is that the CD8 T cell population in these animals has been shown to be contaminated with MHCII-restricted T cells that may substitute for the traditional helper CD4 T cells (337, 364, 365). Therefore, to circumvent this potential problem, we adoptively transferred memory CD8 T cells into uninfected MHC II -/- mice, which also lack CD4 T cells (340). We observed that even in these recipient animals, the donor memory CD8 T cells exhibited homeostatic turnover comparable to that observed in WT and CD4 -/- recipients. However, in contrast with the memory cells transferred into WT or CD4-/- animals, donor memory CD8 T cells slowly declined in their number when transferred into naive MHCII-/- mice. Interestingly, however, both the phenotype of the donor memory cells and the ability to respond to

secondary challenges remained unchanged, supporting the earlier findings from CD4 -/- animals.

It is interesting to speculate on the potential causes for the discrepancy observed in the long-term maintenance of memory CD8 T cells in regard to their total number in CD4 -/- and MHCII -/- animals. As described earlier, several studies have reported that the CD8 T cell population in CD4 -/- mice is contaminated with MHCII-restricted T cells that could behave like traditional helper CD4 T cells (337, 364, 365); therefore, the absence of impairment in the persistence of memory cells in CD4 -/- animals could be due to the help provided by these nontraditional helper T cells. On the other hand, MHC class II molecule is expressed on various cell types (e.g. thymic epithelium, B cells, macrophages, and dendritic cells) and therefore, the observed loss of donor memory CD8 T cells in these animals could be more due to the intrinsic quality of these mice rather than merely due to the absence of CD4 T cells (340). In support, MHCII -/- animals have been shown to spontaneously develop various immune disorders, such as pancreatitis and inflammatory bowel disease (339, 366). Further work will be required to better address this.

In conclusion, our study provides both a much-needed rigorous quantitative analysis of the homeostatic turnover of memory CD8 T cells and an assessment of the requirement for CD4 T cell help during the memory maintenance phase of CD8 T cell immune response. This study will be of great benefit in better understanding the regulations of memory CD8 T cell maintenance and sets the stage for future studies to address how different cytokines (e.g. IL-7 and IL-15) and various niches (e.g. bone marrow) influence this stochastic turnover of memory CD8 T cells.

Materials and Methods

Virus Infection

LCMV Armstrong and recombinant vaccinia virus expressing the GP33 epitope of LCMV (VV-GP33) were propagated, titered and used as previously described (351, 352).

Mice

Six to 8-week old female C57BL/6 (B6), BALB/c, CD4 -/-, and MHCII -/- mice were purchased from Jackson Laboratories (Bar Harbor, ME) or Taconic Farms (Germantown, NY). Thy1.1^{+} P14 transgenic mice with T cells expressing the T cell receptor specific for the D^{b} GP33-41 epitope of LCMV were obtained from Jackson Laboratories and backcrossed to B6 mice in our colony (47). LCMV immune WT P14 chimeric mice were generated by adoptively transferring 1 x 10^{5} naïve Thy1.1^{+} P14 CD8 T cells into congenic B6 mice and subsequently infecting these animals with 2 x 10^{5} pfu of LCMV-Armstrong. For secondary challenge experiments, 3 x 10^{4} immune Thy1.1^{+} P14 CD8 T cells were adoptively transferred into naïve B6 mice and infected with 5 x 10^{6} pfu VV-GP33. All mice were used in accordance with NIH and the Emory University Institutional Animal Care and Use Committee guidelines.

Lymphocyte Isolation and Purification

Single-cell suspensions were prepared from the spleen and from the brachial, inguinal, and mesenteric lymph nodes. Bone marrow was obtained by flushing two femurs with cold RPMI 1640. Total number of cells in bone marrow was calculated as follows: # of cells in two femurs x 7.9 (367). Lymphocytes from blood and liver were obtained as

described in (132). Memory P14 CD8 T cells were purified using anti-CD8 MACS magnetic beads and columns (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of MACS-purified samples was >90%.

CFSE Labeling and Adoptive Transfer

LCMV-specific memory CD8 T cells were labeled with CFSE (Molecular Probes) by incubating at 5 mM in PBS, quenching with FCS, and washing as described previously (353). Approximately 1 x 10^6 memory P14 CD8 T cells or 20-50 x 10^6 total LCMV immune splenocytes (from B6 or Balb/c) were adoptively transferred intravenously (i.v.) into naïve recipient mice.

Cell Surface and Intracellular Staining

All antibodies were purchased from BD biosciences (San Diego, CA), except for anti-mouse IL-7Ra, which was purchased from eBiosciences (San Diego, CA). Cells were stained for surface proteins and intracellular proteins and cytokines as described previously (46).

Preparation of H-2D^b and H-2L^d Tetramers

Tetramers of D^b containing NP396-404 and GP33-41 and L^d containing NP118-126 to quantify CD8 T cells specific for these LCMV epitopes were prepared as previously described (53).

Calculations and Mathematical Modeling

The mean number of divisions and variance in the number of divisions of memory CD8 T cells from the CFSE data was calculated as follows. If $f_n(t)$ equals the fraction of cells having undergone n divisions at time t, then the mean and variance in the number of divisions are given by $\mu(t) = \sum_{n=0}^{\infty} n f_n(t)$, and $\sigma^2(t) = \sum_{n=0}^{\infty} f_n(t)(n - \mu(t))^2$ respectively.

Frequencies rather than absolute numbers were used because the average number of memory CD8 T cells remains constant over time and thus, the measurement of frequencies is more accurate than that of total number of cells.

For a stochastic model of division it can be shown that the frequency of cells with n divisions follows a Poisson distribution. See (361) for details. If we assume both division and death occur at random (i.e. a random birth-death model; RBD), then the number and the frequency of cells with n divisions, x_n, and f_n are given by

$$x_n = f_n e^{(\lambda - d)t} \text{ and } f_n = \frac{(2\lambda t)^n e^{-(2\lambda t)}}{n!},$$

where λ and d are the rate constants for division and death (per unit time). We see that the frequency of cells with n divisions, f_n, follows a Poisson distribution with the mean number of divisions and variance in the number of divisions increasing at rate 2λ.

Figure Legends

Figure 1. Longitudinal analysis of the long-term maintenance of donor memory CD8 T cells. **A and C.** Maintenance of donor memory CD8 T cells in WT recipient mice shown as percentage of total peripheral blood at different time points post-transfer. Data shown are 3 representative experiments out of 7 different experiments in (A) and n=10 in (C). **B.** CFSE profile of the homeostatic turnover of donor memory CD8 T cells maintained in WT recipients at various time points post-transfer. Number shown is the percentage of donor cells in each division. Data shown are 3 representative experiments out of 7 different experiments.

Figure 2. Quantitative analysis of the homeostatic turnover of donor memory CD8 T cells. **A.** Change in the frequency of undivided donor memory CD8 T cells at different time points post-transfer. Solid line represents the best regression line. **B.** Change in both the mean number of divisions and the variance in the number of divisions for donor memory CD8 T cells at different time points post-transfer. Solid lines represent the best regression line for the mean (black) and for the variance (red). **C.** Plot of the CFSE distribution of donor memory CD8 T cell turnover for different time points post-transfer. Solid lines represent the values predicted from a Poisson distribution for days 14 (black), 28 (red), and 60 (green) post-transfer. All data shown are representative of 7 different experiments.

Figure 3. Comparison of the homeostatic turnover of memory CD8 T cells specific for different epitopes of LCMV. **A.** CFSE profile of the homeostatic turnover of donor

naïve ($CD8^{+}CD44^{lo}$) and donor memory ($CD8^{+}CD44^{hi}$ and D^{b} NP396-404^{+}) CD8 T cells at day 21 or day 60 post-transfer. Number shown is the percentage of donor cells in each division. **B.** CFSE distribution of the homeostatic proliferation of L^{d} NP118-126^{+} donor memory CD8 T cells at several time points post-transfer. Number shown is the percentage of donor cells in each division.

Figure 4. Analysis of the role of CD4 T cell help in both the long-term maintenance and the homeostatic turnover of LCMV-specific memory CD8 T cells. **A.** Experimental setup. Fully functional (e.g. $CD127^{hi}$, $CD62L^{hi}$, $Bcl\text{-}2^{hi}$, and IL-2 producing) memory P14 CD8 T cells were purified from WT LCMV immune mice (>60 days post-infection), labeled in vitro with CFSE, and adoptively transferred into either naïve WT, CD4 -/-, or MHCII -/- animals. At different time points post-transfer, individual mice were bled and/or sacrificed, and both the maintenance and the turnover of the donor memory cells were longitudinally assessed. **B.** Longitudinal analysis of the maintenance of the donor memory CD8 T cells in WT (black), CD4 -/- (red), and MHCII -/- (green) animals. Data is shown as a percentage of the total peripheral blood. n=10. **C and D.** Comparison of the percentage (C) and the total number (D) of donor memory CD8 T cells in different peripheral tissues of WT, CD4 -/-, and MHCII -/- animals approximately 60 days post-transfer. Data shown are representative of 5 different experiments. **E.** CFSE profiles of the homeostatic turnover of donor memory CD8 T cells in the peripheral blood of WT, CD4 -/-, and MHCII -/- mice for different time points post-transfer. The numbers shown are the percentage of donor cells in each division. Data shown are representative of 5 different experiments. **F.** Change in the mean number of divisions of donor memory

CD8 T cells at different time points after adoptive transfer. Solid lines represent the best regression lines for the mean in WT (black), CD4 -/- (red), and MHCII -/- animals (green). n=10. G. Plot of the CFSE distribution of donor memory CD8 T cell turnover for different time points post-transfer. Solid lines represent the values predicted from a Poisson distribution for days 14 (black), 21 (red), 35 (green), 48 (blue) and day 60 (light blue) post-transfer in the WT, CD4-/- and MHCII-/- mice shown in Panel A.

Figure 5. Assessment of the role of CD4 T cell help in maintaining the overall quality of fully functional memory CD8 T cells. Recipient WT, CD4 -/-, and MHCII -/- animals were sacrificed at >60 days post initial transfer and both the phenotypic profile (**A**) and cytokine production, after in vitro stimulation with LCMV GP33 peptide (**B**), of the donor memory CD8 T cells were determined. Number shown in (B) is the percent of IFN-γ^+ donor memory CD8 T cells also producing TNF-α or IL-2. Data shown are representative of 5 different experiments. **C.** Experimental setup for recall analysis. At least 60 days post-transfer, recipient WT, CD4 -/-, and MHCII -/- animals were sacrificed and the original donor memory CD8 T cells were once again isolated, purified, and equal number of cells were adoptively transferred into new WT recipients. These recipient animals were then challenged with VV-GP33 and the recall response of the donor memory cells was assessed. Both the percentage in the peripheral blood (**D)** and the total number of the responding donor cells in the spleen (**E**) were assessed approximately 5 days post-challenge (n=3). Data shown are representative of 5 different experiments.

Figure 1.

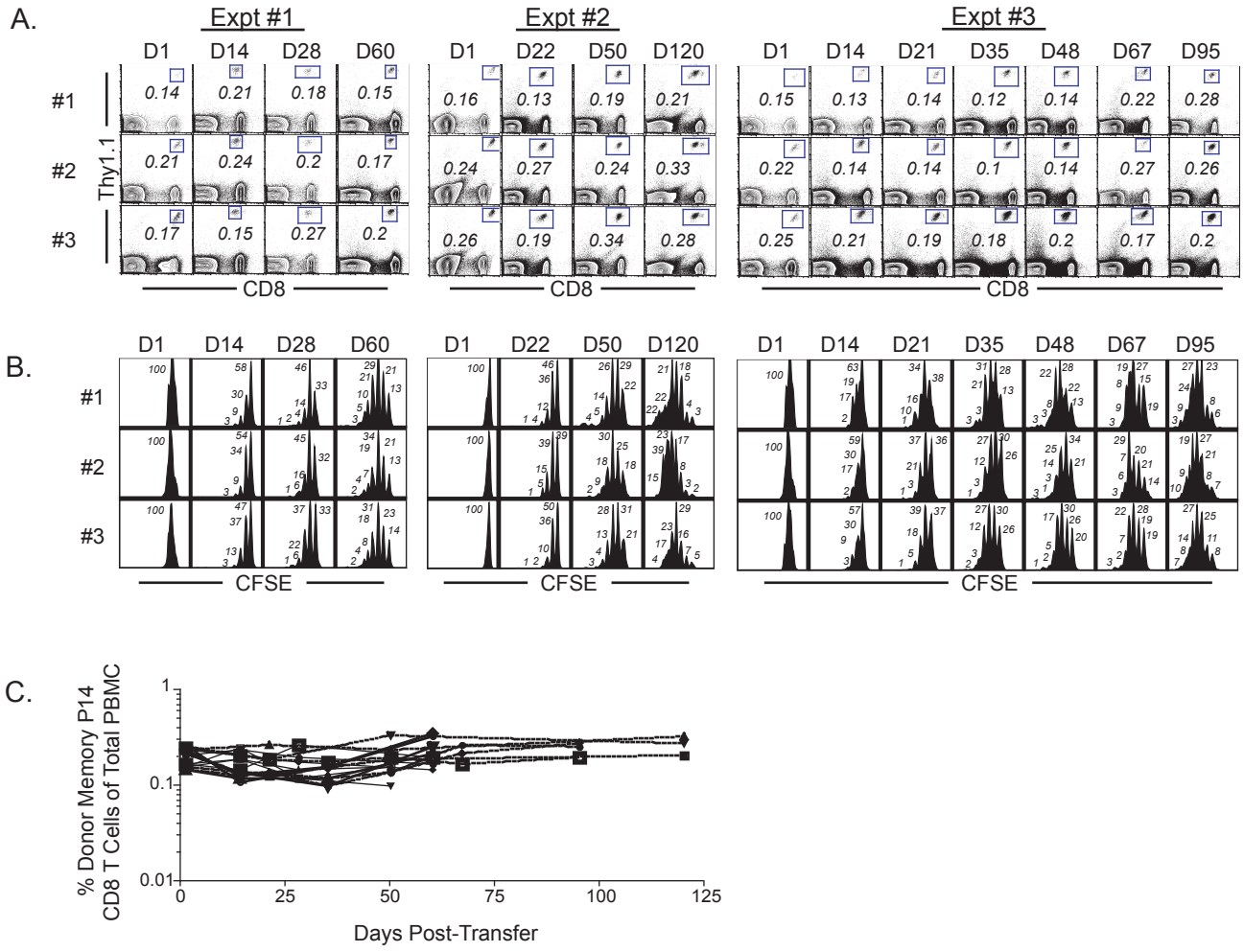

A.
Expt #1
Expt #2
Expt #3
D1 D14 D28 D60
D1 D22 D50 D120
D1 D14 D21 D35 D48 D67 D95
#1
#2
#3
Thy1.1
CD8
B.
CFSE
C.
% Donor Memory P14
CD8 T Cells of Total PBMC
Days Post-Transfer
1
0.1
0.01
0
25
50
75
100
125

Figure 2.

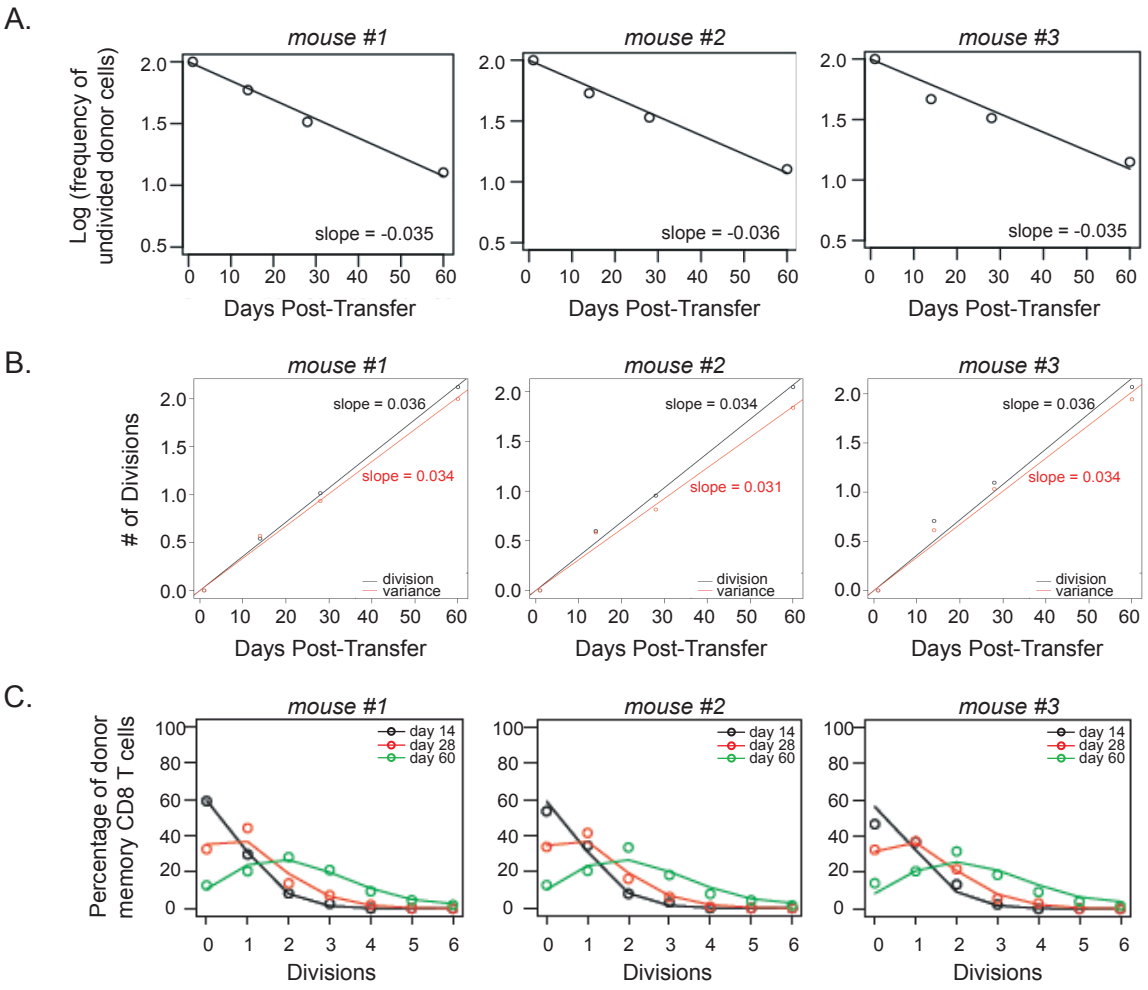

A.
mouse #1
mouse #2
mouse #3
Log (frequency of undivided donor cells)
slope = -0.035
slope = -0.036
slope = -0.035
Days Post-Transfer
B.
# of Divisions
slope = 0.036
slope = 0.034
slope = 0.034
slope = 0.031
slope = 0.036
slope = 0.034
division
variance
C.
Percentage of donor memory CD8 T cells
day 14
day 28
day 60
Divisions

Figure 3.

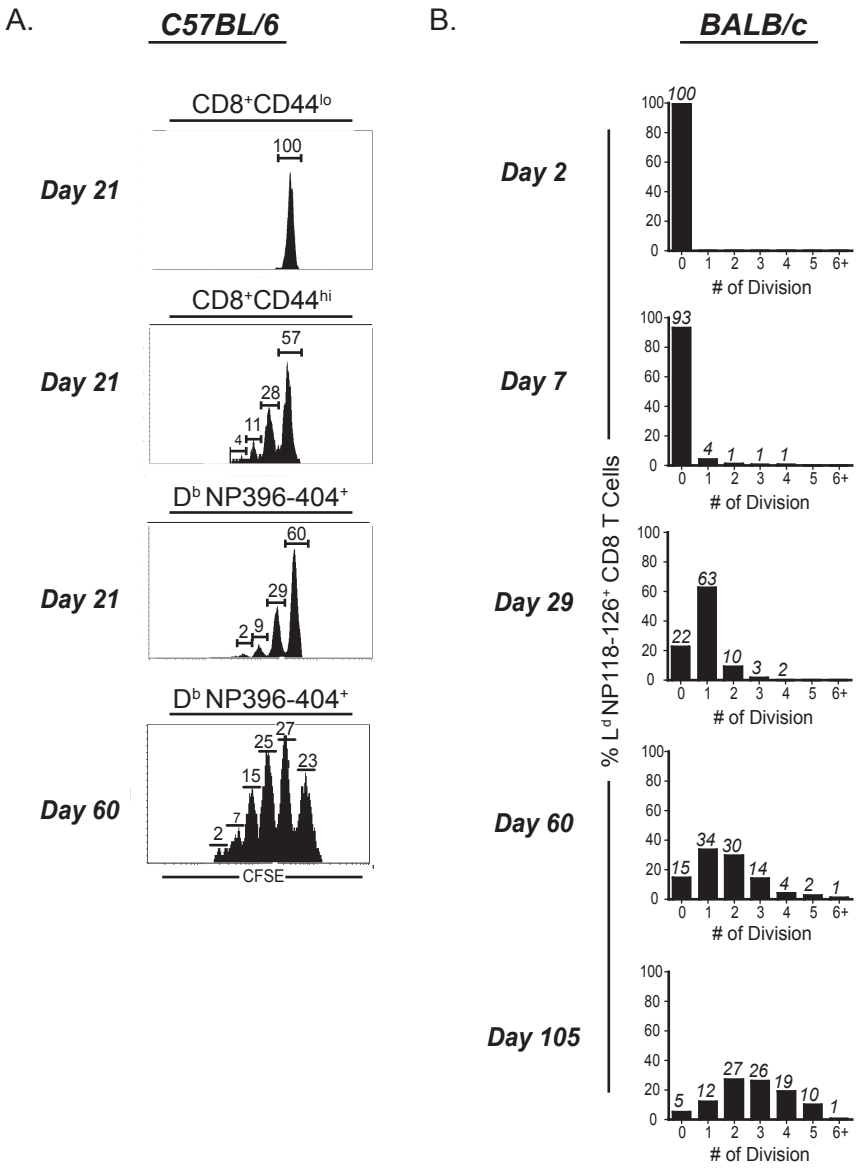

A.
C57BL/6
B.
BALB/c
CD8+CD44lo
CD8+CD44hi
Db NP396-404+
Db NP396-404+
Day 21
Day 21
Day 21
Day 60
CFSE
100
57
28
11
4
60
29
9
2
27
25
23
15
7
2
Day 2
Day 7
Day 29
Day 60
Day 105
% Ld NP118-126+ CD8 T Cells
# of Division
100
93
4
1
1
1
63
22
10
3
2
34
30
15
14
4
2
1
27
26
19
12
10
5
1
0 1 2 3 4 5 6+

Figure 4.

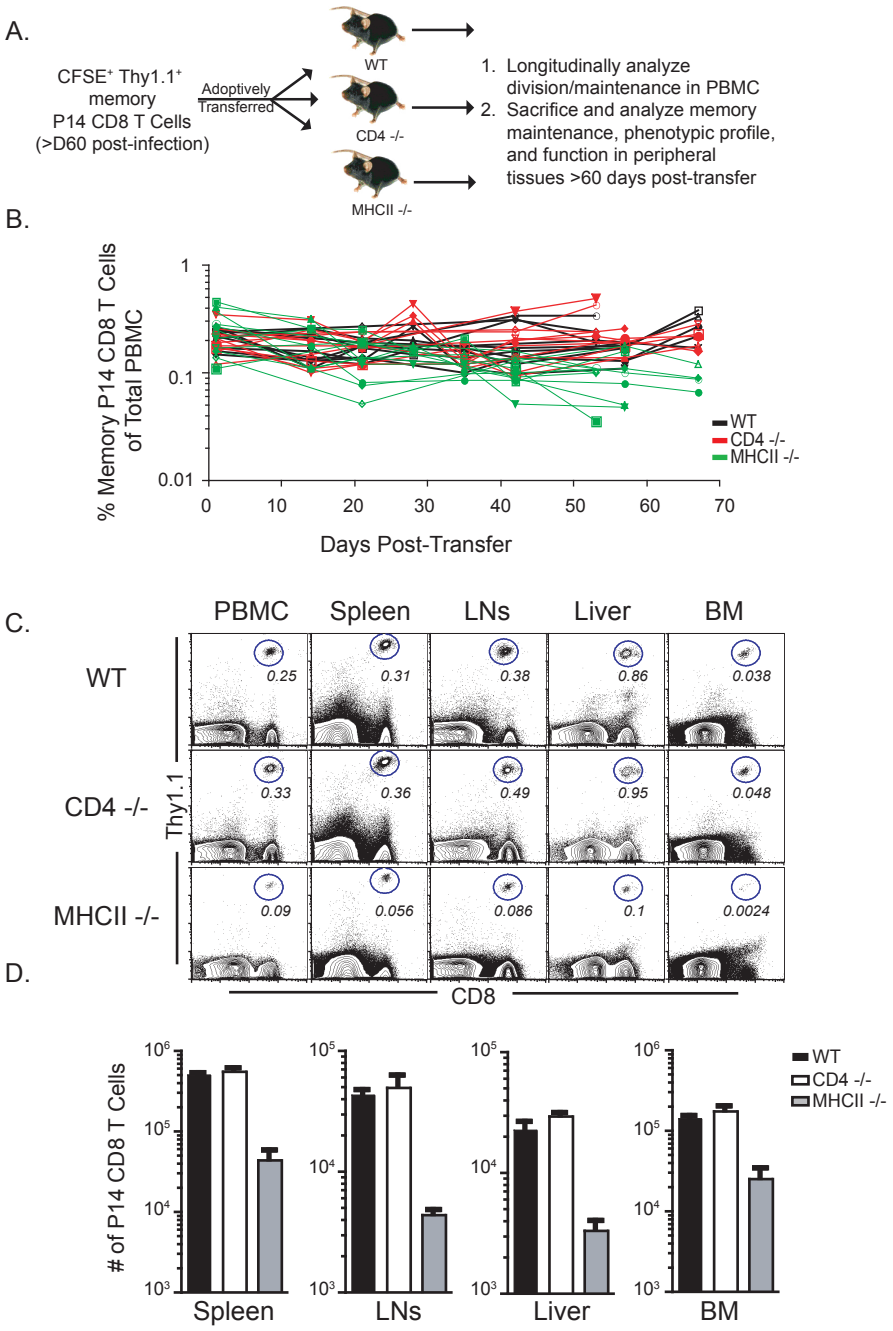

A.
CFSE+ Thy1.1+ memory P14 CD8 T Cells (>D60 post-infection)
Adoptively Transferred
WT
CD4 -/-
MHCII -/-
1. Longitudinally analyze division/maintenance in PBMC
2. Sacrifice and analyze memory maintenance, phenotypic profile, and function in peripheral tissues >60 days post-transfer
B.
% Memory P14 CD8 T Cells of Total PBMC
Days Post-Transfer
WT
CD4 -/-
MHCII -/-
C.
PBMC
Spleen
LNs
Liver
BM
WT
CD4 -/-
MHCII -/-
Thy1.1
CD8
0.25
0.31
0.38
0.86
0.038
0.33
0.36
0.49
0.95
0.048
0.09
0.056
0.086
0.1
0.0024
D.
# of P14 CD8 T Cells
Spleen
LNs
Liver
BM
WT
CD4 -/-
MHCII -/-

Figure 4.

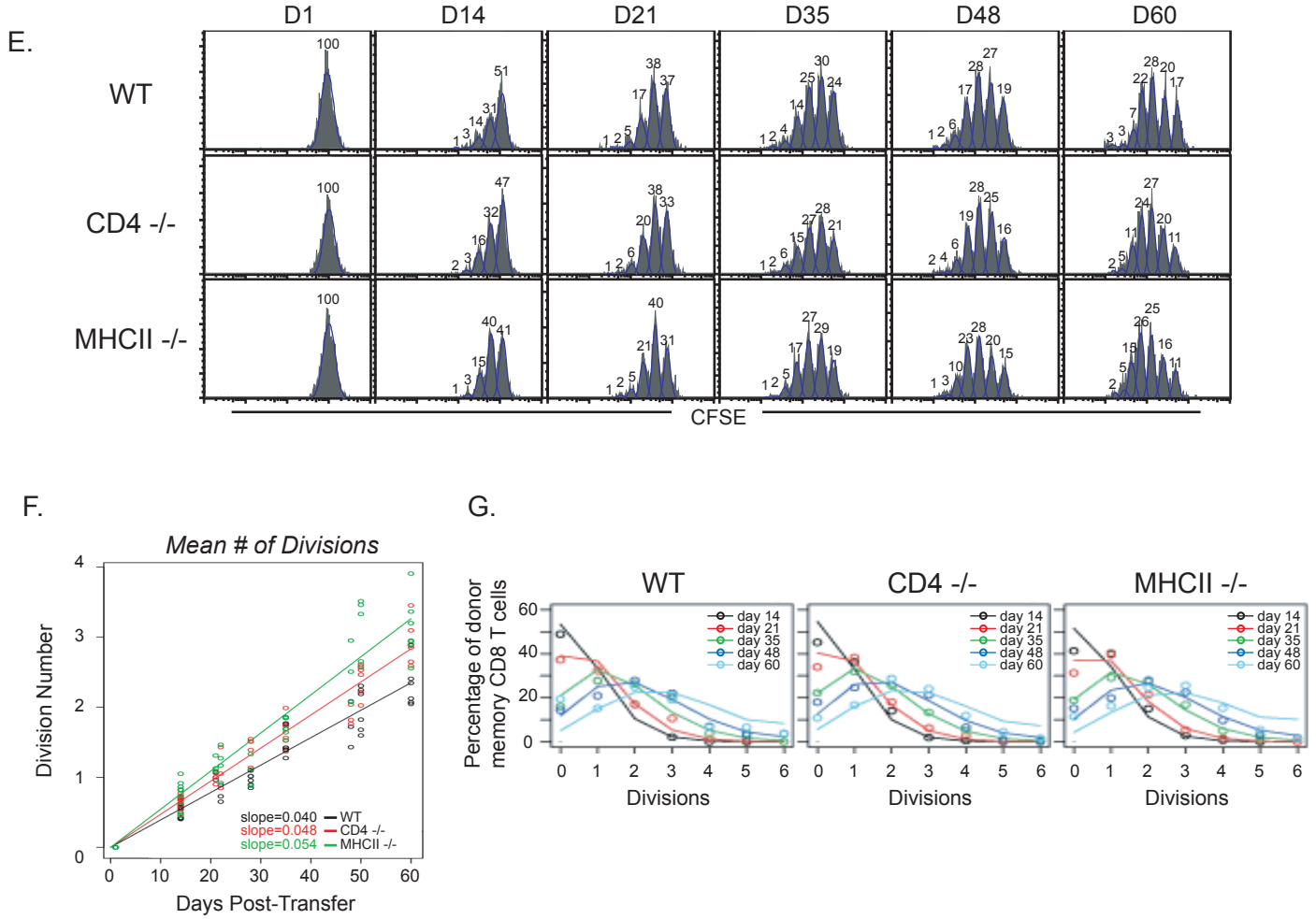
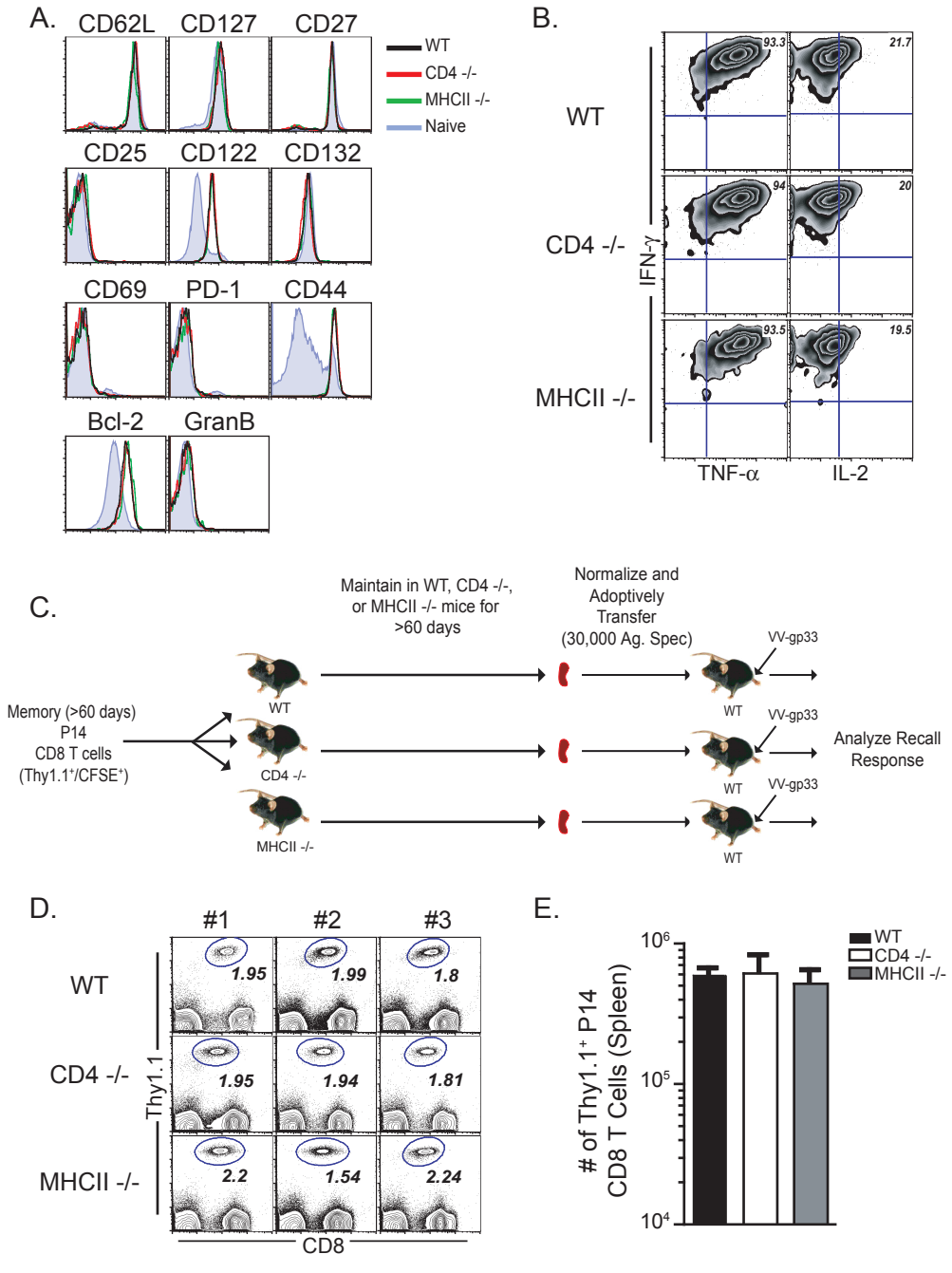

E.
D1
D14
D21
D35
D48
D60
WT
CD4 -/-
MHCII -/-
CFSE
F.
Mean # of Divisions
Division Number
Days Post-Transfer
slope=0.040 WT
slope=0.048 CD4 -/-
slope=0.054 MHCII -/-
G.
WT
CD4 -/-
MHCII -/-
Percentage of donor memory CD8 T cells
Divisions
day 14
day 21
day 35
day 48
day 60

Figure 5.


A.
CD62L
CD127
CD27
CD25
CD122
CD132
CD69
PD-1
CD44
Bcl-2
GranB
WT
CD4 -/-
MHCII -/-
Naive
B.
WT
CD4 -/-
MHCII -/-
IFN-γ
TNF-α
IL-2
93.3
21.7
94
20
93.5
19.5
C.
Maintain in WT, CD4 -/-, or MHCII -/- mice for >60 days
Normalize and Adoptively Transfer (30,000 Ag. Spec)
Memory (>60 days) P14 CD8 T cells (Thy1.1+/CFSE+)
WT
CD4 -/-
MHCII -/-
VV-gp33
Analyze Recall Response
D.
#1
#2
#3
WT
CD4 -/-
MHCII -/-
Thy1.1
CD8
1.95
1.99
1.8
1.95
1.94
1.81
2.2
1.54
2.24
E.
# of Thy1.1+ P14 CD8 T Cells (Spleen)
10^6
10^5
10^4
WT
CD4 -/-
MHCII -/-

Chapter 4: Utilizing an immunosuppressant drug FTY720 to study the migratory properties of virus-specific effector and memory CD8 T cells

Abstract

A hallmark of the adaptive immune system is its ability to mount a much more rapid and potent immune response upon re-exposure to the same antigen in vivo. This enhanced protection is partly the result of both quantitative and qualitative changes that occur as naïve antigen-specific CD8 T cells develop into memory CD8 T cells. Hence, many previous studies have focused on the migratory properties of naïve, effector, and memory CD8 T cells to help identify the mechanisms by which CD8 T cells are able to confer protective immunity. Despite much progress made through these studies, there remain several questions pertaining to CD8 T cell trafficking that have yet to be clearly addressed. For example, although the mechanism by which CD8 T cells enter the lymph nodes (LNs) has been well characterized, the requirements for the egress is less well understood. Furthermore, there is still much debate as to whether memory CD8 T cells continue to re-circulate or are resident within different tissues after their differentiation from effector cells. In this study, we utilized an immunosuppressant drug FTY720 (known ligand of the sphingosine-1-phosphate receptors) as a tool to study CD8 T cell trafficking after acute LCMV infection. We observed that the egress of LCMV-specific effector CD8 T cells, unlike that of their naïve predecessors, occurs independent of sphingosine-1-phosphate signaling. The number of effector T cells in the peripheral tissues of FTY720 treated animals did not differ from the control group at 8 days post LCMV infection. These LCMV-specific cells were not primed in other tissues, where the

egress is less dependent on sphingosine-1-phosphate signaling (e.g. spleen), as experiments with splenectomized mice resulted in similar findings. In addition, we also observed that FTY720 treatment of immune mice results in the sequestration of only a fraction of the total pool of LCMV-specific memory CD8 T cells within the LNs suggesting that memory CD8 T cell population consists of both circulatory and tissue-resident cells. These trafficking properties of CD8 T cells should hopefully provide more insight into the mechanisms by which memory cells confer protective immunity in the context of an acute infection.

Introduction

The ultimate goal of an effective adaptive cellular immune response toward an infection/immunization is to generate long-lived memory CD8 T cells that respond rapidly upon secondary exposure and thereby, provide protective immunity for the host (4). The successful induction of this immune response is critically dependent on the migratory properties of naïve, effector, and memory CD8 T cells. For example, naïve CD8 T cells continuously circulate through the secondary lymphoid tissues (e.g. spleen and LNs) from peripheral blood in search of dendritic cells (DCs) presenting their specific antigen (29, 30). This migration of naïve CD8 T cells in and out of different secondary lymphoid tissues is critical for the induction of an effective immune response. Otherwise, both the migratory limitations (not known to traffic through most common sites of infection – e.g. skin and mucosal tissues) and the low precursor frequencies (approximately 100-200 CD8 T cells specific for a given viral epitope in an entire mouse) of these cells limit the probability of their activation (15, 31, 32). Along the same line, effector CD8 T cells express a unique set of tissue-specific homing receptors (e.g. $\alpha 4\beta 7$ and CCR9 – homing to the mucosal tissues) that allows them to migrate to various peripheral tissues and clear antigen from the host (16). This migratory property of effector cells is also essential for the induction of protective immunity as impaired antigen clearance results in defective memory CD8 T cell development (78). Lastly, upon complete antigen clearance, a subpopulation of effector CD8 T cells in the peripheral tissues differentiates into long-lived memory CD8 T cells. These memory cells are intrinsically more efficient in terms of their responsiveness compared to naïve cells and continue to patrol the peripheral tissues (e.g. intraepithelial lymphocytes, IELs –

gut mucosa) long after antigen clearance (6, 8, 12). This migratory property of memory CD8 T cells is an essential part of their ability to confer protective immunity against secondary challenges. Hence, there has been much interest over the years in better identifying the various factors involved in CD8 T cell trafficking through different lymphoid and non-lymphoid tissues in the hopes that this understanding would help in better understanding the mechanism by which memory CD8 T cells both are generated and confer protective immunity in the context of an acute infection/immunization.

Numerous past studies have successfully identified the requirements for the trafficking of CD8 T cells into the secondary lymphoid tissues. For example, the entry into the LNs occurs primarily via the high endothelial venules (HEVs) and involves a three-step process (rolling, tight adhesion, and transmigration) that is tightly regulated by a family of selectins, integrins, and chemokines (368). Entry into the spleen, on the other hand, occurs independently of the HEVs, but integrins and chemokines continue to play essential roles as in their absence, CD8 T cells fail to accumulate within the white pulp (T and B cell region) (232). Although this has been known for some time, the mechanism by which CD8 T cells exit the secondary lymphoid tissues and migrate back out into the peripheral blood has only now begun to be more carefully elucidated. Recent experiments with an immunosuppressant drug FTY720 have implicated sphingosine-1-phosphate (S1P) signaling as being crucial for the egress of CD8 T cells from the peripheral LNs.

FTY720, 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride, is an immunosuppressant drug chemically derived from fungal metabolite (myriocin/ISP-1) extracted from an herb (Iscaria sinclarii) commonly used by the Chinese for its anti-aging

effects (248). This drug has been shown to be effective in both reducing allograft transplant rejections and onset of many autoimmune diseases in both animals and humans (250). Unlike the more traditional immunosuppressive drugs cyclosporin A and tacrolimus, FTY720 induces its immunosuppressive effects without interfering T lymphocyte activation (251, 252). Initial studies had suggested that FTY720 mediated apoptosis of lymphocytes, but subsequent experiments demonstrated that the blood concentration of FTY720 used in vivo for therapeutic purposes was far less than the amount required to induce apoptosis in vitro (250, 254, 256). Furthermore, experiments have shown that FTY720 acts as an agonist on 4 of the 5 S1P receptors (1, 3, 4, and 5) and induces both the down-regulation and inactivation of the different S1P receptors upon binding to them (262, 263, 296). For example, CD8 T cells isolated from mice treated with FTY720 no longer display chemotactic responsiveness toward S1P in vitro. Therefore, it has been postulated that FTY720 induces its immunosuppressive effects by down-regulating the expression of S1P receptors (primarily S1P receptor 1 – $S1P_1$) and blocking the egress of CD8 T cells from the secondary lymphoid tissues. Adoptive transfer experiments with $S1P_1$ -/- T cells further validated this finding and highlighted the importance of S1P signaling in the egress of lymphocytes from lymphoid tissues as these $S1P_1$ deficient cells accumulated in the secondary lymphoid organs and failed to exit into the periphery (294).

Despite this progress in understanding the different requirements for CD8 T cell trafficking through different tissues, there remain several questions that have yet to be clearly addressed. For example, although both naïve and various transplant-specific CD8 T cells require $S1P_1$ signaling to exit from the lymphoid tissues, it is not yet completely

known whether virus-specific CD8 T cells share this requirement. This would be of great interest to many clinicians as if FTY720 can selectively block the egress of different population of CD8 T cells, it may prove to be a much better immunosuppressive agent compared to either tacrolimus or cyclosporin A. Furthermore, as described earlier, we know that memory CD8 T cells (e.g. IELs) are critical for protective immunity toward secondary exposure by patrolling the peripheral tissues (e.g. mucosal tissues), but there is still much debate as to whether these memory cells do this by continuously re-circulating between the different lymphoid and non-lymphoid tissues or by being resident in different tissues.

In this study, we utilized FTY720 as a tool to study CD8 T cell trafficking after acute LCMV infection in normal mice. We observed that the egress of LCMV-specific effector CD8 T cells, unlike that of their naïve predecessors, occurs independently of S1P signaling. The number of effector T cells in the peripheral tissues of FTY720 treated animals did not drastically differ from the control group at 8 days post LCMV infection. These LCMV-specific cells were not primed in other tissues, where the egress is less dependent on sphingosine-1-phosphate signaling (e.g. spleen), as experiments with both splenectomized mice and influenza virus infection resulted in similar findings. In addition, we also observed that memory CD8 T cells within the gut epithelium do not re-circulate and are instead resident within the tissues. FTY720 treatment of immune mice resulted in the partial sequestration of LCMV-specific memory CD8 T cells from various tissues (e.g. peripheral blood, lung, and spleen) within the LNs, but we observed no significant loss in the number of memory CD8 T cells within the gut epithelium. These trafficking properties of CD8 T cells should hopefully provide more insight into the

mechanisms by which memory cells are both generated and able to confer protective immunity in the context of an acute infection.

Materials and Methods

Virus

LCMV Armstrong and Influenza virus expressing the D^b GP33-41 epitope of LCMV were propagated, titered, and used as previously described (351, 369).

Mice

C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Taconic Farms (Germantown, NY). Thy1.1$^+$ P14 transgenic mice with CD8 T cells expressing the TCR specific for the LCMV epitope D^b-GP33-41 were obtained from The Jackson Laboratory and backcrossed to B6 mice in our colony. Immune and effector P14 CD8 T cells were made by transferring 10^5 naïve Thy1.1$^+$ P14 CD8 T cells into B6 mice i.v. injection and subsequently infected with $2x10^5$ pfu of LCMV-Armstrong. For experiments addressing the role of the spleen as a source of effector CD8 T cells, these mice were infected with 10^3 PFU/nostril of recombinant influenza virus expressing the gp33 epitope of LCMV instead. All mice were used in accordance with NIH and the Emory University Institutional Animal Care and Use Committee guidelines.

Lymphocyte isolation, cell surface and intracellular staining

Lymphocytes were prepared from the blood, spleen, lung, gut epithelium, and bracial and inguinal lymph nodes as described (321). All antibodies were purchased from BD Biosciences (San Diego, CA), except for anti-mouse IL-7Rαantibodies from eBiosciences (San Diego, CA) and KLRG-1 antibodies from Southern Biotech (Birmingham, AL). Cells were stained for surface or intracellular proteins and cytokines

as described previously (161). For intracellular cytokine staining, splenocytes were stimulated with D^b GP33-41 peptide for 5 hours, fixed, permeabilized, and stained as described previously (143).

FTY720 treatment

Mice were exposed ad libetum to drinking water containing dissolved FTY720 at a concentration of 2 ug/mL.

Results/Discussions

Large numbers of LCMV-specific effector CD8 T cells are observed in the peripheral tissues after infection in FTY720 treated animals

The requirement of S1P signaling for the egress of naïve and alloantigen specific lymphocytes from the peripheral LNs has been clearly demonstrated as in vivo administration of FTY720 results in reversible sequestration of these lymphocytes within the LNs and away from the peripheral tissues (256). However, the extent to which infectious pathogen induced effector CD8 T cells rely on S1P signaling for their egress from the secondary lymphoid tissues has not been well characterized. To better address this, we first adoptively transferred naïve Thy1.1^{+} P14 CD8 T cells (specific for the D^{b} GP33-41 of LCMV) into congenic C57BL/6 mice (Thy1.2^{+}). Approximately a day later, these animals were treated with either water alone or with FTY720 and this treatment was continued until the end of the experiments. About 24-48 hours after the beginning of treatment, mice were infected with LCMV-Armstrong. These animals were then sacrificed at 7 days post-infection and both the distribution and the overall quality of the effector CD8 T cells in different tissues was assessed (Fig 1a). Our experimental setup provides an ideal opportunity to directly assess the efficacy of FTY720 treatment as both naïve and LCMV-specific effector CD8 T cells can be tracked within a single animal as the two populations of cells are distinguishable based on their expression of Thy1.1, CD44, and CD62L – naïve CD8 T cells: Thy1.1^{-}, CD44^{lo}, CD62L^{hi}; LCMV-specific effector CD8 T cells: Thy1.1^{+}. In agreement with previous findings (250), in vivo administration of FTY720 resulted in the significant loss of naïve CD8 T cells from the peripheral blood as observed in Fig 1b. In animals treated with water alone, naïve CD8 T

cells constituted nearly 50% of the endogenous (Thy1.1$^-$) CD8 T cells in the peripheral blood. However, the percentage of naïve CD8 T cells in the peripheral blood of FTY720 treated was less than 1% of the endogenous CD8 T cell population. This loss in the peripheral blood (>300 fold) correlated with a significant increase in total number of naïve CD8 T cells in the LNs confirming that the in vivo administration of FTY720 effectively blocks the egress of naïve cells from the peripheral LNs (Fig 1c).

Surprisingly, the effect of FTY720 treatment on the egress of LCMV-specific effector CD8 T cells from peripheral LNs was not nearly as dramatic as compared to naïve CD8 T cells. At 7 days post-infection, we observed a significant population of Thy1.1$^+$ effector P14 CD8 T cells in all tissues examined (peripheral blood, spleen, LNs, lung, and gut/IELs) in mice treated with FTY720 that was comparable to that observed in animals of the control group (Fig 1d and e). However, we did observe a modest decrease (~2 fold) in the total number of effector P14 CD8 T cells in the peripheral blood with a corresponding increase (~3 fold) in the LNs of FTY720 treated animals compared to those animals that were given water alone.

We next compared both the phenotypic expression and the function of these LCMV-specific effector CD8 T cells in the hopes of understanding why a subset of effector CD8 T cells are able to emigrate from the LNs in the absence of S1P signaling whereas others can not. We initially hypothesized that this dependence on S1P signaling may be linked to the overall activation state of the CD8 T cells. Those that had seen antigen or were exposed to inflammation for a longer duration are known to preferentially differentiate into terminal effector cells and perhaps these cells are less dependent on S1P signaling for their egress from the LNs (143, 161). However, we

observed no apparent differences for all phenotypic markers assessed (e.g. CD127, CD62L, Granzyme B, CD27, KLRG-1, PD-1 – Fig 1f; CD25, CD122, CD69, Bcl-2 – data not shown). In addition, LCMV-specific effector CD8 T cells from either of the groups efficiently produced both IFN-γ and TNF-α after in vitro peptide stimulation. All in all, our data suggest that a subset of LCMV-specific effector CD8 T cells does not rely on S1P signaling for their egress from the LNs. It would be interesting in future studies to assess whether there are any inherent differences at the genomic level between these two populations of LCMV-specific effector CD8 T cells.

The egress of LCMV-specific effector CD8 T cells from the LNs can occur in the absence of sphingosine-1-phosphate signaling

An obvious alternate explanation for the presence of LCMV-specific effector CD8 T cells in the peripheral tissues (e.g. peripheral blood, lung, and IELs) of FTY720 treated animals is that these cells had been primed and had emigrated from a tissue where the egress is less dependent on S1P signaling. Along this line, the spleen is a major site of CD8 T cell activation for many systemic infections (e.g. LCMV) (29). Furthermore, the requirement for S1P signaling in the egress of CD8 T cells from the spleen remains controversial (263). Therefore, it may be possible that the LCMV-specific effector CD8 T cells observed in the peripheral tissues of FTY720 treated animals originated from the spleen rather than from the LNs. To address this, we adoptively transferred naïve Thy1.1^{+} P14 CD8 T cells into C57BL/6 mice and began FTY720 treatment as described previously in Fig 1a. However, in this experiment, we infected the animals via intranasal with influenza virus expressing the D^{b} GP33-41 epitope of LCMV rather than with

LCMV-Armstrong as done previously. Intranasal infection of mice with influenza virus results in a very localized immune response with great majority of the CD8 T cell activation occurring within the medistinal LNs.

As observed in earlier experiments with LCMV-Armstrong, large numbers of effector P14 CD8 T cells were observed in all tissues examined (peripheral blood, medistinal LNs, spleen, lung, and IELs) in FTY720 treated animals after influenza virus infection (Fig 2a and b) suggesting that majority of the effector CD8 T cells in the peripheral tissues (e.g. peripheral blood, lung, and IEL) of FTY720 treated animals did not originate from the spleen. This was further validated with splenectomized animals as we also observed a significant number of effector P14 CD8 T cells in the different peripheral tissues of these splenectomized animals treated with FTY720 at 7 days post LCMV-Armstrong infection (Fig 2c). As noted earlier, when the total number of cells was calculated for the different tissues, both in the influenza and in the splenectomy experiments, we did observe a noticeable decrease (influenza expt: ~3.5 fold; splenectomized expt: ~2 fold) in the number of effector P14 CD8 T cells in the peripheral blood of mice treated with FTY720 compared to that observed in control animals. This decrease correlated with an increased accumulation of effector cells within the peripheral LNs suggesting that the dependence on S1P signaling for egress is not uniform among all LCMV-specific effector CD8 T cells. Overall, our data validate our earlier observation that a significant population of effector P14 CD8 T cells can exit from the peripheral LNs in the absence of sphingosine-1-phosphate signaling.

Overall pool of LCMV-specific memory CD8 T cells consists of both re-circulating and tissue resident memory cells

Upon antigen clearance, approximately 90-95% of effector CD8 T cells undergo contraction resulting in a small population of long-lived memory CD8 T cells that behave as sentinels in the peripheral tissues and thus, providing the host with protective immunity against secondary exposure (5). However, it still remains a matter of debate as to whether these memory CD8 T cells do so by continuously re-circulating between blood, lymphoid tissues (e.g. LNs), and non-lymphoid tissues (e.g. mucosal epithelium and lung) or whether by remaining resident within the different tissues. To begin addressing this question, we treated LCMV immune P14 chimeric animals (>60 days post-infection) with FTY720 for approximately 2 days and assessed whether this treatment induced a loss of memory CD8 T cells within the different peripheral tissues. As observed in Fig 3a, FTY720 treatment did result in the loss of memory CD8 T cells in the peripheral blood (~6 fold) and in the lung (~2 fold) with a reciprocal increase in the LNs suggesting that a significant population of memory CD8 T cells in the different peripheral tissues continuously re-circulates and FTY720 treatment efficiently blocked their egress from the LNs. Interestingly, however, this loss of memory CD8 T cells from the peripheral tissues was not complete (as seen with naïve CD8 T cells) as we observed large numbers of memory P14 CD8 T cells both in the peripheral blood and in the lung. Within the gut epithelium, we actually observed no significant loss in memory P14 CD8 T cells with FTY720 treatment. This was not due to the duration of the treatment as prolonging FTY720 treatment to 30 days continuously did not result in the loss of these memory cells from the different tissues (Fig 3b) suggesting that these LCMV-specific

memory CD8 T cells do not re-circulate via the afferent lymphatic system and are more likely resident within these tissues. It should be noted that it may be possible that the subset of memory CD8 T cells that are found in the peripheral tissues of FTY720 treated animals, in fact, are re-circulating via the afferent lymphatic system, but are not sequestered in the LNs after FTY720 treatment because they are not dependent on S1P signaling, as observed with effector CD8 T cells. However, recent experiments by Masopust et al. demonstrate that there are memory CD8 T cells (e.g. within the gut epithelium) that do not re-circulate and are resident within the tissues (321). Further experiments will need to better address this in other non-lymphoid tissues (e.g. lung, liver, skin).

Overall our data provide evidence that CD8 T cells are highly mobile and that the migratory properties vary depending on their differentiation status. Naïve CD8 T cells migrate primarily between the peripheral blood and the lymphoid tissues and their egress from the LNs is highly dependent on S1P signaling. Effector CD8 T cells are capable of migrating to many different lymphoid and non-lymphoid tissues and they seem to be less dependent on S1P signaling for their egress from the LNs. The overall population of memory CD8 T cells consists of both tissue-resident and continuously circulating cells that collectively work to confer protective immunity to the host.

Figure Legends

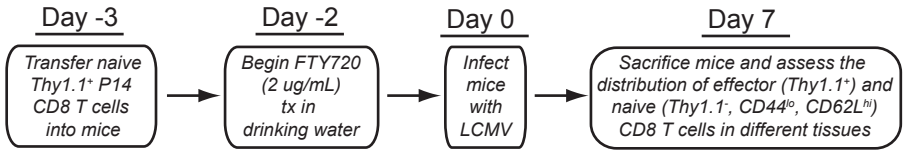
Figure 1. Large numbers of LCMV-specific effector CD8 T cells are observed in the peripheral tissues after infection in FTY720 treated animals. A. Experimental setup. Naïve Thy1.1^{+} P14 CD8 T cells were adoptively transferred into C57BL/6 animals. Approximately a day later, mice were given either water alone (control) or FTY720 in their drinking water. Mice were then infected with LCMV-Armstrong and at day 7 post-infection, the distribution of effector and naïve CD8 T cells in different tissues was assessed. B and C. Comparison of the percentage (B) and of the absolute number (C) of naïve CD8 T cells in different tissues. Data shown are gated on Thy1.1^{-} CD8 T cells. The percent of CD44^{lo} and CD62L^{hi} CD8 T cells is shown in (B). n=6 in (C). D and E. Comparison of the distribution of LCMV-specific effector CD8 T cells in different tissues of FTY720 treated and control animals. The percent of Thy1.1^{+} effector P14 CD8 T cells is indicated in (D). n=6 in (E). F. Phenotypic properties of LCMV-specific effector CD8 T cells in different tissues of control and FTY720 treated animals. Data shown are gated on Thy1.1^{+} CD8 T cells. G. Production of IFN-γ and TNF-α by effector P14 CD8 T cells from control and FTY720 treated animals. Cells from the spleen, LNs, and the liver were incubated with LCMV peptide (D^{b} GP33-41) and BFA for 5 h in vitro. Cells were then stained for extracellular CD8 and intracellular IFN-γ and TNF-α expression. Data shown are gated on Thy1.1^{+} donor P14 CD8 T cells. The percent of IFN-g^{+} Thy1.1^{+} CD8 T cells producing TNF-α is indicated. All data shown are representative of 5 different experiments.

Figure 2. The egress of LCMV-specific effector CD8 T cells from the LNs can occur in the absence of sphingosine-1-phosphate signaling. A and B. Comparison of the percentage (A) and of the absolute number of (B) effector P14 CD8 T cells in different peripheral tissues. P14 chimeric animals treated with FTY720 or water alone were infected with influenza virus expressing the D^b GP33-41 epitope of LCMV. Approximately 7 days post-infection, animals were sacrificed and the distribution of Thy1.1$^+$ P14 CD8 T cells was assessed in different tissues. The percent of Thy1.1$^+$ P14 CD8 T cells in different tissues is indicated in (A). n=6 in (B). C. Comparison of the total number of LCMV-specific effector CD8 T cells in splenectomized mice treated with either water alone or FTY720. n=6. All data shown are representative of 3 different experiments.

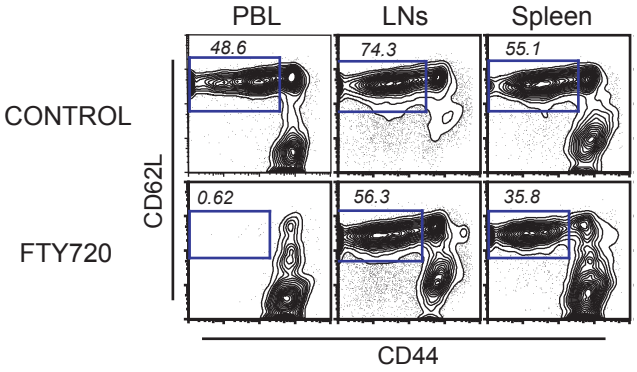
Figure 3. Overall pool of LCMV-specific memory CD8 T cells consists of both re-circulating and tissue resident memory cells. A and B. Naïve P14 CD8 T cells were transferred to naïve C57BL/6 mice and recipients were infected with LCMV. Approximately 60 days later, 2 ug/mL FTY720 was dissolved in drinking water (black) or mice were maintained on normal drinking water (white). (A) Two or (B) 30 days after FTY720 treatment, the number of LCMV-specific P14 memory CD8 T cells was determined in blood (PBL), lymph nodes (LNs), lung, or small intestinal epithelium (IEL). Data shown are representative of 5 different experiments. n=3.

FIGURE 1.

A.


Day -3
Transfer naive Thy1.1+ P14 CD8 T cells into mice
Day -2
Begin FTY720 (2 ug/mL) tx in drinking water
Day 0
Infect mice with LCMV
Day 7
Sacrifice mice and assess the distribution of effector (Thy1.1+) and naive (Thy1.1-, CD44lo, CD62Lhi) CD8 T cells in different tissues

B.


PBL
LNs
Spleen
CONTROL
FTY720
48.6
74.3
55.1
0.62
56.3
35.8
CD62L
CD44

C.

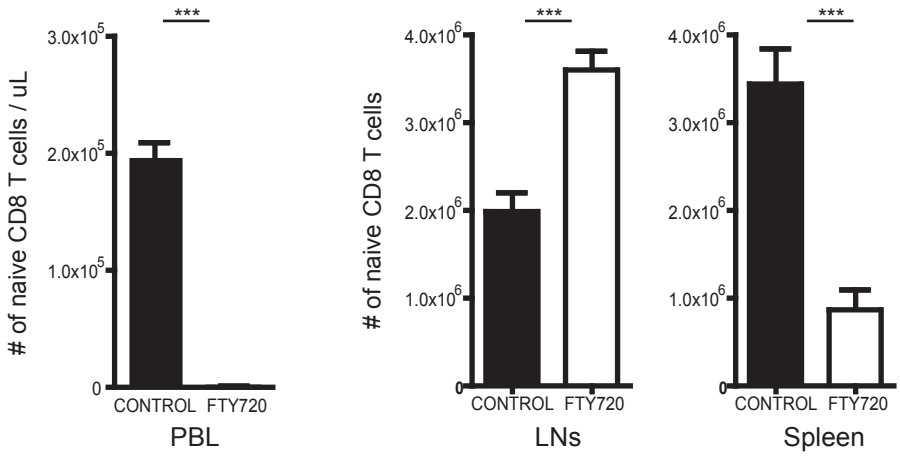
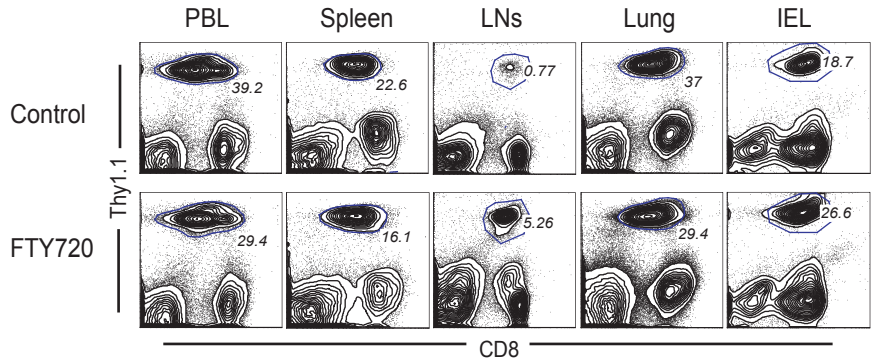

# of naive CD8 T cells / uL
3.0x10^5
2.0x10^5
1.0x10^5
0
***
CONTROL FTY720
PBL
# of naive CD8 T cells
4.0x10^6
3.0x10^6
2.0x10^6
1.0x10^6
0
***
CONTROL FTY720
LNs
4.0x10^6
3.0x10^6
2.0x10^6
1.0x10^6
0
***
CONTROL FTY720
Spleen

FIGURE 1.

D.


PBL
Spleen
LNs
Lung
IEL
Control
FTY720
Thy1.1
CD8
39.2
22.6
0.77
37
18.7
29.4
16.1
5.26
29.4
26.6

E.

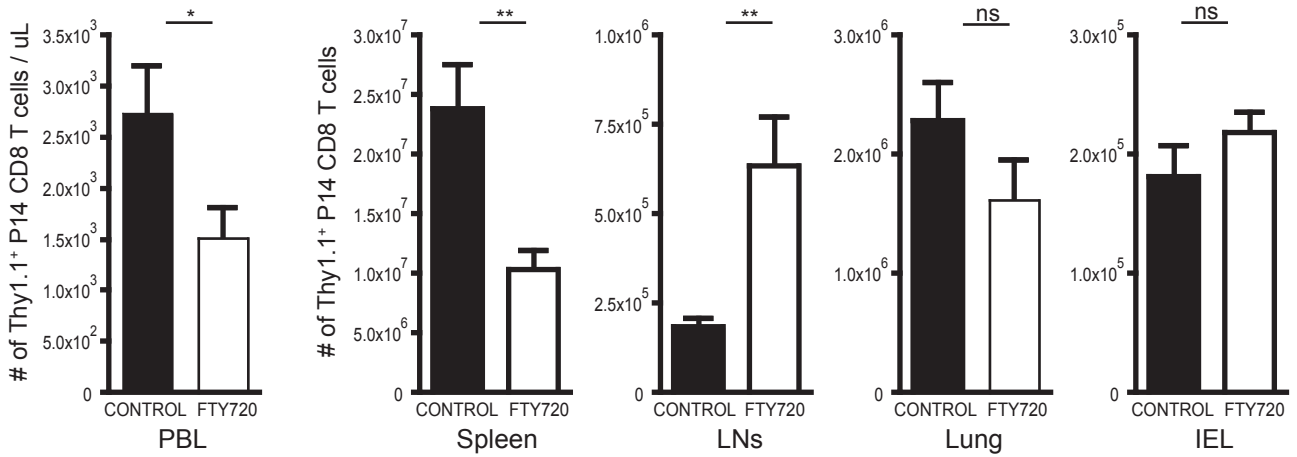
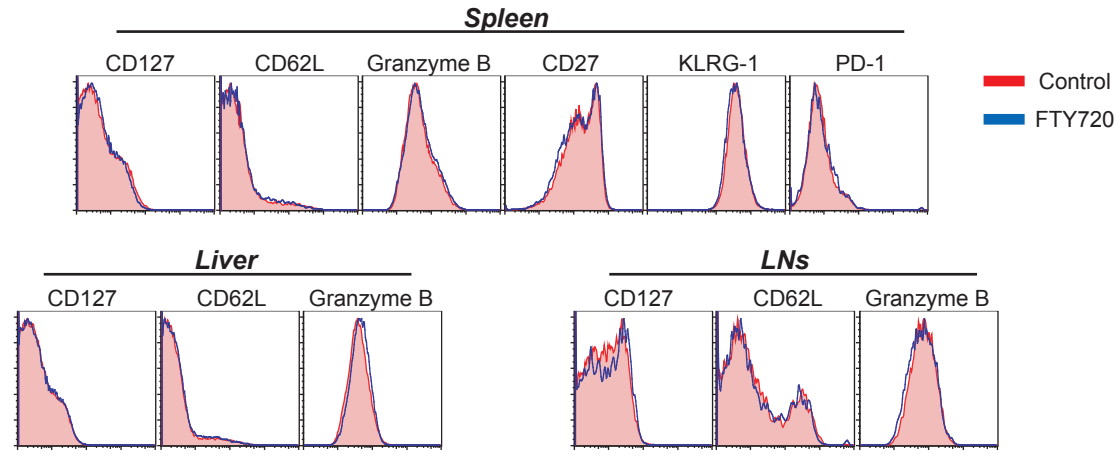

# of Thy1.1+ P14 CD8 T cells / uL
# of Thy1.1+ P14 CD8 T cells
CONTROL
FTY720
PBL
Spleen
LNs
Lung
IEL
*
**
**
ns
ns

FIGURE 1.

F.


Spleen
CD127
CD62L
Granzyme B
CD27
KLRG-1
PD-1
Control
FTY720
Liver
CD127
CD62L
Granzyme B
LNs
CD127
CD62L
Granzyme B

G.

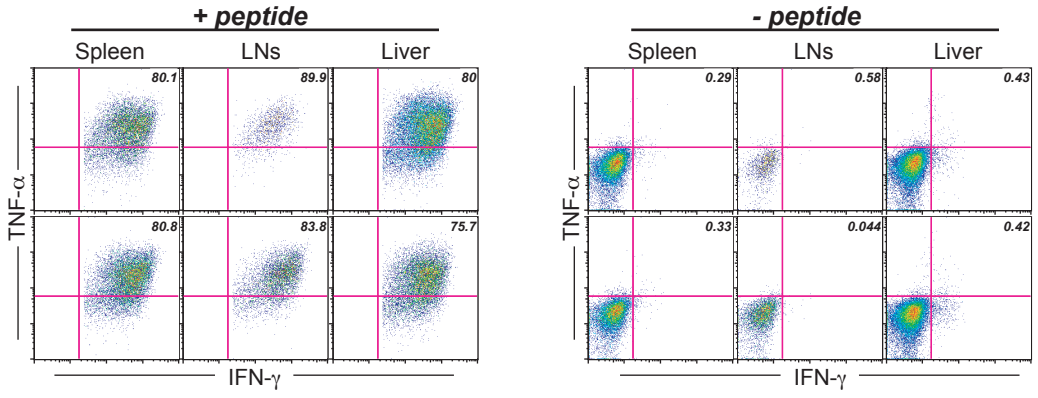

+ peptide
Spleen
LNs
Liver
80.1
89.9
80
80.8
83.8
75.7
- peptide
Spleen
LNs
Liver
0.29
0.58
0.43
0.33
0.044
0.42
TNF-α
IFN-γ

FIGURE 2.

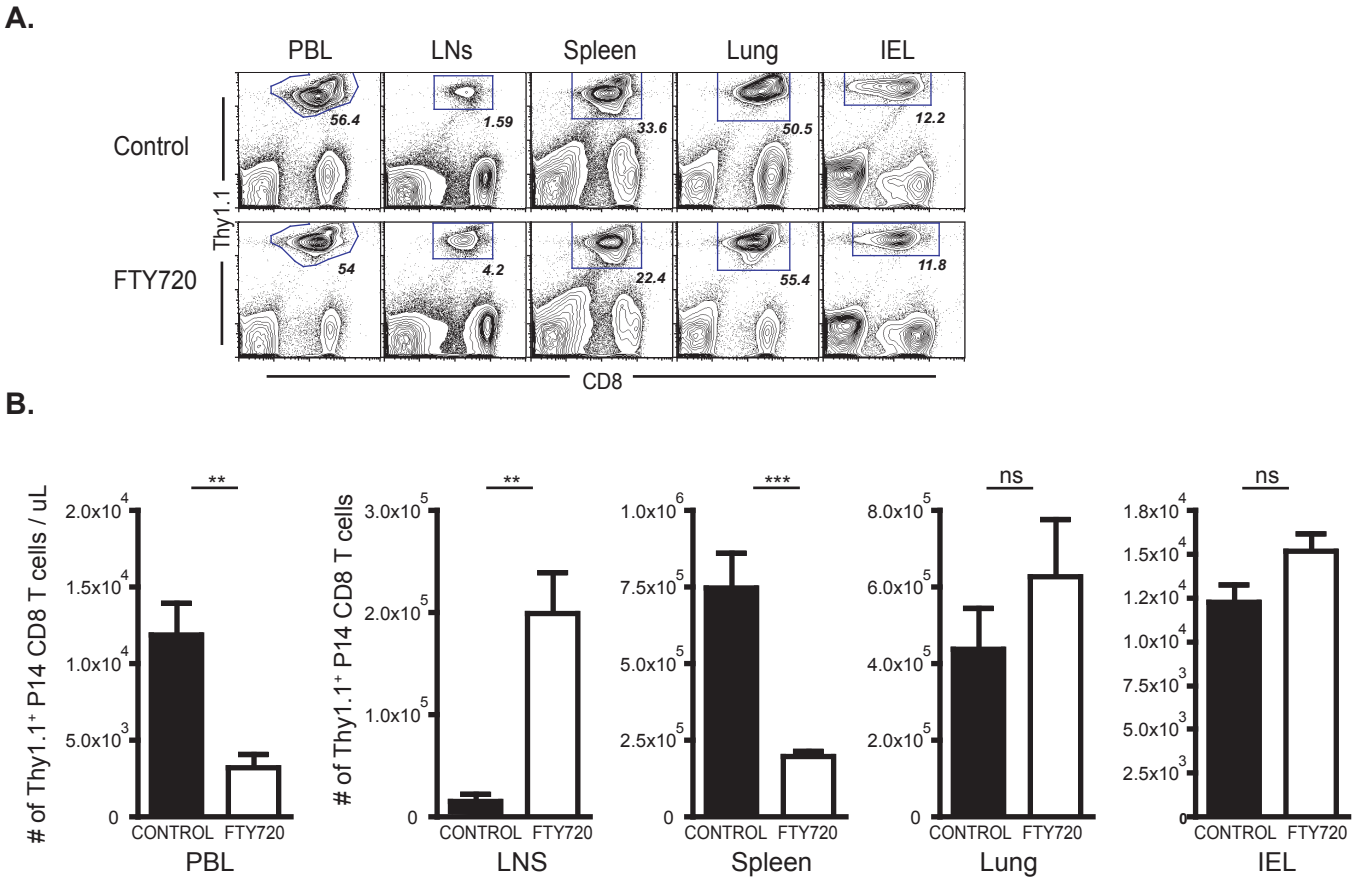

A.
PBL
LNs
Spleen
Lung
IEL
Control
FTY720
Thy1.1
CD8
56.4
1.59
33.6
50.5
12.2
54
4.2
22.4
55.4
11.8
B.
# of Thy1.1+ P14 CD8 T cells / uL
# of Thy1.1+ P14 CD8 T cells
CONTROL
FTY720
PBL
LNS
Spleen
Lung
IEL
**
***
ns

FIGURE 2.

C.

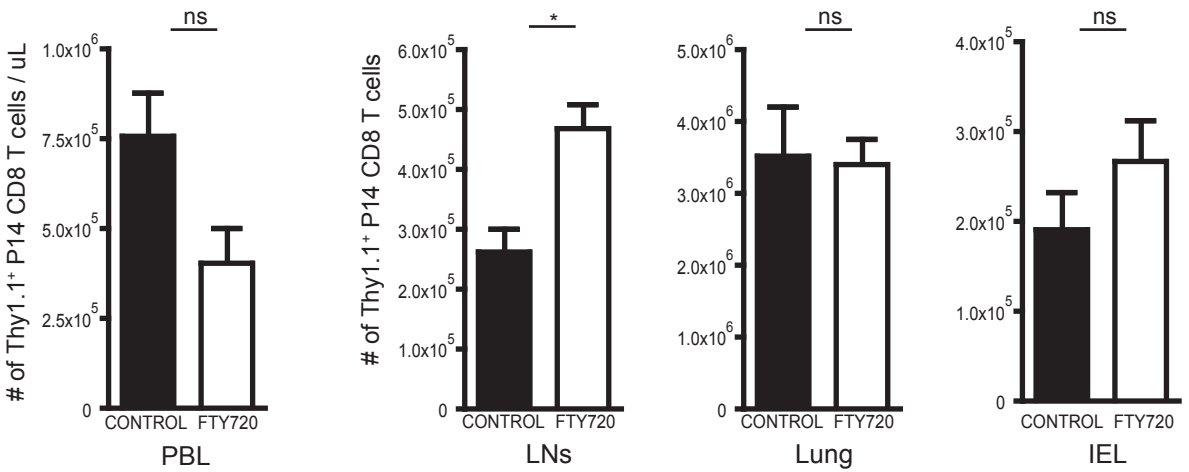
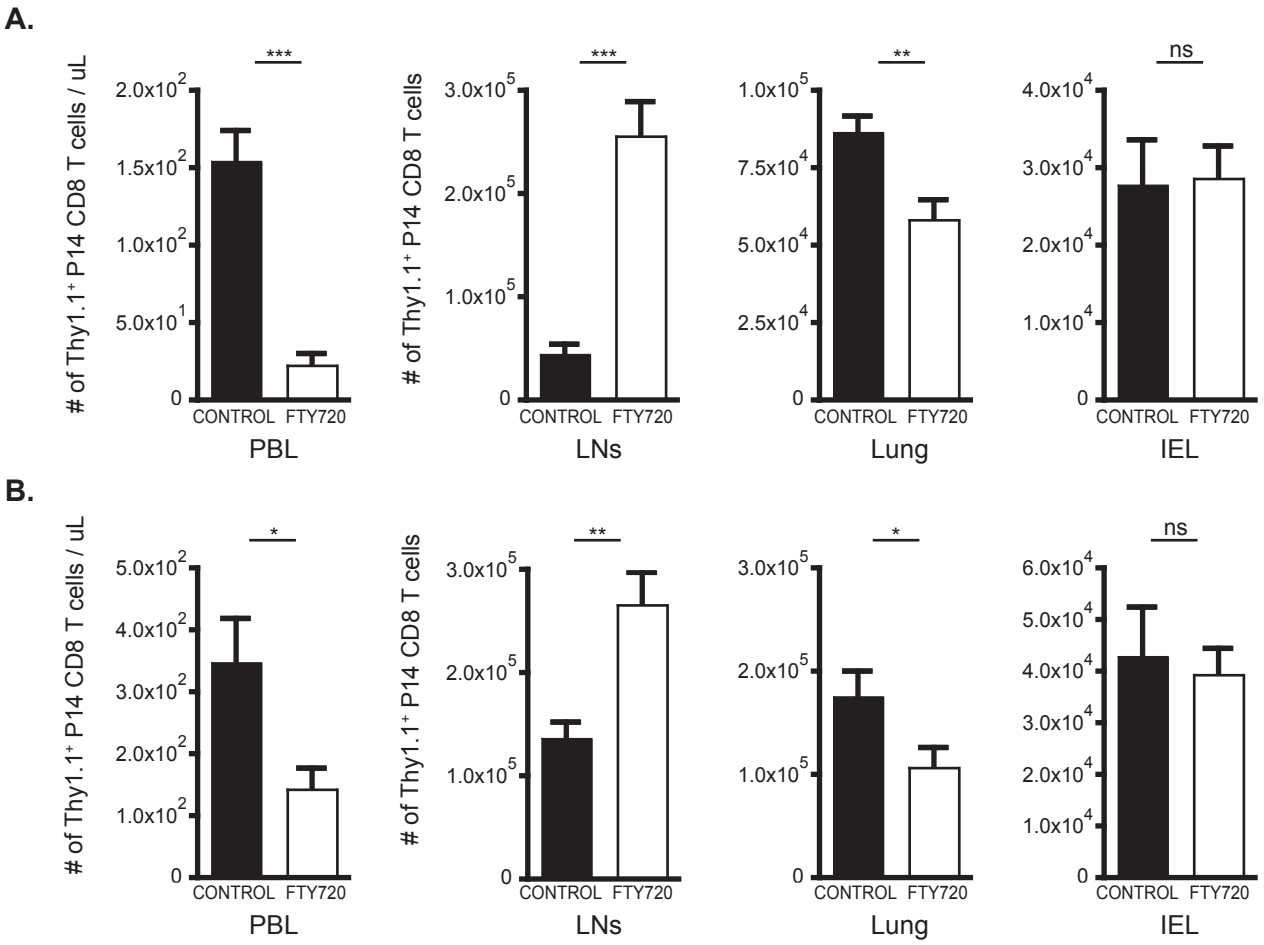

ns
1.0x10^6
7.5x10^5
5.0x10^5
2.5x10^5
0
# of Thy1.1+ P14 CD8 T cells / uL
CONTROL FTY720
PBL
*
6.0x10^5
5.0x10^5
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
# of Thy1.1+ P14 CD8 T cells
CONTROL FTY720
LNs
ns
5.0x10^6
4.0x10^6
3.0x10^6
2.0x10^6
1.0x10^6
0
CONTROL FTY720
Lung
ns
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
CONTROL FTY720
IEL

FIGURE 3.


A.
# of Thy1.1+ P14 CD8 T cells / uL
2.0x10^2
1.5x10^2
1.0x10^2
5.0x10^1
0
***
CONTROL FTY720
PBL
# of Thy1.1+ P14 CD8 T cells
3.0x10^5
2.0x10^5
1.0x10^5
0
***
CONTROL FTY720
LNs
1.0x10^5
7.5x10^4
5.0x10^4
2.5x10^4
0
**
CONTROL FTY720
Lung
4.0x10^4
3.0x10^4
2.0x10^4
1.0x10^4
0
ns
CONTROL FTY720
IEL
B.
# of Thy1.1+ P14 CD8 T cells / uL
5.0x10^2
4.0x10^2
3.0x10^2
2.0x10^2
1.0x10^2
0
*
CONTROL FTY720
PBL
# of Thy1.1+ P14 CD8 T cells
3.0x10^5
2.0x10^5
1.0x10^5
0
**
CONTROL FTY720
LNs
3.0x10^5
2.0x10^5
1.0x10^5
0
*
CONTROL FTY720
Lung
6.0x10^4
5.0x10^4
4.0x10^4
3.0x10^4
2.0x10^4
1.0x10^4
0
ns
CONTROL FTY720
IEL

Chapter 5: Conclusions and Future Directions

Since Edward Jenner's groundbreaking experiment, much progress has been made resulting in the successful development of vaccines towards many human diseases (e.g. mumps, measles, rubella, influenza, diphtheria, etc.) (370). One of the greatest triumphs has been the successful eradication of smallpox during the 1970s. While during the mid-1960s some ten to fifteen million cases of smallpox occurred, today smallpox is an affliction of the past. Unfortunately, hundreds of millions of people worldwide continue to be afflicted with diseases like HIV, Hepatitis B, and HCV, as there are currently no effective vaccines toward these diseases (371-373). Furthermore, many of the current existing vaccines require improvement in their potency/efficacy as some require several booster immunizations to develop protective immunity. Therefore, there has been a great interest in better understanding both how memory CD8 T cells are generated and how these cells provide protective immunity towards secondary challenges. Such knowledge will greatly aid both in improving current vaccines and in developing novel strategies toward diseases to which no current vaccines exist.

In the first study, we addressed the role of CD4 T cells in the development of fully functional memory CD8 T cells. Others had previously suggested that CD4 T cells were critical as memory CD8 T cells generated in CD4 T cell deficient animals were severely compromised in their overall function (212, 213). However, both the mechanism and when these CD4 T cells were required during CD8 T cell immune response remain unresolved. Therefore, we attempted to address these issues, but, unexpectedly, we observed that healthy LCMV-specific memory CD8 T cells could be

generated in the complete absence of CD4 T cells. Effector CD8 T cells, primed in either WT or CD4 -/- animals, developed into fully functional memory CD8 T cells when adoptively transferred into uninfected CD4 -/- animals suggesting that CD4 T cells are not required for memory CD8 T cell development. Instead, we noted impairment in LCMV antigen clearance in CD4 -/- animals and this persistence of antigen was directly responsible for the defect in memory development observed in these animals. Interestingly, however, the level of antigen persistence in these animals was significantly reduced compared to many chronic infections (e.g. Clone-13). Although the antigen was not detected by traditional methods (e.g. plaque assays), through the use of much more sensitive assays (e.g. sensor cells), we observed that there was a clear defect in the clearance of LCMV antigen in CD4 -/- animals. This may explain why many of the earlier studies had instead theorized that CD4 T cells were necessary for the "programming" of memory CD8 T cells as these studies relied mostly relied only on plaque assays to measure virus. Furthermore, this may also potentially explain for all the variations and discrepancies among the different studies addressing the role of CD4 T cells. Depending on the nature of the infection and the experimental system used to represent CD4 T cell deficiency, effector CD8 T cells alone may or may not be sufficient in the complete clearance of antigen (even if it did appear as if antigen was cleared completely by the more traditional and less sensitive assays).

Although our data suggest that CD4 T cells are not directly involved in the development of memory CD8 T cells, we postulate that CD4 T cells do play an important role, as they appear to be essential for the complete clearance of LCMV antigen. Future studies need to address how CD4 T cells aid in the clearance of LCMV antigen.

Although not likely, CD4 T cells may aid effector CD8 T cells in the clearance of virus through direct cytolytic activity (343). Alternatively, CD4 T cells may play an important role in the induction of an effective humoral immune response. These antibodies may be required for the clearance of antigen left behind by effector CD8 T cells. Along this line, it has been previously demonstrated that the therapeutic injections of LCMV-specific antibodies into LCMV-infected CD40 -/- animals results in the complete clearance of viral antigen and the rescue of the defect in memory CD8 T cell development otherwise observed in these animals (345). Furthermore, adoptive transfer of LCMV-specific transgenic CD4 T cells into chronically infected animals resulted in improved antibody response and a subsequent reduction in overall viral burden (R. Aubert, unpublished observations). Accordingly, passive transfer of antigen-specific antibodies may be of great use in improving memory CD8 T cell development in various chronic diseases.

In our second study, we observed that all memory CD8 T cells have the ability to undergo homeostatic turnover further highlighting the importance of this IL-15 mediated division in the long-term maintenance of memory CD8 T cells. Furthermore, we observed that memory P14 CD8 T cells divided at an average rate of 0.02 divisions/day, which translates into a mean time between divisions of 25-50 days (depending on when death occurs). However, future studies should better address whether all memory CD8 T cells divide at this same rate. Our preliminary data suggest that even among memory CD8 T cells specific for a single pathogen, there may be some subtle differences in the rate at which memory CD8 T cells divide. We observed that D^b NP396-404$^+$ memory CD8 T cells divided modestly slower than D^b GP33-41$^+$ and D^b GP276-286$^+$ memory CD8 T cells. Future studies should utilize a system in which all these different memory

CD8 T cells can be studied within a single host to minimize potential outside variables that may influence the rate of the homeostatic turnover of these cells. It would also be of interest to compare the rate of the homeostatic proliferation of memory CD8 T cells specific for different pathogens. This may or may not provide some insight as to why memory CD8 T cells are better generated towards certain pathogens compared to others.

We also observed in this study that the long-term maintenance of memory CD8 T cells (absolute number, homeostatic turnover, and function) is independent of CD4 T cell help. Memory CD8 T cells adoptively transferred into CD4 -/- animals were maintained stably, underwent normal homeostatic turnover, and retained their overall quality (phenotypic expression and function). This is in direct contrast to what others observed when memory CD8 T cells were transferred into MHC class II -/- mice (216). Future studies should work to address to resolve this discrepancy between the two animal models, as it is crucial that some intrinsic quality (besides the CD4 T cell deficiency) of the animal does not bias the data observed. For example, it has been suggested previously that CD4 -/- animals are not ideal models to study CD4 T cell deficiency as the $CD8^+$ population in CD4 -/- mice is heavily contaminated with MHC class II-restricted T cells that may compensate for the absence of the traditional CD4 T cells (365). We, on the other hand, observed that uninfected MHC class II -/- animals contained a greater percentage of CD8 T cells expressing PD-1 compared to WT animals. Along this line, several groups have suggested that MHC class II -/- mice are more susceptible to various autoimmune disorders (e.g. pancreatitis; inflammatory bowel disease) (339, 340). We do not currently know what kind of an influence these observations may have on the CD8 T cell immune response in these animals.

Lastly, we demonstrated in our final study that the egress of LCMV-specific effector CD8 T cells from the peripheral LNs is not dependent on S1P signaling as observed with naïve CD8 T cells. Despite FTY720 administration, we observed a significant number of LCMV-specific effector CD8 T cells in all peripheral lymphoid and non-lymphoid tissues. We had initially hypothesized that these effector CD8 T cells in the peripheral tissues originated from the spleen, as S1P signaling does not seem to be as essential for egress from the spleen. However, both splenectomy and infections with the influenza virus experiments provided concrete evidence that these LCMV-specific effector CD8 T cells exit the peripheral LNs in the absence of S1P signaling. This finding was unexpected as the primary mechanism by which FTY720 promotes transplant tissue survival is by sequestering transplant-specific CD8 T cells within the peripheral LNs (256). Further work needs to be carried out to identify the mechanism by which virally induced effector CD8 T cells are able to emigrate from the peripheral LNs.

Our data from this study also support a model by which early effector CD8 T cells migrate into the intestinal mucosa, then down-regulate $\alpha 4\beta 7$ expression, differentiate into long-lived memory CD8 T cells in situ, and remain resident with the IELs without re-circulating to other tissues. We observed that administration of FTY720 to an immune animal did result in the sequestration of memory CD8 T cells from different tissues (e.g. spleen, liver, lung, and blood) within the peripheral LNs. Interestingly though, there was no significant decrease in the number of memory CD8 T cells in the IEL of FTY720 treated animals compared to WT controls. This suggests that these memory CD8 T cells are non-circulating and remain resident within the IELs. In support, we also observed that nearly all of the naïve CD8 T cells from peripheral tissues were successfully

sequestered within the LNs after FTY720 treatment. However, a significant fraction of memory cells remained within the different tissues (e.g. lung) even after FTY720 administration), albeit less than control animals. Unfortunately, we can not rule out at this time that these memory CD8 T cells do continuously re-circulate, but just not through the peripheral LNs as they lack the necessary receptors. These "resident" memory CD8 T cells expressed high levels of CD62L, but their expression of CCR7 is currently unknown as there are no ideal reagents available at this time to stain for CCR7 expression in mice. Future studies may want to utilize chemotactic assays to measure the responsiveness of these cells towards CCL21/CCL19 as an indirect means of measuring for CCR7 expression. It may also be that this unique subset of LCMV-specific memory CD8 T cells, as observed with effector CD8 T cells, may not require S1P signaling for their egress from the peripheral LNs. Further work will be required to better address this. Overall, this understanding of whether memory CD8 T cells continuously re-circulate or are resident within the various tissues has obvious important ramifications for vaccination. This knowledge may shed much light on how memory CD8 T cells are targeted to different peripheral tissues upon initial antigen encounter and thus, aiding in the development of vaccines towards diseases with preferential sites of infection (e.g. mucosal tissues for HIV).

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