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Antigen load and cytokine requirements for CD8 T cell memory generation

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Author
Dave, Amy V.

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Antigen Load and Cytokine Requirements for CD8 T cell Memory Generation

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Amy V Dave

Committee in charge:
Professor Mitchell Kronenberg, Chair
Professor Stephen Hedrick, Co-Chair
Professor Cornelis Murre
Professor Matthias von Herrath

2009
The Thesis of Amy V Dave is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2009
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VITA

2006- Present  Laboratory Assistant, La Jolla Institute for Allergy & Immunology

2008  Bachelor of Science in Biology, University of California, San Diego

2008  Teaching Assistant, Department of Biological Sciences, University of California, San Diego

2009  Master of Science in Biology, University of California, San Diego

PUBLICATIONS

ABSTRACT OF THE THESIS

Antigen Load and Cytokine Requirements for CD8 T cell Memory Generation

by

Amy V Dave

Master of Science in Biology

University of California, San Diego, 2009

Professor Mitchell Kronenberg, Chair
Professor Stephen Hedrick, Co-Chair

Memory CD8 T cells are an integral part of protective immunity against viral infections, however the factors controlling memory T cell development are not well understood. In the present study, we provide evidence that antigenic load affects P14 T cell expansion positively, but inversely impacts efficacy to memory conversion during the course of acute LCMV. A gradually shift from a CD62L^{lo} (T_{EM}) phenotype to a CD62L^{hi} (T_{CM}) phenotype was observed when P14
T cells were introduced on day 3 p.i., while memory T cells from mice that received P14 cells at the same day of viral infection predominately expressed a CD44$^{hi}$CD62L$^{lo}$ (T$_{EM}$) phenotype with no alteration in the effector CD8 T cell response for P14+ T cells introduced on Day 0 or Day 3 p.i. We also demonstrate that IL-7 treatment at the contraction phase slightly increases the formation of P14 memory cells when introduced early during the course of the anti-LCMV response (same day of infection). Interestingly, anti-CD127 (anti-IL-7R$\alpha$) treatment at the contraction phase significantly enhances P14 memory conversion if cells are introduced late during the course of the anti-LCMV response. We addressed that anti-IL-7R$\alpha$ treatment does not increase the activation of antigen-specific CD8$^+$ T cells upon in vitro stimulation. Identifying the factors controlling the generation of memory CD8 T cells could result in the development of new strategies for enhancing the memory T cell pool and therefore vaccination design.
Introduction

Immunity consists of a complex system of defense mechanisms against the invading pathogens. Many different cell types orchestrate the immune response. At the first line of defense, cells without specificity act immediately conferring partial protection known as innate immunity. These cells (mainly dendritic cells, macrophages, and natural killer cells) participate in the activation of antigen-specific lymphocytes (B and T cells) that meditate a more robust response and persist following pathogen clearance as memory cells (adaptive immunity) (Germain, 1999).

B lymphocytes mature in the bone marrow, whereas T lymphocytes mature in the thymus. Mature lymphocytes circulate through the secondary lymphoid organs, returning to the bloodstream through the lymphatic vessels. The secondary lymphoid tissues include the spleen, which gather antigen from the blood, the lymph nodes, which collect antigen from sites of infection in the tissues, and the mucosal-associated lymphoid tissues (MALT), which collect antigens from the epithelial surfaces of the body. Prior to antigen interaction, naïve T cells migrate through the secondary lymphoid tissues ([Butcher et al., 1996], [Gowans et al., 1964], [Picker et al., 1992], [Sprent et al., 1977]). This continuous journey allows the naïve T cells to rapidly interact with antigens that are presented as peptide fragments bound to the major histocompatibility complex (MHC) molecules on specialized APCs, particularly by dendritic cells (Heath and Carbone, 2001). The MHC molecules can be characterized as MHC class I and MHC class II which stimulate the CTLs and helper T cells. More
specifically, intracellular antigens are processed in the APC into peptides and are presented on MHC class I molecules which are recognized by T-cell antigen receptors on cytotoxic T cells. Extracellular antigens, on the other hand, are processed by the endocytic pathway of the APC and typically bind to the MHC class II molecules which are recognized to the T-helper cells (Steinman, 1991). DCs or other APCs constitutively present self-antigen bound to the MHC. Naïve T cells that receive this low level of self peptide/MHC complex stimulation continue to circulate through the blood and secondary lymphoid tissues (Steinman, 1991). This self-antigen recognition along the IL-7 cytokine is necessary for naïve T cells survival ([Ernst et al., 1999], [Rooke et al., 1997], [Schluns et al., 2000], [Sprent et al., 1994], [Takeda et al., 1996], [Tan et al, 2001], [Tanchot et al. 1997]).

Upon pathogen recognition, APCs upregulate the expression of co-stimulatory molecules such as B7 molecules and display the specific antigen to activate adaptive immunity. DCs also up-regulate the synthesis and surface expression of cytokines, including interleukin (IL-12), and type I interferon (IFN). Presentation of both the specific antigen to the T-cell receptor (TCR) and a B7 molecule to CD28 receptor on the T cell lead to the clonal expansion of T cells and subsequent differentiation into antigen-specific effector T cells ([Curtsinger et al., 2003], [Curtsinger et al., 2005], [Lanzavecchia et al., 2001], [Schmidt et al., 2002], [Valenzuela et al., 2002]). In order to produce a strong primary immune response, proliferation of antigen-specific T cells occurs rapidly resulting in a >10,000-100,000 fold expansion of these cells (also known as the expansion
phase of the immune response) (Jabbari and Harty, 2006). During the expansion phase, CD8 T cells also modify their ability to localize to different tissues based on the changes of the trafficking molecules on their cell surface. More specifically, CD62L and CC chemokine receptor 7 (CCR7), a chemokine receptor that mediates homing to lymph nodes are down-regulated, while CD44, which mediates homing to peripheral tissues is up-regulated. In addition, CD8 T cells adopt effector functions, such as the ability to produce IFNγ and TNF and to exocytose granules of cytolytic enzymes, including perforin and granzyme B (GrB) (Harty et al., 2000). The pathogen can be targeted and directly killed by the cytotoxic T cells or helper T cells. CD8 cytotoxic T cells kill target cells infected with cytosolic pathogens. CD4 T_{H1} cells activate macrophages to kill intracellular parasites, while CD4 T_{H2} cells are important in activating B cells to secrete antibodies. Cytokines such as interferon-γ (IFN-γ) can also be secreted by effector T cells as a signal to destroy the infectious agents. Helper T cells play a role in the growth and activation of cytotoxic T cells by the production of IL-2 as well as the secretion of cytokines and proteins that stimulate or interact with APCs and other leukocytes. As the effector T cells destroy the pathogen during the immune response, T cell interaction with the reduced inflow of antigen bearing APC results in the decreased capacity of the APC to produce stimulatory cytokines (Langenkamp et al., 2000).

Upon viral clearance, the production of mass numbers of effector cells during the primary immune response overburdens the immune system and compromises the response of naïve T cells to new pathogens. In order to prevent
this overcrowding, most (90-95%) of effector T cells are die by the end of the
immune response (also known as the contraction phase of the immune
response) ([Badovinac et al., 2002], [Kaech et al., 2002], [Murali et al., 1998]).
The mechanism by which these effector cells are destroyed is not clearly defined.
Studies have suggested that mature T lymphocyte apoptosis is antigen-driven
and mediated by lymphokine withdrawal which are controlled by the expression
of cytokines such as IL-2 as well as Fas and tumor necrosis factor (TNF),
([Leonardo et. al, 1999], [Razvi et. al, 1995], [Simon et. al, 2000]).

Specifically for CD8 T cells, IFNγ is thought to play a role in the reduction
CD8+ T cells and IFNγ deficient mice display normal clonal expansion of effector
cells during an immune response but poor elimination of these cells (Badovinac
et al., 2000). Other studies suggest that massive cell death of CD8+ T cells
specifically is not dependent on the Bcl-2- regulated apoptosis signaling pathway
which promotes increased expression of Bcl-2 and Bcl-xL ([Petschner et al.,
1998], [Razvi et al., 1995]). In addition, data involving the role of Fas/Fas-ligand
interactions in apoptosis during an acute viral infection has shown that Fas
deficient mice still display apoptotic cells in their spleen suggesting that Fas is
partially, but not exclusively involved in the wide-scale death of T lymphocytes
(Razvi et al., 1995). A small percent of the effector cells, however, survives as
memory cells.

Acute infection of mice with Lymphocytic Choriomeningitis Virus (LCMV)
has been widely used to study CD8 T cell immunity to intracellular pathogens.
LCMV, a single-stranded RNA virus of the family Arenaviridae induces an acute
infection in adult, immunocompetent mice (von Herrath et. al, 2001). Infection with LCMV can provoke antigen-specific T cells to proliferate more than 10,000-fold reaching its peak number on day 8 post infection denoted as the expansion phase of the immune response. Days 8-15 mark the contraction phase of the immune response to LCMV where antigen-specific T cells undergo apoptosis and reduce in numbers. A portion of these cells survives to form long-lived memory CD8 T cells ([Ahmed et al., 1996], [Lau et al., 1994]. Re-encounter with LCMV results in the rapid expansion of cytotoxic T lymphocytes from the memory precursors thus mounting a more rapid and robust immune response ([Choi et al., 2001], [Grayson, et al., 2002], [Tough, et al., 1996]).

Adaptive immunity results in the production of memory T cells that are capable of responding faster and stronger to subsequent infection with the same or related virus (Seder and Ahmed, 2003). Several years ago, it was proposed that memory CD8+ T cells that are generated after acute infection compromise of two subsets: effector memory T- cells (TEM) and central memory T- cells (TCM), which are distinguished by their expression of the homing molecules chemokine receptor CCR7 (CC-chemokine receptor 7) and CD62L (also known as L-selectin). CCR7 and CD62L are necessary for lymphocytes to enter the lymph nodes ([Sallusto et al., 1999], [Unsoeld et al., 2005]. TCM cells typically express both CCR7 and CD62L, while TEM cells generally do not express these molecules (CD62L-CCR7-) ([Sallusto et al., 1999]). CD27 expression, a member of the TNF receptor family, is also used in distinguishing between these two subsets because TCM cells are generally CD27hi, while TEM cells are CD27lo ([Baars et al.,
The expression of these receptors led to the notion that \(T_{CM}\) cells typically localize to the lymph nodes, blood and spleen, while \(T_{EM}\) cells mainly reside in non-lymphoid tissues such as the gut, lung and liver, as well as the blood and spleen (Masopust et al., 2001). In addition, \(T_{EM}\) cells express the molecules necessary for lytic activity: perforin and granzyme B and isolated \(T_{EM}\) cells display ex vivo antigen-specific lytic activity. It is important to note, that \(CD62L^{low}\) CD8\(^+\) T cells in the mouse spleen, however, typically express low levels of lytic mediators despite having a \(T_{EM}\)-cell phenotype ([Masopust et al., 2001],[Masopust et al., 2006], [Sallusto et al., 1999]).

The development of memory T cells into these specific populations continues to be of interest and debate. Several models depicting the lineage of memory T cells have been proposed ([Lefrancois et al., 2003], [Seder et al., 2003], [Wu et al., 2002]). Within all proposals, activation of naïve T cells is necessary to form effector cells that subsequently will develop into memory T cells. One of the models suggests that self-renewing \(T_{CM}\) cells produces \(T_{EM}\) cells that are differentiated and incapable of self-renewal (Fearon et al., 2001). However, no \textit{in vivo} studies have showed that \(T_{CM}\) (CD62L\(^+\)CD8\(^+\)) cells transferred to naïve mice generate \(T_{EM}\) cells (CD62L\(^-\)CD8\(^+\)) cells ([Marzo et al., 2005], [Wherry et al., 2003]). Upon re-infection with a pathogen, both \(T_{CM}\) and \(T_{EM}\) can become effectors \textit{in vivo} in order to mount an effective immune response. The conversion of \(T_{CM}\) back to \(T_{EM}\) seems to require re-exposure to an antigen. Specifically, when CD62L\(^{hi}\) \(T_{CM}\) cells were transferred to C57BL/6 mice, nearly 90% of all LCMV-specific CD8\(^+\) T cells in the spleen, blood, and liver had
become CD62L$^{lo}$ by day 5 post infection (p.i.) in response to acute LCMV. Conversion from CD62L$^{hi}$ to CD62L$^{lo}$ was not observed in the absence of antigen as adoptive transfer of T$_{CM}$ in the spleen, LN, liver and lung retained the same CD62L$^{hi}$ phenotype ([Bouneaud et al., 2005], [Marzo et al., 2005]).

It is also hypothesized that T$_{CM}$ cells and T$_{EM}$ cells develop as separate lineages with frequent self-renewal of T$_{CM}$ and infrequent self-renewal of T$_{EM}$ cells (Bouneaud et al., 2005). Analysis of the TCR$\beta$ repertoire within the peripheral blood mononuclear cells (PBMC) of humans showed that the T cell memory pool represents at least two distinct subsets with the central memory subset more diverse than the effector memory subset as most TCR$\beta$ sequences were found in only in the T$_{CM}$ subset at a given time point (Chtanova et al., 2005).

Another model proposes that self-renewing T$_{CM}$ cells are derived from T$_{EM}$ in TCR-transgenic CD8 memory T cells specific for LCMV glycoprotein 33 (P14). Purified CD62L$^{hi}$ or CD62L$^{lo}$ D$^b$-gp33$^+$ memory T cells adoptively transferred into naïve mice and CD62L expression on splenic D$^b$-gp33$^+$CD8 T cells was determined on day 25. Transferred CD62L$^{hi}$ T$_{CM}$ population retained the same phenotype, but nearly half of the transferred CD62L$^{lo}$ T$_{EM}$ cells had converted to CD62L$^{hi}$ cells, suggesting that the T$_{CM}$ subset can be formed from T$_{EM}$ ([Bouneaud et al., 2005], [Marzo et al., 2005]).

While these memory subsets are typically distinguished primarily based on the phenotype and homing capabilities, it is important to consider that some memory CD8$^+$ T cells do display phenotypic heterogeneity and express a mixed CD62L$^{-}$ CCR7$^+$ phenotype (Baron et al., 2003). Furthermore, T$_{CM}$ cells are not
only present in lymphoid organs but can also be found to reside in non-lymphoid tissues. Carboxyfluorescein succinimidyl ester (CFSE) analysis of cell division in D\(^b\)-gp33–specific T\(_{\text{CM}}\) or T\(_{\text{EM}}\) cells stimulated with gp33 peptide showed that T\(_{\text{CM}}\) had a greater proliferation rate than T\(_{\text{EM}}\). The greater protective immunity displayed by T\(_{\text{CM}}\) may be due to this increase in proliferative capacity, the production of IL-2 as well as the localization of T\(_{\text{CM}}\) to LN (Wherry et al., 2003). However, other studies have suggested that at relatively early time-points after infection, T\(_{\text{EM}}\) exhibit equal proliferative capacities as TCM (Roberts et al., 2005). Moreover, it has been previously shown the increased relative ability of T\(_{\text{EM}}\) to lyse targets in lytics assays. However, data has also supported T\(_{\text{CM}}\) may exhibit a higher cytolytic capacity than TEM (Barber et al., 2003). These differences suggest that memory CD8 T cells are more display more heterogeneity than encompassed in the T\(_{\text{CM}}\) and T\(_{\text{EM}}\) models.

Subsets of memory CD8 T cells that are present during chronic viral infections (where virus persists for a longer time before it gets cleared) in humans and mice exhibit different properties from memory CD8\(^+\) T cells that are generated after acute infection ([Obar et al., 2004], [Sierro et al., 2005], [Wherry et al., 2004]). A paper by Wherry et al. found that CD8 T cells displayed reduced expression of the IL-7 and IL-15 receptors and elevated levels of PD-1 and were unable to undergo homeostatic proliferation and persist long term in the absence of antigen in response to infection with chronic LCMV. In response to acute LCMV, however, CD8 T cells were capable of homeostatic proliferation and expressed high levels of the IL-7 and IL-15 receptor (Wherry et al., 2004).
Understanding memory T-cell development at the molecular level can ultimately be used to create effective vaccines against cancer and infections by manipulating the known memory T-cell pathways. In addition, identification of memory CD8 T cells and their precursors during the effector phase would allow for a better insight in the development of memory T cells and lead to methods that would increase the representation of this population. ([Badovinac and Harty, 2006] and [Kaech et al., 2002]). To date, vaccines contain dead or inactivated organisms therefore taking advantage of the adaptive immune response, which is the ability to mount a greater and faster response to a reoccurring pathogen. Successful vaccination requires the use of viral antigen and adjuvant in order to generate a pool of virus-specific memory T cells capable of rapid activation and antiviral function during the occurrence of an infection.

The development of memory T cells may be influenced by antigen dose, the strength of the interaction of TCR for antigen, APCs and cytokines. Furthermore, the memory T-cell lineage is thought to be imprinted early in the immune response to acute infection (Kaech et al., 2003). Low viral dose, for example, resulted in a more rapid conversion of $T_{EM}$ cells to $T_{CM}$ cells, while high viral dose preferentially generated $T_{EM}$ cells and the emergence of some $T_{CM}$ cells. The time at which a naïve T-cell encounters an infectious response could possibly decide the fate of a memory T-cell into effector or central memory T cells (Marzo et al., 2005). As the amount of antigen decreases during the course of an acute infection, subsequent T cells that arrive late may receive less antigenic stimulation than antigen-specific T cells present earlier at the time of
infection. The decrease in antigenic stimulation may correlate to a decrease in the CD62L down-regulation and thus an increase in the number of T\textsubscript{CM} cells (Lefrançois et al., 2006).

Cytokines play a crucial role in the survival of naïve and memory T cells. Fully understanding the mechanism by which cytokines mediate the homeostasis of memory T cells is important in developing effective vaccines that enhance the development of a larger antigen-specific memory T cell pool (Nanjappa et al., 2008). As mentioned earlier, cytokines can affect T-cell proliferation and survival at many stages of the immune response. IL-15 is thought to play a role in the DC activation during the initiation of the T-cell response. T-cell clonal expansion in response to T cell receptor (TCR) ligation of peptide–MHC might be driven by IL-2. IL-2 is also thought to mediate the late clonal-expansion phase which results in the loss of most antigen-specific T cells. Some of these antigen-specific cells do survive, however, to generate memory T cells. T cells may escape cell death via the cytokines: IL-15 and IL-7 (Schluns et al., 2003). Memory T cells are maintained long term by undergoing a low level basal homeostatic proliferation, which depends on IL-15. IL-7, on the other hand, seems attributed in the survival, rather than the proliferation, of memory T cells. Although, IL-7 is not essential for CD8 T cell growth in response to a viral infection, it is involved in the formation and acute homeostatic proliferation of memory CD8 T cells ([Goldrath et al., 2002], [Tan et al., 2002]).

The common cytokine receptor \(\gamma\)-chain (\(\gamma\)-c) cytokine-receptor family plays an important role during the immune response and in memory T-cell
development and compromise two or three subunits. The structures of IL-7R, IL-2R and IL-15R mediate memory T-cell development and homeostasis. IL-7R possesses the α-chain and γ-chain, while IL-2R and IL-15R are composed of three subunits: IL-2/IL-15Rβ, γc, and unique receptor α chains. The IL-7 receptor α-chain (IL-7Rα, CD127) and IL-15Rα are constitutively expressed by naïve T cells (Schluns et al., 2000). Although naïve T cells do not express IL-2Rα (CD25), T cell activation increases the expression of IL-2Rα. The expression of IL-2Rα decreases before the proliferative peak of the immune response ([Janeway et al., 1994], [Smith et al., 1988]). IL-15Rα and IL-2/IL-15Rβ expression levels are increased after T cell activation, and high levels of expression are preserved throughout the memory-cell stage ([Becker et al. 2002], [Schluns et al., 2002]). T cell activation down-regulates the expression of IL-7Rα and increases in expression as the immune response progresses, reaching high levels on memory CD8⁺ T cells. A study by Kaech et al. showed increased CD127 expression on effector CD8 T cells fated to become memory T cells (Kaech et al., 2003).

Memory CD8⁺ T cells homeostasis is controlled by events that balances a low level of T cell proliferation with survival. The basal level of memory CD8 T-cell turnover (basal homeostatic proliferation) requires IL-15, but not IL-7 (Goldrath et al., 2002). Pathogens can increase the levels of IL-15 production and thus result in the increase memory T cell proliferation. The number of antigen-specific memory CD8 T cells decreases in the absence of either IL-15 or IL-15Rα in response to LCMV. In addition, antigen-specific memory CD8 T cells from normal mice and IL-15- or IL-15Rα-deficient mice were both able to produce
IFN-α and TNF and respond to re-infection (Tan et al., 2002). Thus, IL-15 is involved in the maintenance of antigen-specific memory CD8 T, but not in memory CD8 T-cell function. IL-2 can indirectly inhibit memory T-cell division. IL-2Rα are expressed on CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) suggesting that CD25⁺CD4⁺ T cells are involved in the IL-2-mediated inhibition of the proliferation of memory T cells by an unknown mechanism. Interestingly, transfer of CD4⁺CD25⁺ T cells causes a decrease in the division of memory CD8 T cells (Murakami et al., 2002).

The survival of memory T cells is positively influenced by IL-7, which is produced constitutively by stromal cells present in the thymus, bone marrow, and other lymphoid organs. IL-7 is a major regulator of T cell homeostasis and plays a key role in the survival of T cell precursors, as well as naïve and memory T cells (Kaech et al., 2003). Recently, Nanjappa et al. showed that IL-7 enhanced the formation of memory CD8⁺ T cells when administration was restricted to the contraction phase of the response to acute LCMV infection (Nanjappa et al., 2008). In vivo studies involving blocking IL-7, however, did not uncover a clear role for IL-7 in memory T cell homeostasis ([Goldrath et al., 2002], [Tan et al., 2002]). While in some cases CD8 memory cell proliferation was moderately inhibited, other experiments reflected that blocking IL-7 had no effect (Ku et al., 2000). IL-7 treatment during the expansion phase of the anti-LCMV immune response did not alter the degree of primary T cell expansion as well as the size of CD8 memory T-cells (Nanjappa et al., 2008). This observation may be explained by the fact that during the antigen-driven expansion phase of the
immune response TCR signaling leads to the down-regulation of the expression of IL-7Rα. Thus, CD8 T-cells may be unresponsive to IL-7 due to this down-regulation in the receptor expression. The mechanism by which IL-7 treatment restricted to contraction phase of the anti-LCMV response enhances the CD8 T cell response or the size of CD8 T cell memory has not been clearly defined. IL-7 may suppress the proapoptotic Bcl-2 family member Bim accompanied by the up-regulation of the antiapoptotic molecule Bcl-2 and MCL-1 in order to promote T-cell survival ([Hildeman et al., 2002], [Opferman et al., 2003]). In addition, killer cell lectin-like receptor G1 (KLRG1) hi, but not the KLRG1 lo effector CD8 T cells, were unable to proliferate well to IL-7, which may be due to increased amounts of p27kip (a member of the universal cyclin-dependent kinase inhibitor) in KLRG1 hi cells (Hand et al., 2007).

In this study, we demonstrate that antigenic load affects expansion of P14 T cell receptor transgenic cells specific for the MHC class I restricted GP33 epitope of LCMV positively, but inversely impacts efficacy to memory conversion during the course of acute LCMV. With respect to phenotype, P14 T cells were introduced on day 3 p.i. (late-transferred cells), a gradually shift from a CD62L lo (TEM) phenotype to a CD62L hi (TCM) phenotype was observed, while memory T cells from mice that received P14 cells at the same day of viral infection (early-transferred cells) predominately expressed a CD44 hi CD62L lo (TEM) phenotype. Studying the role of IL-7 cytokine in memory cell development, IL-7 treatment at the contraction phase slightly increases the formation of CD62L lo memory cells when introduced early during the course of the anti-LCMV response (same day
of infection). Interestingly, anti-CD127 (anti-IL-7Rα) treatment at the contraction phase significantly enhances P14 memory conversion (with cells expressing a CD62L$^{hi}$ phenotype) if cells are introduced late during the course of the anti-LCMV response. Overall, our findings provide a better understanding of the factors controlling the generation of memory T cells and may aid in the development of more effective vaccine design.
Chapter 1: Timing matters; naïve T cells recruited at different stages of the acute anti-LCMV response display different activation and memory kinetics

1.1 No effect in the endogenous CD8 LCMV-specific response after the transfer of 2x10^3 CD8 P14 cells.

TCR- transgenic (Tg) T cells have been widely used to study the T-cell response after infection or immunization with a particular agent (antigen). A recent publication by Badovinac et al. suggests that as the initial number of TCR-tg T cells transferred increases, the kinetics, proliferation, effector function and memory generation of CD8 T cells are gradually altered and differ significantly from the endogenous immune response (Bandovinac et al., 2007). Specifically, adoptive transfer of the most commonly used number (~10^6) of TCR-Tg T cells does not mimic the endogenous CD8 T cell response to infection. On the other hand, 5 x 10^3 number of TCR-Tg cells likely represents an appropriate number for P14 studies involving LCMV.

This suggests that low numbers of TCR-Tg cells seem to better mimic the anti-LCMV responses. As T cells proliferate, the expression of cytokine, chemokine, and homing receptors alters and the immune response can be influenced by the endogenous levels of these soluble mediators. Increasingly, the naïve antigen-specific T cell pool at initio may reduce the amount of these soluble mediators and therefore shift the immune response to non-physiological responses. This concept may explain the differences in the observed memory T cell conversion based on the number of TCR-Tg T cells transferred. Transfer of large number LCMV- or OVA-specific TCR-Tg T cells increased the ability of T_{EM}
cells to convert to $T_{CM}$ cells, while low numbers of these transferred cells displayed stable $T_{EM}$ and $T_{CM}$ CD8 T cell subsets suggesting that $T_{CM}$ and $T_{EM}$ subsets may not be similar in all settings (Bandovinac et al., 2007).

Based on the publication by Badovinac et al., we wanted to better mimic the natural CD8 T cell response. Thus, relatively small numbers ($2\times10^3$) of TCR-Tg LCMV-specific CD8 T cells ($GP_{33-41}$ P14 specific) were transferred on day 0 or day 3 from infection with $10^4$ PFU LCMV Arm into C57BL/6 mice. For tracking purposes, GFP expressing ($GFP^+$) cells from P14 mice crossed to GFP Tg mice were used. For better controlling the experimental outcome, mice that received P14 GFP$^+$ cells on day 0, received P14 GFP$^-$ cells on day 3, while mice that received P14 GFP$^+$ cells on day 3, received P14 GFP$^-$ cells on day 0 (table 1). Same numbers of cells were transferred at all times. As shown in figure 1, introduction of $2\times10^3$ P14 cells did not significantly prohibit the formation of endogenous $GP_{33-41}$-specific effector cells as measured by pentamer staining on day 8 and day 45 post infection (p.i.). Thus, introduction of low numbers of P14 T cells likely represents an accurate approach to better understand the endogenous T cell response to acute LCMV.

1.2 Reduced activation followed by minimal contraction due to lower antigen recognition does not affect memory T cell conversion

The time at which a naïve T-cell encounters an infectious response could determine memory conversion. Thus, our first aim of the project was to define the role of antigen load on the development of memory CD8 T cells during the acute LCMV immune response. For this purpose, $2\times10^3$ of TcR-Tg LCMV-specific CD8
T cells (GP<sub>33-41</sub>) were transferred on day 0 (denoted as the early-transferred group) or day 3 (late-transferred group) following infection with 10<sup>4</sup> PFU LCMV Arm into C57BL/6 mice. Mice that received P14<sup>+</sup> GFP<sup>+</sup> cells on days 0 or 3 from infection were analyzed on days 8, 15, 45. The percentage and total number of transferred P14 GFP<sup>+</sup> CD8 T cells in the spleen, blood, and mln for the early and late-transferred groups are shown in Graph 1.A/B.

**Table 1.1**: Experimental Approach. For the purposes of studying the generation and survival of memory T cells, the protocol listed in the table was implemented. Early transferred P14 cells were treated with recombinant human IL-7 (rhIL-7), while late transferred P14 cells were treated with blocking IL-7Rα antibody. Both treatment approaches were conducted at the transition window from peak to contraction phase of the CD8 endogenous response, days 8-14 p.i.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cells transferred</th>
<th>Treatment</th>
<th>Dose</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (early)</td>
<td>2x10&lt;sup&gt;3&lt;/sup&gt; GFP&lt;sup&gt;+&lt;/sup&gt; P14 cells on Day 0</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>2x10&lt;sup&gt;3&lt;/sup&gt; GFP&lt;sup&gt;-&lt;/sup&gt; P14 cells on Day 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (late)</td>
<td>2x10&lt;sup&gt;3&lt;/sup&gt; GFP&lt;sup&gt;-&lt;/sup&gt; P14 cells on Day 0</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>2x10&lt;sup&gt;3&lt;/sup&gt; GFP&lt;sup&gt;+&lt;/sup&gt; P14 cells on Day 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C (early)</td>
<td>2x10&lt;sup&gt;3&lt;/sup&gt; GFP&lt;sup&gt;+&lt;/sup&gt; P14 cells on Day 0</td>
<td>rhIL-7</td>
<td>3µg/mouse</td>
<td>Days 9-11-13 p.i.</td>
</tr>
<tr>
<td></td>
<td>2x10&lt;sup&gt;3&lt;/sup&gt; GFP&lt;sup&gt;-&lt;/sup&gt; P14 cells on Day 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D (late)</td>
<td>2x10&lt;sup&gt;3&lt;/sup&gt; GFP&lt;sup&gt;-&lt;/sup&gt; P14 cells on Day 0</td>
<td>Anti-IL-7Rα, CD127</td>
<td>100 µg/mouse</td>
<td>Days 8-10-12-14 p.i.</td>
</tr>
<tr>
<td></td>
<td>2x10&lt;sup&gt;3&lt;/sup&gt; GFP&lt;sup&gt;+&lt;/sup&gt; P14 cells on Day 3</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
**Figure 1.1:** No significant effect in the endogenous \( \text{GP}_{33-41} \) LCMV-specific response after the transfer of low number of P14 Tg cells. C57B/6 mice were infected with \( 10^4 \) PFU LCMV. Spleens from mice receiving \( \text{P14}^+\text{GFP}^+ \) on day 0 or 3 post infection (p.i) according to the protocol described in table 1 were collected and analyzed by pentamer staining on day 8 p.i. (A) and day 45 p.i. (B) after gating in CD8\(^+\)GFP\(^-\)CD19\(^-\) population. The total number of antigen-specific cells per spleen was calculated by multiplying the percent of CD8/GP33pentamer double positive cells by the total number of cells isolated from the spleen of each mouse. Representative data are from one out of two experiments. Differences are not statically significant.
The data in Graph 1.1 A/B shows greater P14 T cell expansion on day 8 (which marks the peak of the LCMV-specific clonal expansion), when cells were introduced early during the course of the immune response as depicted by the percentage of antigen-specific GFP+ CD8 T cells in the blood and spleen and the total number of GFP+ CD8 T cells in the spleen. In the mln, on the other hand, the frequency of P14 GFP+ CD8 T cells were not greater in the early-transferred group. Overall, GFP+P14+ T cells that are present at the initial phases of the viral infection display a greater expansion than cells introduced day 3 post infection. This expansion is likely related to the fact that LCMV antigen decreases after 3 days from LCMV infection. On day 15, which marks the contraction phase of the anti-LCMV immune response, there is a greater loss in the percentage of CD8 T cell that were transferred early compared to the late-transferred group. This suggests that the greater P14 T cell expansion displayed by the early-transferred group is followed by a greater contraction as well.

1.3 Introduction of low numbers of naïve P14 LCMV-specific CD8 Tg T cells at a later stage of the anti-LCMV immune response generates a higher ratio of memory per effector T cells.

The relative contribution to the memory CD8 T cell pool of cells recruited early or later in the anti-LCMV response was different. This is better determined when the fold of change for the percentage and total GFP in CD8 T cells from d8 p.i. was calculated for the spleen, mln, and blood compared to mice that received P14 cells on the same day of infection (Graph 1.2). A high degree of variation among samples was seen in the group of mice that received P14 cells
Graph 1.1: Greater P14 T-cell expansion on D8 when cells are introduced early during the course of the immune response. Spleen, blood and mesenteric lymph node (mln) cells from mice receiving P14\(^+\)GFP\(^+\) on day 0 or 3 from infection according to the protocol described in table 1 were analyzed on days 8, 15 and 45 p.i. with flow cytometry. The GFP\(^+\) cell percentage within the total CD8 population is depicted in the first 3 horizontal graphs (A), whereas in the bottom two (B), the total number of GFP\(^+\) cells per tissue is depicted. The GFP\(^+\)CD8\(^+\) percentage for each individual tissue was multiplied with its respective total lymphocyte number. Trypan blue staining was used to exclude dead cells prior to counting.
on day 3 p.i. (late). Here we found that naïve T cells recruited on day 3 p.i. due to lower antigen load or other factors (such as cytokine shifts due to viral infections) have higher chances of becoming memory T cells.

In order to address the development of memory T-cell subsets based on the time at which naïve T cells encounters an infectious response, we characterized T\text{CM} cells and T\text{EM} cells based on their expression of CD44 and CD62L. We chose to observe the expression of CD62L since it is a key marker that separates the effector and central memory T cell subsets along with the expression of CD44 because it is expressed at high levels in all effector and memory T cells but not in naïve T cells. Phenotypic analysis of memory T cells on days 15 and 45 from the spleen and blood of the early-transferred group displayed high levels of CD44 and low levels of CD62L expression, suggesting that these cells predominantly fall within the T\text{EM} population. Memory T cells located in the mln, however, preferentially expressed high levels of both CD44 and CD62L on days 15 and 45, indicating that these cells fall within the T\text{CM} population. It is important to note that between day 15 and day 45, there was a decrease in the percentage of the T\text{CM} subset in the mln (from 73.6% to 57.1%). Interestingly, phenotypic characterization of the highly survived memory cells from the spleen and blood of the late-transferred group showed a conversion from the T\text{EM} population (CD44\text{hi}CD62L\text{lo}) on day 15 to the T\text{CM} population (CD44\text{hi}CD62L\text{hi}) on day 45. Memory T cells located in the mln, however, preferentially expressed high CD44 and CD62L levels on days 15 and 45 with a
Graph 1.2: Increased memory conversion when naïve P14 Tg cells are introduced at the peak of the immune response, on day 3 post LCMV infection. Spleen, blood and mesenteric lymph node (mln) cells from mice receiving P14*GFP* on day 0 or 3 from infection according to the protocol described in table 1 were analyzed on days 8, 15 and 45 p.i. with flow cytometry. The fold of change of the GFP* cell percentage within the total CD8* population is depicted in the first 3 horizontal graphs (A), whereas in the bottom two (B), the fold of change in the total number of GFP* cells per tissue is depicted. The GFP*CD8* percentage for each individual tissue was multiplied with its respective total lymphocyte number. Trypan blue staining was used to exclude dead cells prior to counting.
greater percentage of the $T_{CM}$ on day 45 (90.6% to 91.2%).

Altogether, in this study we showed that when P14 T cells are introduced early during the course of anti-LCMV response, a much greater expansion is observed on day 8 followed by a distinct and dramatic contraction phase. A much less expansion was seen which was followed by almost no contraction when GFP$^+$P14 T cells were introduced at a later time point of the effector phase. Nevertheless, on a per cell basis, a much larger proportion converted and contributed to the memory cell pool. Notably, when P14 cells were introduced on day 3 p.i., a gradually shift from a CD62L$^{lo}$ phenotype to a CD62L$^{hi}$ ($T_{CM}$) phenotype was observed in the spleen and the blood, while memory T cells from mice that received P14 cells at the same day of viral infection predominately expressed a CD44$^{hi}$CD62L$^{lo}$ ($T_{EM}$) phenotype.

These findings suggest that antigenic load affects the expansion and contraction phase of an anti-LCMV response positively whereas it is inversely related to memory conversion.

1.4: No alterations in the effector function of CD8 T cells introduced early or late during the course of the immune response.

As effector T cells differentiate into memory cells, they retain the potential to rapidly produce IFN-$\gamma$ and TNF-$\alpha$ when exposed to antigen. We wanted investigate whether this effector function differed for the early-transferred group and the late-transferred group using antigen-induced secretion of IFN-$\gamma$ and TNF cytokines as a measure of effector function. As shown in figure 1.3, intracellular cytokine staining following stimulation with class I-restricted epitope GP33
showed no difference in IFNγ and TNF production on Days 8 and 45 for P14 T cells introduced early (on day 0) or late (day 3) post infection with acute LCMV. These results are in agreement with recent studies in mice that showed that following restimulation with cognate antigen, memory T cells displaying either a CD62L$_{lo}$ or CD62L$_{hi}$ phenotypes are equally good at rapidly producing IFN-γ and TNF-α (Unsoeld et al., 2002).
Figure 1.2: P14 cells introduced on day 3 p.i. display a gradually shift from a CD62L\textsuperscript{lo} phenotype to a CD62L\textsuperscript{hi} phenotype, while P14 cells introduced on the same day of infection predominately express a CD44\textsuperscript{hi}CD62L\textsuperscript{lo} (T\textsubscript{EM}) phenotype.

C57BL/6 mice were adoptively transferred with CD8 T cells isolated from uninfected naïve P14XT-GFP mice on day 0 and day 3 and infected with LCMV arm on day 0. On days 15 and 45, GFP+ CD8 T-cells from the spleen, blood and mln of infected mice were examined for the phenotype of transferred cells. A representative dot plot of CD44 and CD62L expression by GFP+CD8 gated cells is shown for the early-transferred group (A) and the late transferred group (B) from two independent experiments.
**Figure 1.3:** No alteration in the effector CD8 T cell response for P14+ T cells introduced on Day 0 or Day 3 p.i. Spleens were collected and analyzed 8 and 45 days after infection from the early-transferred group and late-transferred group. Spleen cells were cultured with the LCMV MHC-I peptide GP33 before staining for intracellular IFNγ and TNF. The percentage (A) and total number (B) of TNF/IFNγ double positive cells are shown. The total number of antigen-specific cells per spleen was calculated by multiplying the percent of TNF/IFNγ double positive cells by the total number of cells isolated from the spleen of each mouse. A representative dot plot of TNF and IFNγ expression by GFP+CD8 gated cells is shown (C). Representative data are from one out of two experiments. Differences are not statistically significant.
Chapter 2: The role of IL-7 in the generation of memory CD8 T cells

2.1: rhIL-7 treatment slightly increases memory generation when conducted at the end of the expansion phase in mice receiving P14 cells at the same day of infection (early)

In order to identify the parameters that are important in driving CD8 T cell memory conversion when naïve P14 cells are introduced at an early stage (same day of infection), we treated mice 3 times with rhIL-7 (3 µg/mouse) every 2 days from days 8-14 p.i. which marks the transition window from the peak to contraction phase of the CD8 endogenous response. Interestingly, as shown in Graph 2.1, and in agreement with previously published results, greater memory cell conversion was seen especially in the mln after rhIL-7 treatment. In addition, as shown in Graph 2.1, P14 CD8 T cells did not contract to the same extent due to IL-7 treatment in almost all tissues examined by day 15 p.i. Furthermore, phenotypic analysis of memory T cells on days 15 and 45 from the spleen and blood of the early-transferred group and the early-transferred group treated with rhIL-7 displayed high levels of CD44 and low levels of CD62L expression, indicating that these cells predominantly fall within the T_{EM} population. Memory T cells located in the mln for both non-treated and treated groups, however, preferentially expressed high levels of both CD44 and CD62L on days 15 and 45, suggesting that these cells fall within the T_{CM} population (Figure 2.1).
**Graph 2.1:** Increased memory conversion after rhIL-7 treatment at the early contraction phase when naïve P14 Tg cells are introduced at the same day with LCMV infection. Spleen, blood and mln cells from mice receiving P14*GFP* on the same day with infection followed by rhIL-7 treatment according to the protocol described in table 1 were analyzed on days 8, 15 and 45 p.i. with flow cytometry. The GFP* cell percentage within the total CD8* population is depicted in the first 3 horizontal graphs (A), whereas in the bottom two (B), the total number of GFP* cells per tissue. The GFP*CD8* percentage for each individual tissue was multiplied with its respective total lymphocyte number. Trypan blue staining was used to exclude dead cells prior to counting.
2.2 Anti-IL-7Rα (anti-CD127) treatment conducted at the end of the expansion phase in mice receiving P14 cells 3 days after infection (late) greatly enhances memory conversion

We demonstrated earlier there is a greater P14 memory conversion on a per cell basis present at d8 p.i. when GFP⁺ P14 T cells were introduced at a later time point of the effector phase during the course of an acute LCMV immune response. More particularly, when P14 cells were introduced on day 3 p.i., greater percentage of cells present on day 8 p.i. converted into memory T cells compared to P14 cells introduced at the same day of viral infection. Thus, our results prompted us to investigate the signals that are necessary to enhance memory conversion in the late P14⁺ T cell transfers. Initially we hypothesized that the greater memory conversion seen in mice that received naïve P14 cells on day 3 (acute phase) of the LCMV-specific immune response described in Graph 1.2 was either due to differences in antigen load (less on day 3 p.i.) or due to greater IL-7Rα levels expressed by P14 cells entering the response at that later time point. In order to address our second hypothesis, naïve P14 cells were introduced on day 3 p.i subsequent to infection with LCMV. Next, mice were treated 4 times with anti-CD127 (100 µg/mouse) on days 8-14 p.i. which falls during the transition window from the peak to contraction phase of the CD8 endogenous response (Table 1.1). To our surprise, as shown in Fig. 2.1, a much greater memory cell conversion was seen especially in the spleen and blood of mice receiving cells late and treated with anti-CD127. In addition, as shown in
Fig. 2.1, P14 CD8 T cells expanded to a greater extent rather than contracted by day 15 p.i. due to anti-IL-7Rα treatment.
Graph 2.2: Increased memory conversion after anti-CD127 treatment at the early contraction phase when naïve P14 Tg cells are introduced at the same day with LCMV infection. Spleen, blood and mln cells from mice receiving P14⁺GFP⁺ on the same day with infection followed by anti-CD127 treatment according to the protocol described in table 1 were analyzed on days 8, 15 and 45 p.i. with flow cytometry. The GFP⁺ cell percentage within the total CD8⁺ population is depicted in the first 3 horizontal graphs (A), whereas in the bottom two (B), the total number of GFP⁺ cells per tissue. The GFP⁺CD8⁺ percentage for each individual tissue was multiplied with its respective total lymphocyte number. Trypan blue staining was used to exclude dead cells prior to counting.
This observation suggests that when naïve cells are recruited later during the course of the anti-LCMV response when antigen load is reduced, anti-CD127 treatment enhanced proliferation and reduced contraction, thus enabling higher memory conversion. Phenotypic characterization of the highly survived memory cells from the spleen and blood of the late-transferred group and the late-transferred group treated with anti-CD127 displayed a conversion from the $T_{EM}$ population ($CD44^{hi}CD62L^{lo}$) on day 15 to the $T_{CM}$ population ($CD44^{hi}CD62L^{hi}$) on day 45. Memory T cells located in the mln for both non-treated and treated groups, however, preferentially expressed high levels CD44 and CD62L ($T_{CM}$) on days 15 and 45 (Figure 2.1).

Altogether, our results suggest that IL-7 treatment at the contraction phase slightly increases the formation of P14 memory cells when introduced early during the course of the anti-LCMV response (same day of infection). These are confirmatory results since the IL-7-mediated effect has been shown previously (Nanjappa et al. 2007). Anti-CD127 treatment at the contraction phase, however, significantly enhances P14 memory conversion if cells are introduced late during the course of the anti-LCMV response (day 3 p.i.) (Graph 2.3). Previously published studies were conducted with cells transferred at an early stage of infection and showed no clear effects of anti-CD127 in LCMV-specific memory conversion.
**Graph 2.3:** Increased memory conversion after rhIL-7 treatment or anti-IL-7Ra blockade at the contraction phase depends on the timing of P14 cell introduction in the course of anti-LCMV response. Spleen, blood and mln cells from mice receiving P14*GFP* cells and treated with rhIL-7 or anti-CD127 according to the protocols described in table 1 were analyzed on days 8, 15 and 45 p.i. with flow cytometry. The fold of change of the GFP* cell percentage within the total CD8* population is depicted in the first 3 horizontal graphs (A), whereas in the bottom two (B), the fold of change in the total number of GFP* cells per tissue is depicted. The GFP*CD8* percentage for each individual tissue was multiplied with its respective total lymphocyte number. Trypan blue staining was used to exclude dead cells prior to counting.
Figure 2.1: Both anti-CD127 treated and non-treated late-transferred groups display a gradually shift from a CD62L\textsuperscript{lo} phenotype to a CD62L\textsuperscript{hi} (T\textsubscript{CM}) phenotype, while rh-IL-7 treated and non-treated early-transferred groups predominately express a CD44\textsuperscript{hi}CD62L\textsuperscript{lo} (T\textsubscript{EM}) phenotype. Purified P14+ CD8 T cells were adoptively transferred on day 0 and day 3 and infected with LCMV arm on day 0. Subsequently, the early transferred group was left untreated or treated with rhIL-7, while the late-transferred group was left untreated or treated with anti-CD127. On days 15 and 45, GFP+ CD8 T-cells from the spleen, blood and mln of infected mice were examined for the phenotype of transferred cells. A representative dot plot of CD44 and CD62L expression by GFP+CD8 gated cells is shown for the early-transferred group, early-transferred group treated with rhIL-7, the late transferred group, and the late transferred group treated with anti-CD127 on days 15 (A) and 45 (B) from two independent experiments.
2.3: Anti-IL-7Rα (anti-CD127) treatment does not increase the activation of antigen-specific CD8\(^+\) T cells upon \textit{in vitro} stimulation.

The anti-IL-7Rα treatment has been described to neutralize the IL-7 cytokine levels \textit{in vitro} and \textit{in vivo}. Nevertheless, in order to address whether the enhanced memory conversion observed after anti-IL-7Rα (anti-CD127) treatment in mice receiving P14 cells 3 days after infection was due to a direct effect of the antibody on the IL-7R expressed by those naïve T cells, we performed an additional \textit{in vitro} activation assay. More specifically, we analyzed the ability of anti-CD127 to further activate the antigen-specific response of the P14 CD8 T cells \textit{in vitro}. For this purpose, naïve P14+ CD8 T cells were purified and labeled with 5 µM CFSE. The CFSE-labeled target cells were cultured in the presence of T cell-depleted spleen APCs loaded with 0.05, 0.5, or 5µg/ml GP33 at a 1:3 ratio. Anti-CD127 (10 µg/ml) was added on days 1 and 2. Activation of the CD8 T cells was evaluated 3 days later by assessment of CFSE dilution. As shown in Figure 2.2, we did not find any effect of the anti-CD127 treatment on CD8 T cell activation. In the absence of GP33, P14+ CD8 T cells did not show any signs of proliferation as expected. These results suggest that anti-CD127 treatment does not directly affect the activation of antiviral, GP33-specific CD8 T cells \textit{in vitro} upon binding to the IL-7R.
Figure 2.2: Anti-IL-7Rα (anti-CD127) treatment does not increase the activation of antigen-specific CD8\(^+\) T cells upon \emph{in vitro} stimulation. Naïve P14+ CD8+ T cells were purified and labeled with 5 µM CFSE. The CFSE-labeled target cells were cultured in the presence of T cell-depleted spleen APCs loaded with 0µg/ml (A), 0.05 (B) or 5µg/ml GP33 (C). Anti-CD127 (10 µg/ml) was added on days 1 and 2. Activation of CD8\(^+\) T cells was evaluated 3 days later by the assessment of CFSE dilution.
2.4: Discussion

The immunological environment following exposure to pathogens is critical since it can affect memory development and the ability of a pathogen to persist. The results of our study allow us to propose that naïve T cells recruited at different stages during the acute anti-LCMV response follow different activation and memory kinetics. Specifically, antigenic load affects the expansion and contraction phase of the CD8 immune response to LCMV positively whereas it is inversely related to memory conversion. The greater P14 expansion observed at the peak of the clonal expansion phase when cells were introduced early during the course of the anti-LCMV immune response may be attributed to the fact that as the amount of antigen increases during the course of an acute infection, T cells that are introduced early may receive more antigenic stimulation than antigen-specific T cells present later during the course of the infection. The increase in antigenic stimulation may correlate with greater expansion of P14 CD8 T cells. Another explanation for this phenomenon is that as the levels of antigen increases, there is greater interaction between T cells and antigen-bearing APCs as T cells travel through the secondary lymphoid tissues. Therefore, T cells that encounter more antigen-bearing APCs may receive enhanced T-cell stimulation though the interaction of multiple APCs resulting in the induction of greater cell divisions.

Brief exposure to antigen by cells that are introduced late during the course of the anti-LCMV response is not sufficient for these cells to proliferate and differentiate into effector cells, and could be inadequate to activate the
various death pathways that are required for the contraction of effector cells thus leading to a greater memory conversion. This proposal may explain the observation that a much less expansion was seen which was followed by almost no contraction when P14 cells were introduced at a later point of the effector phase. On the other hand, subjecting the early-transferred cells to prolonged antigenic stimulation may lead to the “exhaustion” of these effector cells subsequently activating the death pathway, leading to less memory-cell generation.

While antigen load seems to correlate with the expansion of CD8 T cells positively, the less memory conversion observed for the cells introduced early may also be influenced by the IL-7 receptor expression. Kaech et al. suggested that CD127 (IL-7Rα) expression is a marker that identifies early CD8 T cells destined to become memory CD8 T cells. Expression of the IL-7Rα is down-regulated by T-cell activation and may correlate with reduced expression of the anti-apoptotic molecule Bcl-2. Perhaps longer antigenic stimulation may have resulted in the prolonged down-regulation IL-7Rα and Bcl-2 in the early-transferred group compared to late-transferred group during the course of the LCMV immune response leading to greater deletion of antigen-specific memory T cell precursors for the early-transferred group.

While we have demonstrated that the time at which a naïve T enters the LCMV immune response may control memory T cell development, other factors could influence its generation. The role of the strength of the interaction of TCR for antigen, APCs and cytokines should be considered for future experiments.
The mechanism by which IL-7 treatment restricted to contraction phase of the anti-LCMV response enhances the CD8 T cell response or the size of CD8 T cell memory has not been clearly defined. Previously published studies have suggested that IL-7 may suppress the proapoptotic Bcl-2 family member Bim accompanied by the up-regulation of the antiapoptotic molecule Bcl-2 and MCL-1 in order to promote T-cell survival ([Hildeman et al., 2002], [Opferman et al., 2003]). Our observation can provide an explanation for the mechanism by which IL-7 affects memory conversion: it inhibits activation induced cell death (AICD). This result is also in agreement with previously described IL-7-mediated effects on CD8 T cell memory conversion. Thus, IL-7 is important for acute homeostatic proliferation and survival of memory cells but not for the basal turnover that sustains memory numbers (Goldrath et al., 2002). Recently, it has been suggested that the biological activity of IL-7 in vivo is greatly increased by association with anti-IL-7 mAb (Boyman et al., 2008). Treatment with rhIL-7 alone seems to only show subtle effects on the contraction and memory formation of the early-transferred cells. Thus, future experiments should try to enhance these effects by increasing the activity of IL-7 in vivo using IL-7/M25 complexes.

Interestingly, when naïve cells were recruited late during the course of the anti-LCMV response anti-CD127 treatment enhanced proliferation and reduced contraction, thus enabling higher memory conversion. We addressed that anti-CD127 does not enhance activation of T-cells expressing IL-7Ra in vitro. Perhaps, anti-IL-7R could enhance memory conversion because it limits cells to respond to other homeostatic cytokines such as IL-15 or IL-2 in vivo that may
promote greater memory T cell proliferation. Alternately, highly CD127 expressing naïve P14 CD8⁺ T cells activated on day 3 p.i. by lower levels of antigen could be less susceptible to inhibitory signals that affect their death, i.e. express lower levels of PD-1 and CTLA-4. This phenomenon, in conjunction with the anti-CD127 treatment perhaps leads to higher memory cell conversion by an unknown mechanism.

Overall, our findings show that the timing at which a naïve T cell enters the immune response affects memory conversion. In addition, we addressed the multifaceted role of cytokine IL-7 in memory development and found that while IL-7 treatment for early-primed cells enhanced memory T cell formation, anti-IL-7R treatment for the late-primed cells also increased memory T formation. Thus, our studies provide a better understanding of the factors controlling the generation of memory T cells. Further research is needed to better define the mechanisms that control the formation of CD8 memory T cells in order to achieve the development of more effective vaccine designs.
2.5 Methods

Mice

Six- to 8-wk-old male C57BL/6 mice were purchased from Jackson Laboratory. Naïve TCR transgenic GFP+ P14 mice on the C57BL/6 background were bred and were used to obtain GP33-specific CD8 T cells. P14 TCR-Tg mice were backcrossed to Tg-GFP mice to derive P14XT-GFP double Tg mice. Expression of both transgenes was confirmed by flow cytometry by testing for GFP, Vα2, and Vβ8.3 expression. All mice were maintained under specific-pathogen-free conditions at La Jolla Institute for Allergy and Immunology.

CD8^+ T cell negative selection

CD8 T cells were purified from the splenocytes of P14xT-GFP mice by negative selection using purified rat-anti mouse monoclonal antibodies specific for B cells (anti-B220), CD4+ T-cells (anti-CD4a), dendritic cells (anti-CD11c), anti-FcγRII (clone 2.4G2), mouse MHC class II (anti-mouse MHC Class II I-A/I-E) and macrophages (anti-CD11b). All antibodies were from BioLegend (San Diego, CA, USA). CD8 T cells were then purified by magnetic separation using the Dynabeads Sheep anti-Rat IgG (Invitrogen). Cells were washed with Dynal Buffer (1X PBS containing 2% FBS and 2.5 mM EDTA [Invitrogen]). 2x10^3 of the naïve GFP+ P14 CD8 T cells were adoptively transferred into the tail vein on day 0 or day 3 p.i.

Virus
Mice were infected with $10^4$ PFU of LCMV strain Armstrong 53b by i.p. injection.

**IL-7 treatment**

Recombinant human IL-7 was obtained from R&D Systems. Recombinant IL-7 was diluted in sterile PBS containing 0.1% of bovine serum albumin, and mice received i.p. injections of IL-7 at a dose of 3µg/mouse on days 9-11-13 p.i. Purified NA/LE rat anti-mouse CD127 (Clone SB/14; BD Biosciences Pharmingen) was diluted in sterile PBS and mice received i.p. injections of anti-IL-7Rα, CD127 at a dose of 100µg/mouse on days 8-10-12-14 p.i.

**Flow cytometry**

Single cell suspensions of lymphocytes were prepared from the spleen, blood and mln from all untreated and treated groups. After a 2.4G2 block step, cells were stained with the conjugated antibodies for cell surface markers. PE-conjugated H2-Db/GP33 pentamers were purchased from ProImmune. Directly conjugated antibodies, CD8-PerCP (BD Pharmingen), CD44-APCCy7 (e-Bioscience), CD62L-APC (BD Pharmingen), CCR7- PeCy7 (e-Bioscience), CD25-PB (Biolegend), and CD127-PE (BD Pharmingen) were used. For surface staining, cell suspensions were incubated at 4°C for 30 min. After surface staining, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich).

For intracellular stains, single cell suspensions were restimulated for 3 hours with 1 µg/ml MHC class I-restricted viral peptide GP33-41 (from Abgent). Cells were stained for surface expression of CD4 and CD8, fixed, permeabilized, and stained for intracellular IFNγ and TNF. After staining with the fluorescently
labeled mAbs or pentamer, cells were processed on LSR II (BD Biosciences) and results were analyzed using FlowJo (Tree Star).

**CFSE labeling**

Naïve P14+ CD8 T cells were purified from the spleen of C57BL/6 mice (using purified monoclonal antibodies as described earlier) and incubated with 5 μM CFSE. The CFSE-labeled target cells were cultured in the presence of T cell-depleted spleen APCs (using Dynabeads mouse pan T (Thy1.2)) loaded with the GP33 peptide in complete RPMI medium+ 5% FBS (TDS: T cell ratio= 3.1). Anti-CD127 (10 μg/ml) was added on days 1 and 2. The cells were cultured for 3 days, and cell division was assessed by the CFSE dilution profile of the labeled target cells acquired on LSR II.

**Statistical analysis**

Data are expressed as a mean ± SD. The statistical significance of the difference between means was determined using the two-tailed Student's t-test. *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
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