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Regulation of CD8 T Cells during Chronic Viral Infection

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Regulation of CD8 T Cells during Chronic Viral Infection

By

Erin Elizabeth West
B.S., University of Illinois, 2002

Advisor: Rafi Ahmed, Ph.D.

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Abstract

Regulation of CD8 T Cells during Chronic Viral Infection

By Erin E. West

CD8 T cells play an important role in controlling viral infections, however in the case of chronic infections, where antigen persists, CD8 T cells become dysfunctional, or exhausted. There has been much emphasis in understanding the difference in CD8 T cell responses during acute and chronic infection to better design vaccines and therapeutic strategies for chronic infections, although important questions remain.

First, a critical aspect of designing a T cell based vaccine for chronic diseases is understanding memory CD8 T cell responses to persistent antigen re-stimulation. Although, naïve and memory CD8 T cell responses have been well defined during rapidly cleared infections, less is known about memory responses during antigen persistence. To address this, we compared the responses of memory and naïve CD8 T cells to acute and chronic lymphocytic virus infection (LCMV). Memory cells dominated over naïve cells and were protective when present in sufficient numbers to quickly reduce infection. In contrast, memory cells were rapidly lost when infection was not quickly reduced, unlike naïve cells. This loss of memory CD8 T cells was due to a block in sustaining cell proliferation, selective regulation by the inhibitory receptor 2B4, and increased reliance on CD4 T cell help.

Secondly, new therapeutic strategies are needed to control chronic infections. Herein, we show that IL-2 therapy can act directly on exhausted CD8 T cells during chronic viral infection, increasing their numbers and function, while converting their

phenotype towards one associated with decreased exhaustion, including decreased expression of multiple inhibitory receptors, increased expression of CD44 and T-bet, and upregulation of IL-7Rα (CD127), a marker of functional memory cells. Surprisingly, these enhancing effects of IL-2 therapy were not correlated with significantly decreased viral loads. However, combining IL-2 therapy with blockade of the inhibitory PD-1 pathway had striking synergistic effects, resulting in both enhanced T cell responses and decreased viral load.

Overall, these studies emphasize the importance of designing vaccines that elicit effective CD4 T cell help and rapidly control infection and, secondly, demonstrate that combined IL-2 therapy and PD-1 blockade may be a useful regimen for treating human chronic infections and cancer.

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Chapter 1: Introduction

LCMV as Model System for Studying Acute and Chronic Viral Infections

Infectious diseases remain a health threat worldwide, even in countries with high standards of health care. Few specific treatments for viral infections exist and the best hope of protecting individuals has been through the development and use of prophylactic vaccines. However, effective vaccines against many chronic infections, such as Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV), are lacking. A better understanding of the CD8 T cell responses during chronic infections will help us to better design T cell vaccines against these persistent infections and also can help us to design therapies to combat these infections once they have taken hold. Use of animal models of chronic infections are a starting point to help us understand the unique challenges the immune system faces during antigen persistence and therefore increase our knowledge of how to design protective vaccines and therapeutics to combat these infections.

Lymphocytic choriomenengitis virus (LCMV) is a non-cytopathic arenavirus that induces a strong CD8 T cell response in mice. CD8 T cells are the major mediators of viral control in this viral infection (1, 2), making this a great model to study CD8 T cell responses to a virus. Furthermore, multiple strains of LCMV exists that result in differing lengths of infection, allowing easy comparison between acute infections, where virus is cleared within 1-2 weeks, and chronic infections where virus persists in the host. Herein, I will focus on two strains of LCMV, the Armstrong strain, which results in an rapidly cleared acute infection, and the clone-13 strain which results in prolonged viremia for ~2

months and reservoirs of the virus remain in the kidneys and brain for the life of the animal (3, 4). Neonatal infection of mice with LCMV results in mice that are lifelong carriers of the virus and allows for transmission of the virus *in utero* to their pups, also rendering the pups lifelong carriers of the virus (5, 6). The clone-13 strain of the virus was initially isolated from the spleens of these mice congenitally infected with LCMV Armstrong (3, 7). LCMV Armstrong and clone-13 differ in only 2 amino acids, where in LCMV clone 13 a leucine is mutated to phenylalanine at amino acid 260 of the glycoprotein(8) and a lysine is mutated to glutamine at amino acid 1079 of the polymerase (8, 9). By use of reassortant viruses of LCMV Armstrong and clone-13 containing each of these mutations alone, it has been shown that the single mutation in the glycoprotein of LCMV clone-13 confers higher binding affinity to it's cellular receptor α-dystroglycan (10). Furthermore, while it is unclear how the mutation in the polymerase alters viral persistence, it has been shown that macrophages infected with the reassortant containing both mutations, in the glycoprotein and the polymerase, are able to produce more viral progeny on a per cell basis (9). Therefore, it is likely that these two mutations cooperate with one another to increase viral persistence. Moreover, these mutations also seem to alter the tropism of LCMV, as the clone-13 strain has been reported to have increased tropism for dendritic cells (11-13) and also targets fibroblastic reticular cells in the spleen (14). Importantly, the two amino acid differences between LCMV Armstrong and clone-13 lie outside of the mapped CD8 T cell epitopes, allowing for easy comparison of CD8 T cell responses to both viral strains (15).

Lastly, while LCMV clone-13 infection results in prolonged viremia for ~60 days, depleting mice of CD4 T cells before infection with LCMV cl-13 results in high viremic

load lasting for life of the animal(16, 17) which may be a better model for highly persistent human infections, such as HIV. Overall, the LCMV model allows for a good comparison of CD8 T cell responses during both acute and chronic infection and has helped shape our understanding of how CD8 T cells function differently in these two situations. Therefore, LCMV provides us with a model system to start developing therapeutics and T cell based vaccines to chronic infections.

CD8 T Cell Responses during Acute Infection

CD8 T cells provide a crucial line of defense against intracellular pathogens, such as viruses and intracellular bacteria. During a cellular immune response a small number of CD8 T cells specific for an epitope (e.g. ~100-200 for LCMV GP33) expand and become effectors (18, 19). If the pathogen is rapidly cleared, within the first few weeks, such as in an acute infection, these effector cells undergo substantial contraction resulting in only 5-10% remaining as a stable memory CD8 T cell population. These memory CD8 T cells provide protection to re-infection by the same pathogen due to their rapid cytokine secretion and cytotoxic potential, increased tissue distribution and precursor frequency, and overall ability to respond to re-infection faster than naïve CD8 T cells (20-24). Technical advances, such as the development of tetramers(3), marking T cells by "cellular barcoding"(25), and two-photon microscopy(26) have all increased our ability to study and understand pathogen-specific CD8 T cell responses and memory formation.

A great body of work has been devoted to better understanding CD8 T cell responses and development of CD8 T cell memory in situations of limited antigen exposure, such as vaccination or acute infection, partially in the hopes of better

understanding: 1. What makes an effective CD8 T cell response? and 2. How to elicit "good" memory cells that are capable of protecting the host upon infection (after vaccination) or re-infection with the same pathogen. Another area of great interest is determining what defines a "good" memory cell. A general definition of memory CD8 T cells is T cells that persist long after antigen clearance (while typical effectors die off during the contraction phase), those that respond more rapidly to secondary infection, and are those that can maintain relatively stable numbers without antigen stimulation.

Multiple studies have paved the way to understanding that memory cells have distinct properties compared to naïve or effector CD8 T cells. Kaech et. al showed that the molecular profile of memory CD8 T cells is distinct from that of naïve and effector cells, where memory CD8 T cells have distinct gene profiles for genes related to cell migration, cell division, and signal transduction. Furthermore, day 8 effector cells do not have the same survival, and are unable to homeostatically proliferate and control infection as well as memory CD8 T cells (24). Moreover, direct comparison of naïve and memory CD8 T cells within the same host during acute infection have shown that the memory cells respond quicker to viral infection and undergo less contraction than naïve cells after subsequent infection with LCMV, vaccinia, listeria or activation with peptide pulsed dendritic cells (27, 28). Also, memory CD8 T cells are thought to be more sensitive to antigen than naïve cells, and Kersh et. al showed that increased sensitivity of memory CD8 T cells to antigen is not the result of increased T cell receptor (TCR) sensitivity, but due to changes in their lipid rafts that increases their ability to induce phosphorylation of downstream signaling molecules such as LAT, ERK, JNK and p38, which augments signaling (29). In addition to enhanced signaling after TCR stimulation,

another feature that may allow memory CD8 T cells to respond to infection faster than their naïve counterparts is the fact that they may proliferate faster upon antigen encounter. An initial study using transferred naïve and memory CD8 TCR transgenic cells specific for a male HY antigen, showed *in vivo* that memory CD8 T cells entered into their first division ~15 hours earlier than naïve CD8 T cells (30). However, in contrast, Carbone and colleagues showed that memory and naïve CD8 T cells in vivo proliferated equally during Herpes Simplex Virus infection and accumulated to similar numbers (31). Therefore, memory CD8 T cells may proliferate faster to antigen than naïve cells, but this is still a matter of debate. Lastly, a hallmark property of memory CD8 T cells is their ability to homeostatically proliferate to maintain stable numbers for long periods of time. Originally it was thought that memory CD8 T cells are maintained by low residual levels of antigenic stimulation (32), however more recent studies have shown that turnover is antigen and TCR independent (33-35), and instead is dependent upon the cytokines IL-7 and IL-15 (36-41).

To better understand CD8 T cell differentiation into memory cells, an understanding of the signals regulating this process is necessary. Classical activation of naïve CD8 T cells is based on a 3-signal model, where the T cell needs: 1. TCR activation by ligation to its corresponding MHC I bearing the specific antigenic peptide 2. Costimulation through receptors such as CD28 on the T cell binding to CD80/86 on the antigen presenting cells, and 3. Stimulus by cytokines. This model is a simplistic view of CD8 T cell activation, as there are many signals and receptors that collectively influence the overall signals that T cells receive upon activation and some of these will be discussed later. However, the strength of signal that a T cell receives seems to play a role

in determining cell fate. In this vein, a high dose of continuous stimulation, such as what occurs during chronic infection, results in non-functional T cells that do not develop memory characteristics (42), and this will be discussed in detail in a later section of the introduction. Furthermore, recent studies have provided evidence that T cells being recruited into the response later in infection (which limits antigenic stimulation) (43-45) or that early termination of antigen encounter (46) increases the number of memory precursors. Implying a role for simulation intensity in determining CD8 T cell fate. In addition, two recent reports have demonstrated a role for mammalian target of rapamycin (mTOR), a down stream signal of TCR, cytokine signaling and co-stimulatory receptors, in altering CD8 T cell differentiation. Inhibiting mTOR, either by the drug rapamycin or by knocking down components of the mTORC1 complex after CD8 T cell activation, enhances memory CD8 T cell development from the effector pool (47, 48). However inhibiting mTOR too early can inhibit T cell activation and expansion (47). This again emphasizes the idea that CD8 T cells need enough stimulation to become effectors and develop into memory, but lower levels of stimulation favor memory CD8 T cell development compared to high levels of stimulation. As naïve CD8 T cells require multiple signals for their activation, other signals besides direct TCR signaling can influence CD8 T cell differentiation, including environmental signals such as specific transcription factors and cytokines.

The role of transcription factors in the differentiation of CD8 T cells during both acute and chronic infections has been of recent focus. The transcription factors, T-bet, Blimp-1 and Eomes have been shown to play an important role in the effector function of CD8 T cells and determining cell fate, where increased levels of T-bet or Blimp-1

promote a terminally differentiated effector state (49-54). Furthermore, inflammation can influence these transcription factors, as for example; inflammatory cytokines, such as IL-12, can increase T-bet levels (51, 55). In addition to transcription factors, the field has been interested in determining surface markers that help distinguish cells that are fated to become memory CD8 T cells versus terminally differentiated effector cells during acute infections. Two markers of great interest are IL-7 Rα (CD127) and KLRG-1. CD127 is expressed by a small population of effectors at the peak of the response (~5-20%), and these cells have been shown to be substantially more likely to become memory CD8 T cells than their counterparts expressing low levels of CD127 (40, 56, 57). Besides CD127, KLRG-1 has also been used as a marker to help distinguish terminal effector vs. memory CD8 T cell fate (46, 51). As early as 4 days after infection KLRG-1 high and intermediate expressing cells can be determined, and separate transfer of the two populations shows that the majority of KLRG-1 high expressing cells become terminal effectors, while the KLRG-1 intermediate expressing cells tend to become memory CD8 T cells (46).

Complicating our understanding of memory CD8 T cells further is that these cells are a non-homogenous population. In general, there are two main populations of memory CD8 T cells: central memory (Tcm) and effector memory (Tem) CD8 T cells ($CD62L^{hi}$ $CCR7^{hi}$ and $CD62L^{lo}$ $CCR7^{lo}$, respectively)(58). Effector memory cells have decreased proliferative potential and may be shorter lived than their central memory counterparts (59), however they are widely distributed through the tissues of the body, while central memory cells are generally confined to lymphoid organs (60, 61). This heterogeneity of memory CD8 T cells also increases complication in understanding how to design CD8 T

cell vaccines. Is it better to design a vaccine that elicits effector memory CD8 T cells that are on guard at the site of infection, but have less proliferative potential, or central memory cells that are capable of more proliferation and perhaps have increased survival?

CD8 T Cell Responses during Chronic Infection

Functional memory CD8 T cells develop after quickly resolved infections, however, in contrast, in the case of high antigen persistence the responding CD8 T cells are unable to differentiate into effective memory and are retained in a relatively non-functional exhausted state. Recently, exhaustion has been characterized as a loss of function with exhausted CD8 T cells having a distinct molecular signature from naïve, memory, and effector CD8 T cells (42, 62). This exhaustion was first described in chronic LCMV infection in mice where it was observed that LCMV-specific CD8 T cells were unable to produce cytokines (63), however more recently this same exhausted state has been described in human chronic viral, bacterial and parasitic infections and also in human cancer (42, 64). The loss of function of in exhausted CD8 T cells occurs in a hierarchical manner, with loss of IL-2 production, *ex-vivo* cytotoxic killing ability, and proliferative potential occurring in the early stages, loss of TNF-α production occurring in the middle, and loss of IFN-γ production occurs during the final stages (42). In cases of extreme exhaustion, cells can be physically deleted, such as the NP396 epitope during chronic LCMV infection (4, 63, 65). During acute infection there is often is a hierarchy of immunodominance that is established and maintained throughout the response, however this hierarchy can be altered during chronic infection. For example, during acute LCMV infection D^bNP396 cells dominate over D^bGP33 and D^bGP276 specific cells,

while D^bNP396 cells are deleted during chronic LCMV infection and D^bGP276 and D^bGP33 cells dominate the response (4, 66-68). The immunodominance of the D^bNP396 epitope during acute infection may be due to the fact that more NP396 appears to be presented on the surface of infected cells (4) which may be the result of more rapid transcription of the nucleoprotein (NP) compared to the glycoprotein (GP) after LCMV infects a cell, due to the ambisense nature of the LCMV genome. This indicates that the deletion of the NP396 response during chronic LCMV infection may be due to increased TCR signalling, resulting in the most extreme form of exhaustion, cell deletion.

Even late during a persistent infection, these exhausted CD8 T cells do not develop into cells with memory CD8 T cell properties. The exhausted CD8 T cells downregulate important memory CD8 T cell markers, such as CD127 (69) and are unable to undergo normal homeostatic proliferation to IL-7 and IL-15 (70). In contrast, exhausted CD8 T cells are dependent upon antigen for their turnover and survival, and do not survive when transferred into uninfected mice, unlike memory CD8 T cells (71). Moreover, exhausted CD8 T cells are molecularly distinct from memory CD8 T cells. Recent studies have shown that exhausted CD8 T cells display a unique molecular pattern, indicating that they are a distinct lineage of CD8 T cells (62). Genes involved in many systems/pathways were changed in exhausted cells compared to naïve, effector or memory cells such as those encoding inhibitory receptors, those related to metabolism, chemotaxis, migration, and adhesion, and a distinct transcriptional profile was maintained (62). Furthermore, since the process of T cells anergy can also result in non-functional T cells, it is important to note that exhaustion appears to be molecularly distinct from anergy (62), indicating that these are two different processes. Here, I will focus on the

regulation of exhausted cells by inhibitory receptors, transcription factors, and CD4 T cell help.

Immunoregulatory or inhibitory receptors play a major role in immune processes, including prevention of autoimmunity and regulation of self-tolerance, along with playing a very important role in T cell exhaustion during chronic antigen stimulation (64). A defining feature of exhaustion in mice, primates and humans, is the co-expression of multiple inhibitory receptors, such as PD-1, CTLA-4, 2B4 (CD244), CD160, Lag-3 and Tim-3 (72). Furthermore co-expression of multiple inhibitory receptors on the same cell is linked to an increased state of exhaustion (42). However, since many inhibitory receptors are transiently upregulated on T cells after acute antigen stimulation, expression of inhibitory receptors alone does not indicate exhaustion (42). Herein, will mainly focus on PD-1 and 2B4.

The most well characterized inhibitory receptor involved in exhaustion is Programmed death-1 (CD279 or PD-1). PD-1 is member of the CD28 family that is upregulated on T and B cells upon stimulation of the T or B cell receptor. Its' two ligands are PD-L1, which has a widely distributed expression, and PD-L2, which is mostly expressed on antigen-presenting cells such as dendritic cells. An initial study by Barber et. al, showed an important role for PD-1 in exhaustion of CD8 T cells (73). Blockade of the PD-1 pathway during chronic LCMV in mice resulted in decreased viral burden and the "re-invigoration" (increased numbers and function) of virus-specific CD8 T cells. PD-1 plays a central role in other human and animal models of chronic infection, as confirmed by subsequent studies in HIV, HBV, HSV, HCV and SIV (42). Furthermore, more recently, multiple inhibitory receptors have been shown to be co-expressed with

PD-1 and play a role in CD8 T cell dysfunction. As co-expression of multiple inhibitory receptors is associated with a more exhausted state (42, 74), simultaneously blocking other inhibitory receptors at the same time as PD-1, such as Tim-3 (75, 76), Lag-3 (74), and CTLA-4 (77, 78) further enhances the recovery of CD8 T cell function.

Another less well-characterized immunoregulatory receptor involved in exhaustion is 2B4 (CD244). 2B4 is a member of the signaling lymphocyte activation molecule (SLAM)-related membrane receptor family and is mainly expressed on natural killer cells, some αβT cells and monocytes. The receptor for 2B4, CD48 is widely expressed on all hematopoetic cells (79-81). The gene encoding 2B4, *Cd244*, was found to be highly upregulated on exhausted CD8 T cells during chronic LCMV infection (62). However, not much is known about the role of 2B4 in the process of CD8 T cell exhaustion. To complicate matters, 2B4 has been most well studied in natural killer cells where it has been shown to have both an activating and inhibitory role (82). The ability of 2B4 to convey an inhibitory versus an activating signal is dependent upon the expression levels of 2B4 and the adaptor molecule signaling lymphocyte activation molecule-associated protein (SAP); where an inhibitory signal results with high levels of 2B4 receptor crosslinking in the context of low levels of SAP (81, 82). Due to this dual nature of 2B4, there has debate as to whether 2B4 plays an activating or inhibitory role on exhausted CD8 T cells. However, the high expression levels of 2B4 and the relatively low levels of SAP (62), as seen by gene expression profiles, indicate that 2B4 is likely to play an inhibitory role on exhausted CD8 T cells. Recently reports detailing human chronic diseases, such as HCV, HBV, HIV HTLV-1 and melanomas, have shown an increased surface expression of 2B4 on exhausted CD8 T cells, which is consistent with

the finding of both 2B4 mRNA and protein being upregulated during mouse chronic LCMV infection (83-87). Furthermore, a recent study showed that *in vitro* blockade of CD48, the ligand of 2B4, resulted in increased cytokine production by exhausted CD8 T cells; thus, indicating that 2B4 plays an inhibitory role (74). Moreover, two recent human studies have demonstrated that 2B4 expression on HCV-specific CD8 T cells is associated with decreased proliferation and increased T cell differentiation (83) and that blockade of 2B4 increased HBV-specific CD8 T cell cytotoxicity and proliferation (86), again indicating that 2B4 is working in an inhibitory fashion. While the molecular mechanism for 2B4 mediated regulation of exhausted CD8 T cells has not been defined, studies in both animal models and humans imply that the blocking of 2B4 on exhausted T cells may be beneficial in the setting of chronic infection.

While it is clear that inhibitory receptors play an important role in CD8 T cell dysfunction during chronic infection, also the amount of antigen stimulation (as described above), transcriptional state of the cells, CD4 T cell help, and environment can influence this process. Here, I will first focus on the role of transcription factors in T cell exhaustion. Recently, a significant amount of work has been done to better understand the molecular and transcriptional profiles of CD8 T cells during differentiation. As described previously in the section about CD8 T cell responses during acute infection, multiple transcription factors have been shown to play a role in CD8 T cell development into effector and memory CD8 T cells. A few studies have explored the transcriptional profiles of exhausted memory CD8 T cells (62, 88). The levels of both Blimp-1 and T-bet influence CD8 T cell differentiation during acute infection, with higher levels of either one enhancing the differentiation of CD8 T cells into terminal effector cells rather than

memory cells (51, 52, 54). During chronic infection, very high levels of Blimp-1 are found in exhausted cells that co-express multiple inhibitory receptors and Blimp-1 was shown to increase the expression of some inhibitory receptors, such as PD-1 and Lag-3 (88). In contrast to Blimp-1, high expression of T-bet during chronic infection results in more functional CD8 T cells that express less inhibitory receptors (89). This indicates that Blimp-1 and T-bet may important roles in the stages of CD8 T cell differentiation, including the state of exhaustion. It also highlights the fact that individual transcription factors, e.g. T-bet, may play differential roles in CD8 T cells during acute and chronic infection. In addition to these transcription factors that play a well defined role in CD8 T cell differentiation during acute infection, BATF (90), a downstream player of the PD-1 pathway, and the transcription factor NFATc1 (62, 91, 92),which may regulate PD-1 expression (93), also appear to promote dysfunction.

While inhibitory receptors and transcription factors play an important role in the process of CD8 T cell exhaustion, CD4 T cell help also plays a vital role. Depletion of CD4 T cells before infection with LCMV cl-13, results in high viremia lasting for the life of the animal, unlike normal cl-13 infection in which viremia lasts for ~60 days, indicating that CD4 T cells play an important role in controlling persistent LCMV infection (16, 17). Other studies have supported this, and shown a critical role for CD4 T cells in long-term control of mouse and human chronic infections (94, 95). Furthermore, lack of CD4 T cell help results in more extreme exhaustion (42, 73) (Chapter 3). Recently, multiple reports have shown that IL-21 produced by CD4 T cells is important for the maintenance of functional CD8 T cell responses during chronic LCMV infection

(96-98) indicating one mechanism in which CD4 T cell help can sustain CD8 T cell function.

Increased antigenic stimulation, inhibitory receptors, transcriptional programming, and CD4 T cell help are only some of the factors influencing exhaustion. The next section will focus on methods to reverse exhaustion and will better illustrate how the environment, such as cytokines and regulatory cells, also shape the process of exhaustion.

Reversing Exhaustion

Since chronic infections are a major health burden world-wide and few prophylactic vaccines exist for these infections, it has been of great interest to determine whether therapies or treatments can help reverse the state of T cell exhaustion and allow for clearance of the chronic infection. I will focus mainly on chronic viral infections, as this is area in which a large portion of the research has been conducted, although many of these findings may also apply to other situations of persistent antigen, such as parasitic infections, intracellular bacterial infection, and even to some cancers. There are multiple strategies that have been employed for reversing exhaustion or treating chronic infections (42, 99). One strategy is to inhibit negative regulatory factors such as inhibitory receptors, regulatory cells and immunosuppressive cytokines. A second strategy is to boost the immune system and CD8 T cells through therapeutic vaccination or cytokines. These two strategies will be discussed below.

Negative regulation of the immune response is a common theme in chronic infections. While this thesis has focused on CD8 T cells, it is important to note that other

aspects of the immune system, such as CD4 T cells, innate immune cells and B cells are also dysregulated during many chronic infections (64). In the section above about CD8 T cell responses during chronic infection, it was described how inhibitory receptors can negatively regulate CD8 T cell responses during chronic infection and blocking the function of many of these inhibitory receptors leads to increased CD8 T cell function and decreased viral loads in multiple chronic infection models. However, inhibitory receptors are not the only negative regulators at play during chronic infection. CD4 T regulatory cells (Tregs) suppress immune responses during chronic infection, and this has best been characterized in parasitic infections (100). However, recent data has also suggested a role of Tregs in chronic Friend leukemia virus infection (101). Furthermore, a study by Punkosdy et al. (102) and preliminary data by Penaloza-MacMaster et. al. shows that depletion of Tregs during chronic LCMV infection results in increased CD8 T cell responses. In contrast, another study has suggested that Tregs play a minor role in chronic LCMV infection (103). The role of Tregs in chronic LCMV infection is thus an open area of study and debate. However, in human chronic viral infections, such as HCV and HIV, and in some cancers and parasitic infections, T regulatory cells have been shown to suppress the immune response (64). Additionally, there has been a lot of recent focus on CD8 regulatory T cells and immunosuppressive macrophage populations that may also play a role during chronic infections (104) (105, 106). Overall, much more work needs to be done to understand the contribution of regulatory cells to the immunosuppression seen during chronic infection, and to help us better understand if it may be possible to manipulate these cells to reduce their negative regulation of immune responses during chronic infection.

In addition to inhibitory receptors and regulatory cells, immunoregulatory cytokines, such as TGF-β and IL-10, have been shown to regulate immune responses during chronic infections (42). Blocking the ability of CD8 T cells to receive TGF-β signaling by infecting mice engineered to express a dominant negative TGF-β Receptor resulted in increased CD8 T cell survival and function, indicating that TGF-β regulates CD8 T cells during chronic LCMV infection (107). In addition, increased levels of IL-10 has been noted in multiple chronic infections(108), and *in vivo* blockade of IL-10 signaling results in more functional T cell responses and better viral control during mouse LCMV infection (109-111), while *in vitro* blockade improves CD4 T cell responses in human HCV(112) and HIV (113). These are just some examples of how negative regulators of the immune system can impact chronic infection and show that blocking these immunoregulatory cells/cytokines may be a robust therapeutic approach for treating chronic infections.

The second approach for treating chronic infections is using a therapeutic vaccine or cytokines to positively affect the immune response. During chronic infections a reduction in the immune response may be partial due to non-optimal presentation of the antigen on antigen presenting cells. Therapeutic vaccination hopes to increase antigen levels and thus, possibly increase the presentation of the antigen in an optimal manner and thus allow for better T cell responses (99). Initial studies using therapeutic vaccination during chronic infection, ended with rather non-promising results (99). One study involving therapeutically vaccinating mice chronically infected with LCMV, with a recombinant vaccinia virus expressing the GP33 epitope of LCMV, did result in a slight increase in the CD8 T cell response, however the results were not striking (114).

Interestingly, the mice that responded the best to the therapeutic vaccine were those mice that had the lowest viral loads, indicating that perhaps therapeutic vaccination works best in situations of lower viral load (114), an idea that was also confirmed in a human study (115). This lead multiple groups to combine therapeutic vaccination along with other treatment regimens, such as PD-1 blockade or IL-10 blockade, that are known to reduce the immunosuppressive environment and viral loads during chronic infection. Treatment of mice with therapeutic vaccination plus blockade of these inhibitory signals, resulted in greater efficacy (110, 116). These data indicate that therapeutic vaccination alone may not be a successful treatment for chronic infections, but therapeutic vaccination along with other regimens that reduce viral loads or the immunosuppressive environment may be a more promising strategy.

While therapeutic vaccination is one way to augment T cell responses during chronic infection, using stimulatory cytokines is another approach that has had some success. Many γC cytokines, whose receptors share a common γC receptor component, such as IL-2, IL-7, IL-15, and IL-21 have a known role in positively regulating T cell development and homeostasis (117). Due to their known and important effects on T cells, these cytokines have been used as a therapy for chronic infection, in the hopes of increasing antiviral T cell responses. IL-15 and IL-7 play an important role in T cell development and the homeostasis of memory CD8 T cells (117). *In vitro* administration of IL-15 has been shown to decrease apoptosis and enhance HIV specific-CD8 T cells (118, 119), while the results from *in vivo* administration of IL-15 is less clear-cut. In one study IL-15 was given *in vivo* in combination with anti-retroviral drugs and therapeutic vaccination during SIV infection, and there appeared to be little effect on the SIV-

specific T cells, and an increase in viral load after cessation of anti-retroviral therapy (120). While these results were not promising, another study showed that *in vivo* administration of IL-15 with or without anti-retroviral drugs in chronically infected SIV monkeys resulted in increased SIV-specific CD4 and CD8 T cells, without affecting viral load (121). Overall, at this point in time, it is unclear whether IL-15 has a positive therapeutic effect during chronic infections and more studies need to be done to examine this.

Unlike the conflicting data seen with IL-15 administration, recent studies indicate that IL-7 therapy has a positive effect during chronic infection. Multiple studies have shown that IL-7 administration during chronic SIV infection increases virus-specific CD4 and CD8 T cells, but resulted in no reduction of viral loads (122-124). Recently two reports have shown that IL-7 administration during chronic LCMV infection decreases T cell exhaustion and can reduce viral loads (103, 125). It is somewhat surprising that IL-7 is able to act on exhausted CD8 T cells during chronic infection, since these cells, unlike memory CD8 T cells, do not express the IL-7Rα chain (CD127). Treatments that increase CD127 expression on CD8 T cells during chronic infection, such as IL-2 treatment (Chapter 3), may further increase the effectiveness of IL-7 therapy during chronic infection. Overall, these data indicate that IL-7 may play an important role in reversing CD8 T cell exhaustion.

Of recent interest is the role of the γC cytokine IL-21 in chronic infections. *In vivo* administration of IL-21 has been shown to increase CD8 T cell responses during chronic LCMV infection and reduce viral loads (96-98). Furthermore, CD4 T cells producing IL-21 are associated with reduced viral loads and increased CD8 T cell responses during

chronic HIV infection (126, 127), and increased IL-21 producing CD8 T cells are found in elite controllers of HIV infection (128), indicating a role for IL-21 in human chronic diseases. Since CD4 T cells are thought to be the main source of IL-21, an important aspect of CD4 T cell help of CD8 T cells during chronic may be related to IL-21.

Lastly, the γC cytokine IL-2 has been used as a therapy for metastatic cancers and chronic infections. IL-2 therapy will be a major focus of chapter 3. IL-2 is known to have both positive, such as proliferation, and negative effects, such as activation induced cell death (AICD), on CD8 T cells (129-131). *In vivo* and *in vitro* administration of IL-2 can promote terminal differentiation of CD8 T cells when given early during acute infections (132-136). As early as day 3.5 post- acute LCMV infection, the high affinity IL-2Ra chain (CD25) is heterogeneously expressed on LCMV-specific CD8 T cells (132). Kalia et. al show that the cells that express high levels of CD25 develop into terminal effectors, while the CD25 low expressing population can go on to form memory CD8 T cells after the effector stage (132). Furthermore, a study by Pipkin et. al, indicate that IL-2 induces a distinct transcriptional program in CD8 T cells, and that inflammation can influence the effects of IL-2 (133). Taken together, these data show that IL-2 plays an important role in regulating CD8 T cell differentiation during acute infections.

Since IL-2 can also have positive effects on the generation and maintenance of CD8 T cells, such as increasing proliferation and anti-apoptotic proteins, e.g. Bcl-2, there has been an interest in using IL-2 therapy to increase CD8 T cell responses in viral infections and cancer. An earlier study by Blattman et. al showed that the timing of low dose IL-2 therapy was vital during acute infection. Treatment during the effector stage of the response to acute LCMV resulted in decreased survival of CD8 T cells, however IL-2

treatment during the normal period of contraction resulted in increased CD8 T cell numbers, due to increased proliferation and decreased cell death (134). This study indicates that the timing of IL-2 therapy is vitally important. Furthermore, Blattman took this study one step farther by showing that low dose IL-2 treatment could increase CD8 T cell responses and decrease viral load during chronic LCMV infection, indicating that low-dose IL-2 treatment may be a viable therapy for chronic infections (134). It is important to note that this was accomplished with low dose IL-2 treatment, as lowering the dose of IL-2 treatment may help reduce toxicity issues, such as vascular leakage (137). IL-2 administration alone during metastatic renal cell carcinoma (138) or IL-2 with or without peptide vaccination in metastatic melanoma patients (139, 140) has resulted in clinical improvement. However, the current studies employing IL-2 therapy during chronic HIV infections in humans are not as clear-cut. Initial studies showed very limited success of IL-2 therapy combined with anti-retroviral drugs during chronic HIV infection (141-146). However one study indicated greater success when IL-2 therapy and anti-retroviral drugs were combined with therapeutic vaccination (147). Unfortunately, several small studies indicated that this success might not last after termination of anti-retroviral drug administration (148-150). In contrast IL-2 plus anti-retroviral drug therapy and therapeutic vaccination has resulted in increased CD8 T cell responses and decreased viral loads during chronic SIV infection (151, 152). Overall, these studies form a confusing picture for the justification of the use of IL-2 as a therapy for chronic infections. However, since IL-2 therapy has been shown to be beneficial during chronic LCMV and SIV infection, it still may be a promising therapeutic treatment for chronic infection.

In particular, there has been a recent focus in combining immunotherapies to combat chronic infection, since complete success (viral clearance) is not generally achieved by use of one therapeutic alone in situations of persistent high viral load. The strategy of combining immunotherapies has increased success, as simultaneously blocking multiple inhibitory receptors or blocking an inhibitory receptor or immunosuppressive cytokine plus giving a therapeutic vaccine, has resulted in a better outcome than these treatments given alone (42, 99). Furthermore, combining treatments may allow for success while decreasing toxicity issues, since it may make it possible to achieve positive results using lower amounts of the more toxic therapeutic. Therefore, combining the blockade of negative regulators (such as inhibitory receptors) and stimulating the immune system with either cytokines or therapeutic vaccination, is an attractive strategy for combating chronic infections.

Overall the regulation of CD8 T cells during chronic infection is a complicated processed that is governed by multiple factors. Many questions remain and need to be answered to help us understand this regulation in order to design better vaccines and therapeutics. Comparison of CD8 T cells in acute versus chronic infection helps allow us to better delineate the unique factors regulating these cells during sustained antigenic stimulation. While a lot of work has gone into understanding the primary CD8 T cell response during acute and chronic infection, less is understood about the secondary response. Since ideally we will create vaccines that produce memory CD8 T cells capable of combating infection, we need to better understand how these memory CD8 T cells respond during chronic infection (secondary response). Therefore, the second chapter of this thesis will begin to address this question. How does the response of

memory CD8 T cells compare to naïve CD8 T cells in chronic versus acute infection? How are these memory CD8 T cells differentially regulated during chronic infection? Can this help us understand how to create a more successful CD8 T cell based vaccine? Moreover, since prophylactic vaccines are not our only arsenals against chronic infections, the third chapter will address combined therapies for combating chronic infection. Does combination of a positive signal (IL-2), and blocking of an inhibitory receptor (PD-1) lead to a reversal of exhaustion in CD8 T cells and an increase in viral control?

Chapter 2: Tight Regulation of Memory CD8$^+$ T Cells Limits their Effectiveness during Sustained High Viral Load

Summary

To design successful vaccines for chronic diseases, an understanding of memory CD8$^+$ T cell responses to persistent antigen re-stimulation is critical. However, most studies comparing memory and naïve cell responses have only been performed in rapidly cleared acute infections. Herein, by comparing the responses of memory and naïve CD8$^+$ T cells to acute and chronic lymphocytic choriomeningitis virus infection, we show that memory cells dominated over naïve cells and were protective when present in sufficient numbers to quickly reduce infection. In contrast, when infection was not rapidly reduced, due to high antigen load or persistence, memory cells were quickly lost, unlike naïve cells. This loss of memory cells was due to a block in sustaining cell proliferation, selective regulation by the inhibitory receptor 2B4, and increased reliance on CD4$^+$ T cell help. This emphasizes the importance of designing vaccines that elicit effective CD4$^+$ T cell help and rapidly control infection.

Introduction

Memory $CD8^+$ T cells can provide efficient protection to re-infection due to their increased cytotoxic potential, cytokine secretion, and ability to respond to re-infection faster than naïve $CD8^+$ T cells. Recent studies have focused on better delineating what qualities memory cells need in order to be protective and highly functional, as well as how to better design vaccines to elicit memory cells with these properties (20-23, 153). Because memory $CD8^+$ T cells can provide quick and effective elimination of intracellular pathogens, vaccines designed to generate virus-specific memory $CD8^+$ T cells represent an attractive strategy for combating human viral and intracellular bacterial infections, including persistent infections such as HIV, HCV and tuberculosis. Importantly, multiple studies have indicated that virus-specific $CD8^+$ T cell function and proliferation are associated with decreased SIV or HIV viral loads thus, indicating that virus-specific $CD8^+$ T cells can help control SIV and HIV infection (154-156). However, studies have not been rigorously performed comparing the protective abilities and qualities of memory versus naïve $CD8^+$ T cells during chronic infections. Because chronic antigen stimulation has been shown to be detrimental to $CD8^+$ T cells, understanding the response of memory $CD8^+$ T cells to persistent antigen re-stimulation is important for rational vaccine design for chronic infections.

During chronic antigen stimulation, $CD8^+$ T cells undergo exhaustion, characterized by decreased proliferative capacity, loss of cytokine secretion, reduced cytotoxic killing abilities, and phenotypic changes, such as an increase in inhibitory molecule expression (4, 70). Upregulation of multiple inhibitory molecules has been shown to play a major

role in the process of $CD8^+$ T cell exhaustion during chronic infection. In particular, the inhibitory molecule programmed death 1 (PD-1) has been shown to play a central role in the process of $CD8^+$ T cell exhaustion, and blocking PD-1 can partially rescue exhausted $CD8^+$ T cells by increasing both their numbers and anti-viral function (73, 157). Furthermore, other inhibitory receptors, such as lymphocyte activation gene (Lag-3) and T cell immunoglobulin and mucin domain-containing molecue-3 (Tim-3) have been shown to synergize with PD-1, and co-blockade studies have resulted in enhanced restoration of function to exhausted $CD8^+$ T cells (74, 76). Another inhibitory molecule upregulated by exhausted $CD8^+$ T cells is 2B4 (CD244), however the role of this molecule in T cell exhaustion is not well understood. Most research on 2B4 has focused on its' role on natural killer cells and recent reports have provided conflicting views as to whether 2B4 plays an inhibitory or stimulatory role on $CD8^+$ T cells (62, 74, 83, 86, 158-160). Our understanding of $CD8^+$ T cell exhaustion and the roles of inhibitory receptors in this process are mainly based on studies of the primary T cell response. However, since vaccination results in a pool of pathogen-specific memory $CD8^+$ T cells, it is important to better understand how inhibitory molecules affect the secondary response of pre-existing memory $CD8^+$ T cells in the setting of chronic infection.

Another important aspect of T cell vaccine design is understanding the role of $CD4^+$ T cell help in the generation of functional $CD8^+$ T cell responses. While $CD4^+$ T cell help has been shown to be important during $CD8^+$ T cell primary responses to generate quality memory cells in multiple acute viral and bacterial infections (22, 161, 162), the relative importance of $CD4^+$ T cell help in primary and secondary $CD8^+$ T cell responses in acute

versus chronic infection has not been addressed. Therefore, understanding both the response of memory $CD8^+$ T cells to persistent antigen re-stimulation and the role of $CD4^+$ T cell help may be key in designing successful vaccines for chronic diseases. In the present study we addressed these questions using the lymphocytic choriomenengitis virus (LCMV) model to compare naïve and memory $CD8^+$ T cell responses during acute and chronic infection.

Results

Memory cells are selectively lost during high antigen load or antigen persistence

To examine the effects of chronic versus acute antigen re-exposure on naïve and memory $CD8^+$ T cells, we utilized the mouse LCMV $CD8^+$ TCR transgenic P14 system (cells specific for LCMV D^b-restricted epitope GP 33-41). Using this transgenic system allowed us to eliminate differences in TCR avidity and specificity between naïve and memory cells and allow for comparisons on a per cell basis. Furthermore, by co-transferring both the memory and naïve cells into the same mice, we were able to eliminate possible differences in environment. We altered the duration of antigen stimulation by infecting mice with either the Armstrong strain (Arm) of LCMV which results in an acute infection that is cleared by day 8-10 post-infection (p.i), or clone-13 strain (cl-13) which differs from Arm by only two amino acids and results in a highly disseminated viral infection with ~ 2 months of viremia (3, 4). The memory P14 cells used in these adoptive transfer experiments were highly functional memory cells from LCMV Armstrong immune mice that produced TNF-α, IL-2 and IFN-γ upon *ex-vivo* re-stimulation with their cognate GP33 peptide. Furthermore, 80-90% of these memory cells had the central memory phenotype; $CD44^{hi}$ $CD127^{hi}$ $CD62L^{hi}$ (Figure S1 A and B). By 7 days post-infection (p.i.), the transferred memory T cells (secondary effectors) dominated over the transferred naïve T cells (primary effectors) in the blood during acute LCMV infection, as previously described (27, 28, 163, 164) (Figure 1A). In striking contrast, when the mice were infected with the LCMV chronic strain, cl-13, primary effectors outnumbered secondary effectors at day 7, and this difference became even more drastic as the infection progressed (Figure 1A). Importantly, similar results were obtained when

the cells were transferred separately into different mice (data not shown). Moreover, transferred memory cells were also rapidly lost, unlike naïve cells, in the tissues after chronic infection ($p<0.01$ in spleen and $p<0.005$ in liver and bone marrow) (Figures 1B and 1C). These data show that secondary effectors are more detrimentally impacted by high antigen load or antigen persistence than primary effectors.

We wanted to eliminate the possibility that the loss of secondary effector T cells during chronic infection was due to differences in tropism between the Arm and cl-13 strains of LCMV. Although these two strains differ by only two amino acids, the increased receptor binding affinity of the cl-13 strain alters the viral tropism, allowing it to infect cell types that the Arm strain does not (13, 14, 165). Therefore, we transferred naïve and memory P14 T cells into mice and then infected them with either a low or high dose of LCMV cl-13. Mice infected with a low dose of cl-13 ($2X10^2$ pfu) had no detectible viremia at day 4 p.i. and the infection was rapidly cleared as an acute infection, unlike the viral persistence seen in mice infected with the high dose ($2X10^6$ pfu) of cl-13 (data not shown). Infection with the low dose of cl-13 resulted in the secondary effector P14's outnumbering the primary effectors (Figures 1B and C), similar to that in acute Arm infection. Furthermore, by altering the dose and route of Armstrong infection, we changed the length of antigen presence by a few days, but this also resulted in differential survival or loss of transferred memory $CD8^+$ T cells. With the typical acute dose of Arm ($2X10^5$ pfu) given i.p. or iv., secondary effector cells predominated, as shown previously. However, when the higher dose of Arm ($2X10^6$ pfu) was given i.v. the situation was changed, and the primary effector cells dominated over the secondary effector cells

(Figure S1C). It should be noted that this effect was less drastic than that seen after chronic cl-13 infection, where antigen amounts were much higher and the duration of viral persistence was longer. Taken together, these data show that high antigen load or persistence rather than viral tropism affect memory cell expansion and/or survival.

Next, we asked whether this observation was applicable to non-transgenic memory $CD8^+$ T cells. We sorted and transferred either LCMV-specific D^bGP33 $tetramer^+$ memory $CD8^+$ T cells (specific for the same epitope as P14 T cells) or memory P14 T cells along with an equal number of naïve P14 T cells. We observed the same phenomenon of selective loss of the memory cells with both the non-transgenic memory T cells and the transgenic memory P14 cells during chronic infection, while both persisted during acute infection (Figure S1D, data not shown).

Central and effector memory cells generated by multiple vaccines are lost during antigen persistence

Because different vaccines or viruses can result in memory cells of varying phenotypes and function, we asked whether the initial vaccine or virus used to create the memory cells impacted the ability of memory cells to persist during chronic antigen exposure. To address this, we generated memory P14 cells using multiple vaccines or viruses: LCMV, an adenovirus vector expressing the LCMV glycoprotein (GP), or vaccinia virus expressing the LCMV GP33 epitope. It is important to note that memory $CD8^+$ T cells can be divided into two main subsets, central and effector memory T cells, that have different proliferative abilities and effector properties upon re-stimulation, and differing

anatomical locations (58-60, 166). To control for differences due to altered ratios of central to effector memory $CD8^+$ T cells generated by different viruses or vaccines, we sorted the memory P14 cells into $CD62L^{hi}$ central (Tcm) or $CD62L^{lo}$ effector (Tem) memory cell populations and co-transferred each of them individually with naïve P14 T cells (Figure 2A). Because central memory cells (Tcm) have more proliferative capacity than effector memory cells (Tem)(59), we found, as expected, that only Tcm cells dominated the naïve P14 cells during acute infection (Figures 2B-D). However, after chronic infection, both Tcm and Tem cells were preferentially lost in the blood and tissues no matter which vaccine or virus was used to initially create them (Figures 2B-D). Thus, even purified highly proliferative central memory $CD8^+$ T cells were unable to persist during chronic antigen stimulation. Furthermore, this is applicable to memory cells generated by an adenovirus vector, vaccinia virus or LCMV Arm.

Lastly, to verify that the loss of secondary effectors is related to antigen persistence and not something specific to the LCMV system, we co-transferred naïve and memory P14 cells and immunized the mice with a persistent adenovirus 5 vector expressing the LCMV glycoprotein. Intramuscular injection of E1-deleted replication deficient adenoviral vectors expressing a transgene into mice results in detectable amounts of the transgene for 5-6 weeks post-injection, indicating that these vectors result in persistence of a low amount of protein antigen (167). As seen after chronic LCMV infection, memory cells were selectively lost during persistent adenovirus infection (Figure S2), thus, verifying that this phenomenon is not specific to the LCMV system, but is related to antigen chronicity.

Secondary effectors have a block in sustaining cell proliferation

We wanted to determine whether the relative decrease in numbers of secondary effectors to primary effectors was due to a difference in initial recruitment of the cells, increased cell death, and/or decreased proliferation of the secondary effector P14 T cells. By day 4 during acute LCMV infection, there were already significantly more secondary effectors than primary effectors in the tissues ($p<0.01$ in spleen, $p<0.05$ in liver) (Figures 3A and 3B). In contrast, at day 4 during chronic LCMV infection there were equal numbers of transferred memory and naïve cells in the spleens and livers (Figures 3A and 3B). This indicates that although secondary effectors are selectively lost during antigen persistence, their initial recruitment is similar to primary effectors. To understand the possible role that cell proliferation and death play in the loss of secondary effectors, mice were given BrdU i.p. for 6 hours at days 6 and 8 post-infection and cell proliferation was assessed by BrdU incorporation. By day 6 p.i., there was a reduction in the proliferation of the secondary effectors, with only ~21% of the secondary effectors incorporating BrdU compared to ~39% of primary effectors (Figure 3C); an effect that was maintained at day 8 p.i. (~20% of secondary and ~43% of the primary effectors were $BrdU^+$) (Figure 3C). Interestingly cell death of the secondary effectors was either similar at day 6 p.i. (~14% of secondary and ~18% of primary effectors were $7AAD^+$), or reduced at day 8 p.i. (~12% of secondary and ~25% of the primary effectors were $7AAD^+$), compared to primary effectors (data not shown). These data indicate that the decreased number of secondary effectors is due to decreased proliferative abilities compared to primary effectors in the context of antigen persistence.

We next sought to characterize the major differences between naive and memory cells at a molecular level. We compared the gene expression profiles of naive (primary effector) $CD8^+$ T cells with memory (secondary effector) $CD8^+$ T cells at day 8 post-infection in the setting of chronic infection, and tested for enrichment of classes of genes corresponding to major biological processes in each cell type. We found that there was highly significant upregulation of sets of genes related to cell cycle and proliferation in primary effectors compared to secondary effectors in chronic infection (Fig 3E). In addition, genes related to RNA processing, amino acid synthesis, mitochondrial metabolism and DNA repair were highly upregulated in primary vs. secondary effector T cells in chronic infection. In contrast, there was significant upregulation of cell cycle dependent kinase inhibitors (CDKi's) CDKN1A (p21/Cip) ($P = 0.002$, FDR = 0.18) and CDKN2B (p15) ($P = 0.002$, FDR = 0.18) in secondary effectors compared to primary effectors in chronic infection. Some evidence of increased expression of proliferation-associated genes was also evident in the primary vs secondary effector comparison in acute infection, however, CDKi's were not upregulated in secondary effectors in acute infection. Thus in chronic infection, the global expression pattern of primary effectors was consistent with more robust proliferation and metabolic activity than secondary effector $CD8^+$ T cells.

2B4 selectively regulates memory cells during chronic infection

We then asked whether the preferential loss of memory cells during chronic infection could be contributed to increased $CD8^+$ T cell exhaustion of the memory cells compared to naïve cells. From day 5 to day 14 post-chronic infection, both the primary and secondary P14 T cells acquired a decreasing potential to make TNF-α, IFN-γ, and IL-2 after *ex-vivo* peptide re-stimulation with cognate GP33 peptide, indicating that both are undergoing the typical $CD8^+$ T cell exhaustion seen in chronic infection (Figure S3). To evaluate the development of the exhausted state at a global level, we analyzed gene expression profiles from primary and secondary $CD8^+$ T cells at day 8 post-acute or chronic infection. We compared these profiles with published microarray data of exhausted $CD8^+$ T cells at ~D21 during chronic LCMV infection (62). Gene-set enrichment analysis showed highly significant upregulation of the exhausted $CD8^+$ T cell gene signature during chronic infection in both the primary and secondary effectors (Figures 4A and 4B). The most enriched genes from the exhausted signature in both primary and secondary effectors were highly similar (Figure 4C) and, with few exceptions, these genes were expressed at the same magnitude amongst the primary and secondary effector samples (Table S1). These findings suggest that both memory and naïve cells develop the molecular and functional properties of T cell exhaustion to a similar degree and argue against a more extreme exhausted phenotype in secondary effectors.

In order to identify candidate mechanisms involved in the selective loss of secondary effectors in chronic infection, we identified genes selectively upregulated in that

population (compared to naïve cells after chronic infection, and naïve cells and memory cells after acute infection). Among the most differentially upregulated genes in secondary effectors in chronic infection, we found several inhibitory receptors (Figures 4D and 4E). While PD-1 and Lag-3 were expressed at equivalent levels in primary and secondary effectors during chronic infection ($p>0.05$), 2B4 and Tim-3 were among the most highly upregulated in secondary effectors (4.4 fold increase in secondary vs. primary effectors in chronic infection, $P=0.002$ and 2.2 fold increase, $P=0.002$, respectively) (Figure 4E). It is important to note that all of these inhibitory receptors were also highly expressed on primary effectors after chronic infection, as compared to acute infection. However, expression of these receptors was highest on secondary effectors after chronic infection (Figure 4E).

To understand the role of inhibitory receptors in the loss of memory cells during antigen persistence, we performed *in vivo* functional studies. Blocking PD-1 or Lag-3 signaling by administering PD-L1 or Lag-3 blocking antibodies to mice infected with chronic LCMV after adoptive co-transfer of memory and naïve P14 cells did not lead to the increased survival of memory cells over naïve cells (data not shown). Therefore, while the inhibitory receptors PD-1 and Lag-3 play a role in the functional exhaustion of both the secondary and primary virus-specific effector $CD8^+$ T cells during persistent antigen stimulation, these receptors do not inhibit memory cells more than naïve cells. These results were consistent with the microarray data, since neither PD-1, nor Lag-3 was differentially expressed between primary and secondary effector $CD8^+$ T cells during

chronic infection. Thus, PD-1 and Lag-3 do not appear to play a role in the selective loss of secondary effectors during chronic antigen stimulation.

Next, because 2B4 was the inhibitory receptor most differential upregulated on memory cells compared to naïve cells, we sought to determine whether the increased expression of 2B4 played a functional role in the loss of secondary effectors during chronic infection. Because the available antibodies used to block 2B4 *in vitro* are cell depleting *in vivo*, we bred 2B4-deficient mice (*Cd244*$^{-/-}$) with LCMV-specific P14 TCR transgenic mice to obtain 2B4-deficient P14 TCR transgenic mice (*Cd244*$^{-/-}$ P14). We transferred memory and naïve wild-type or *Cd244*$^{-/-}$ P14 T cells into naïve mice and then subsequently infected the mice with chronic LCMV. The phenotype and function of the naïve *Cd244*$^{-/-}$ P14 cells versus wild-type naïve P14 cells as well as the memory *Cd244*$^{-/-}$ P14 cells versus wild-type memory P14 cells were identical pre-transfer (data not shown). Interestingly, the transferred 2B4 deficient-memory cells were able to persist during chronic infection, unlike wild-type (2B4 sufficient) cells ($p<0.01$ at days 7 &14, and $p<0.005$ at day 23), and there was no observable difference between the wild-type and 2B4-deficient naïve cells ($p>0.05$) (Figure 4F and 4G). These data document a role for 2B4 in the preferential loss of secondary effectors during antigen persistence

Memory cells are more dependent on CD4 T cell help than naïve cells during antigen persistence

We have established that inhibitory receptors play an enhanced role in regulating memory cells during chronic infection. Therefore, we next wanted to find a way to overcome the

increased regulation of these cells and enhance memory cell persistence during chronic infection. Because $CD4^+$ T cell help has been shown to be important for $CD8^+$ T cell function during viral infections (22, 42, 161, 162) we investigated whether increased LCMV-specific $CD4^+$ T cell help could rescue the transferred memory cells during chronic infection. To answer this question, we co-transferred LCMV GP61 epitope-specific $CD4^+$ T cells (Smarta) at the time of naïve and memory $CD8^+$ T cell co-transfer into normal $CD4^+$ intact B6 mice. After infection with chronic LCMV, secondary effectors in the mice receiving Smarta cells outnumbered the primary effectors in multiple tissues and the blood ($p < 0.005$) (Figures 5A-C). Overall, this resulted in greater than a 3 fold increase of memory cells in the spleens of mice receiving Smarta cells compared to those without, while the number of naïve cells remained similar between the two groups during chronic infection ($p<0.005$) (Figure 5B). These data indicate selective rescue of the transferred memory cells. The increase in secondary effectors was only seen during chronic infection, as neither the primary nor secondary effector $CD8^+$ T cell numbers changed with Smarta cell co-transfer during acute infection (Figures 5A-C). Furthermore, the rescue of the $CD8^+$ T cells by co-transfer of Smarta cells during chronic infection was not due to reduced viral loads at this early time point after $CD4^+$ T cell transfer, as both the chronically infected mice receiving Smarta cells and those without Smarta cells had serum viral titers between 10^5 and 10^6 $\log_{10}$ pfu/ml at day 6.5 p.i. (data not shown). Thus, our data indicate that during prolonged high antigen stimulation the memory $CD8^+$ T cells are more reliant on $CD4^+$ T cell help than naïve cells.

To understand the mechanism in which $CD4^+$ T cell help recues memory $CD8^+$ T cells during chronic infection, we first asked whether this rescue was linked to the increased immunoregulation of memory cells by 2B4. To address this, we assessed 2B4 expression on memory $CD8^+$ P14 T cells in chronically infected mice after transfer of Smarta cells. 2B4 was expressed at equivalent levels on memory $CD8^+$ T cells at day 6.5 post-infection whether or not they were rescued by added $CD4^+$ T cell help (Figure S4A), thus, suggesting that 2B4 does not play a role in the rescue of memory cells by $CD4^+$ T cell help during chronic infection, and instead these are two independent mechanisms.

Secondly, because CD40 and CD40L interactions have been shown to be important for $CD4^+$ T cell help of $CD8^+$ T cells (168, 169), we asked the question of whether CD40:CD40L interactions were playing a role in the rescue of memory $CD8^+$ T cells by CD4 T cell help. We co-transferred memory and naïve $CD8^+$ P14 T cells along with Smarta cells and blocked this pathway by the use of an antibody that blocks CD40L (MR1). We administered MR1 one day before infection with chronic LCMV (D^-1), on the day of infection (D0) and every three days thereafter, and sacrificed the mice at day 6.5 post-infection. The blockade of CD40:CD40L interactions in mice that did not receive Smarta cells resulted in a slight reduction of both memory and naïve P14 T cells, as it inhibits endogenous $CD4^+$ T cell help (Figure 5D). However, the number of naïve P14 T cells did not differ between the mice treated with MR1 and those treated with MR1 that received Smarta cells. The blockade of CD40:CD40L interactions resulted in a significant reduction in the expansion of memory P14 T cell numbers seen after Smarta cell transfer ($p<0.001$), showing that a mechanism in which $CD4^+$ T cell help rescue.

Furthermore, blockade of CD40L did not significantly affect Smarta cell expansion at this early time point (Figure S4B). Thus, our data indicates that during prolonged high antigen stimulation memory $CD8^+$ T cells are more reliant on $CD4^+$ T cell help than naïve cells and CD40:CD40L interactions may be involved.

The ability of naïve and memory $CD8^+$ T cells to control acute versus chronic infection

In order to better understand how the increased regulation of memory cells during chronic infection impacts protective immunity, we assessed the ability of memory and naïve cells to control LCMV infection. To address this, we performed two sets of experiments. In one experiment, we transferred a constant number of naïve or memory P14 T cells and varied the dose of LCMV infection, while in the second, we varied the number of naïve and memory P14 T cells transferred and infected the mice with the chronic dose of LCMV cl-13. To begin, we transferred $3X10^4$ naïve or memory P14 T cells (~ $3x10^3$ of each cells after accounting for an ~10% take of the cells post-injection) into mice that were subsequently infected with increasing doses of LCMV cl-13 and assessed viral titers in the spleens at days 2, 3 and 5 post-infection. By day 5 p.i. memory P14 T cells significantly decreased viral load in mice infected with $2x10^2$ and $2x10^4$ pfu of cl-13. However, in contrast, these low numbers of memory and naïve P14 T cells were incapable of reducing virus levels in mice infected with higher and more persistent doses of cl-13 ($2x10^5$ and $2x10^6$) (Figure 6A). These data indicate that on a per cell basis, memory cells are better than naïve cells at controlling acute or low dose infections,

however small numbers of memory or naïve cells are unable to control high dose and/or persistent infections.

Next, to determine the ability of naïve and memory cells to control virus in the case of high viral load, we titrated the number of memory and naïve P14 $CD8^+$ T cells that we transferred into mice that were subsequently infected with a chronic dose ($2X10^6$ pfu) of LCMV cl-13, and assessed viral titers in the serum. No reduction in viremia was seen in any group at day 4 post-infection. However, by day 8 p.i., the transfer of greater numbers of P14 T cells, $1x10^5$ or $2.5x10^5$, resulted in a larger expansion of memory cells in the PBMC and increased reduction of viral titers, in contrast to naïve cell transfers (Figures 6B and C). These data indicate that in cases where there are sufficient numbers of functional memory cells to rapidly reduce or clear the virus, memory $CD8^+$ T cells are more efficient than naïve cells. In contrast, in instances with lower numbers of naïve or memory cells present, where virus was not quickly reduced, the naïve cells outnumbered memory cells at day 8 post-infection, but there was little effect on viral load by either naïve or memory cells (Figures 6B and C). By day 14 p.i. there was a small, but significant, decrease in the viral titers of the mice receiving $1x10^3$ naïve P14 T cells compared to memory cells ($p=0.0327$), (Figure 6D), indicating that in some situations of high viral load where virus is not rapidly eliminated, naïve cells can be more effective at slightly reducing viral loads than memory cells. Taken together, these data show that memory cells provide effective control of low dose infection, and can provide control of high dose infections in situations where they are present in large enough numbers to rapidly control viral loads, thwarting the virus from persisting. However, in instances

where memory cells are unable to quickly clear infection, the cells undergo regulatory mechanisms and are rapidly lost.

Discussion

In this study, we reveal the unexpected limitations of memory $CD8^+$ T cells in the context of high viral load or persistent antigen. Memory cells provide quick and effective responses in the case of short-lived antigen stimulation. However, in stark contrast, our data show that memory cell function and survival is tightly regulated during high antigen load or antigen persistence. Unlike naïve $CD8^+$ T cells, memory cells have more TCR signaling molecules clustered at the TCR, increased loci accessibility of genes involved in effector functions, and amplified immediate cytokine production, creating a hyperresponsive state (170). Thus, increased regulation of memory cells compared to naïve cells may be an evolved mechanism to prevent excessive immunopathology in the host. Two recent reports by Hinrichs et al. show that adoptive transfer of effector cells derived from naïve rather than memory $CD8^+$ T cells mediate superior anti-tumor immunity in a mouse model, and secondly that after in vitro stimulation effector cells derived from naive human $CD8^+$ T cells have an increased proliferative capacity and are less terminally differentiated than effectors derived from memory human $CD8^+$ T cells, and, thus, primary effectors may be a superior cell population to use for adoptive immunotherapy (171, 172). Our data support the concept that secondary effectors are more terminally differentiated than primary $CD8^+$ effector T cells during chronic antigen stimulation. An increased expression of NK cell receptors are found on terminally differentiated or senescent human T cells (173), and in parallel, we found that NK cell receptors such as KLRG1 were up-regulated on secondary effectors during chronic infection.

Another property associated with senescence or terminal differentiation is reduced proliferative capacity. In this study, we demonstrate that virus-specific memory and naïve $CD8^+$ T cells are initially recruited similarly during situations of high antigen load. However, since in low dose acute infection, the memory cells already outnumber the naïve cells at this time point, this may suggest that the memory cells are already undergoing regulatory mechanisms before, or as early as, day 4 post-infection in situations of high dose infection. Moreover, we show both experimentally and by gene profiling, that after this equal initial recruitment of naïve and memory cells, memory cells have less proliferative capacity than naïve cells early in chronic infection. Thus, overall, constant antigen stimulation, like that seen in a high dose infection or antigen persistence, may quickly drive memory cells to a more terminally differentiated state.

The data presented here show that both primary and secondary effector $CD8^+$ T cells are prone to $CD8^+$ T cell exhaustion during chronic infection, as indicated by the microarray data and the decreased ability of the cells to make cytokines as infection progressed. Although the inhibitory receptors PD-1 and Lag 3 do not appear to play a role in the selective loss of secondary effectors, our data implicate 2B4 as an immunomodulatory molecule that regulates memory cell expansion or survival during persistent antigen stimulation. Consistent with 2B4 mRNA and protein being up-regulated on exhausted $CD8^+$ T cells during chronic LCMV, increased surface expression on virus-specific $CD8^+$ T cells is found during human chronic diseases, such as HCV, HBV, HIV, HTLV-1 and melanomas (83-87). The role of 2B4 on $CD8^+$ T cell responses has not been well studied. In addition, the dual stimulatory and inhibitory role that 2B4 is known to play on NK

cells further complicates our understanding of this molecule (82). 2B4 has been reported to be positively correlated with good human CTL function (159). Yet, in contrast, other studies indicate an inhibitory role for 2B4 on $CD8^+$ T cells in both human and mouse chronic infections (74, 83, 86). Furthermore, work performed with natural killer cells has shown that inhibitory function of 2B4 is related to increased 2B4 expression, increased receptor crosslinking, and lower expression of signaling lymphocyte activation molecule-associated protein (SAP) (82). Therefore, the fact that our gene profiling of primary and secondary effector $CD8^+$ T cells at day 8 post-acute and chronic LCMV infection showed that 2B4 is most highly up-regulated on the secondary effectors during chronic infection, and yet SAP expression was similar among all cell classes (primary and secondary effectors in both acute and chronic infection), is consistent with a negative regulatory role for 2B4 on memory cells during antigen persistence. In conclusion, we have found an important role for 2B4 in selectively affecting the survival and/or expansion of secondary $CD8^+$ T cell effectors during chronic infection, implying that a better understanding of the role of 2B4 on $CD8^+$ T cells is needed and may be helpful for vaccine design.

While it is well established that $CD4^+$ T cell help is most important during primary responses to create productive memory cells, our work now highlights the selective increased dependence of memory recall responses on $CD4^+$ T cell help in the context of antigen persistence. It is well known that $CD8^+$ T cells require $CD4^+$ T cell help during some mouse and human infections, including chronic infections (16, 22, 42, 161, 162). However, mainly these studies have addressed the role of $CD4^+$ T cell help during the generation of primary $CD8^+$ T cell responses and how this loss of $CD4^+$ T cell help during

initial priming affects the recall responses of these cells, rather than focusing on whether $CD4^+$ T cell help is important during memory recall responses. Surprisingly, our data indicate that memory virus-specific $CD8^+$ T cell responses are more reliant on $CD4^+$ T cell help than naive virus-specific $CD8^+$ T cell responses during antigen persistence. These data indicate that $CD4^+$ T cell help may be an important component of vaccine strategy for chronic infections. Thus, there is a need to better understand the mechanism and requirements for $CD4^+$ T cell help of secondary $CD8^+$ T cell responses during chronic infection to aid in optimal vaccine design for these infections.

These data presented in this paper have important implications towards the design of T cell vaccines against chronic infections and tumors. While memory $CD8^+$ T cells are much more effective than naïve cells at controlling various acute infections, our data show that they are tightly regulated in situations of high viral load or antigen persistence. When left with low numbers of antigen and/or pathogen-specific memory $CD8^+$ T cells post-vaccination, and subsequent infection resulting in high doses of antigen or persistent antigen that is not rapidly controlled, our data indicate that these memory cells are unable to persist due to increased regulatory mechanisms. Furthermore, these memory cells are more reliant on $CD4^+$ help, implying that an important component of vaccine design in situations of high antigen chronicity may be the ability to elicit and maintain large numbers of antigen-specific $CD4^+$ T cells. However, our data also indicate that designing vaccines which result in large pools of highly functional memory $CD8^+$ T cells that rapidly lower antigen loads can provide viral control of high dose or persistent infections, an idea supported by previous experiments in SIV and LCMV (174, 175). Moreover, two

recent human HIV vaccine trials, the Merck STEP trail and Thai RV144 trail, show little efficacy in reducing the set-point of infection during chronic infection, however in the RV144 Thai trial some efficacy is seen in the form of decreased acquisition of infection (176, 177). Lastly, a recent vaccine study in the SIV model shows efficacy when virus was rapidly controlled early after acquisition (178). These studies emphasize the importance of designing vaccines that quickly control or prevent chronic infections. In cases where infection is rapidly controlled, memory cells are unlikely to become subject to this tight regulatory control. Thus, the number and quality of memory $CD8^+$ T cells elicited by a vaccine may greatly impact the success of vaccines for high dose or persistent infection. Furthermore, designing vaccines that elicit large pools of $CD4^+$ T cell help and/or increasing strategies to overcome immunoregulation by inhibitory receptors, may be necessary for a successful vaccine outcome.

Experimental Procedures

Mice and Infections

Six-week old female C57BL/6 mice were purchased from the Jackson Laboratory. P14 TCR transgenic mice and Smarta TCR transgenic mice were bred in house. 2B4 deficient (*Cd244*$^{-/-}$) P14 TCR transgenic mice were made by breeding P14 TCR transgenic mice with *Cd244* knockout mice (*CD244*$^{-/-}$). Cd244$^{-/-}$ mice were generated on using C57BL/6 ES cells (Bruce 4) by replacement of exon 2 and 3 by a LoxP site and were kept on the C57BL/6 background (S. Calpe et. al, manuscript in preparation). All *Cd244*$^{-/-}$ P14 mice were analyzed for P14 TCR expression and genotyped before use to make sure that both alleles of 2B4 were interrupted. For acute infections mice were infected with either $2x10^5$ pfu Arm ip or $2x10^2$ pfu cl-13 iv, unless otherwise noted. For chronic infections mice were infected with $2x10^6$ pfu cl-13 iv. For persistent Ad-5 GP infection, mice were infected with 10^{10} vp Ad-5 GP im. Ad-5 GP was kindly provided by G.A. Spies and M.J. McElrath (Fred Hutchinson Cancer Research Center). Virus levels were assayed by plaque assays as previously described (4). All mice were used in accordance with the Emory University Institutional Animal Care and Use Committee Guidelines.

Cell transfers

We created memory cells using the LCMV-specific TCR transgenic system as described (24). Briefly, we transferred a small number of P14's (~$1x10^5$), unless otherwise noted, and then infected the mice with $2x10^5$ pfu Arm ip. For the comparison of multiple vaccines/viruses, $1x10^4$ P14 were transferred in to mice that were then infected with

$2X10^5$ pfu Arm LCMV ip or $1x10^6$ pfu VV-33 ip or 10^9 vp of AD-5 GP im. Memory cells were isolated at >45 days p.i. using $CD8^+$ T cell isolation kit (Miltenyi biotech) or alternatively by sorting on $CD8^+$ $Thy1.2^-$ $CD62L^+$ or $CD62L^-$ cells on a BD FACS Vantage or BD Aria. $1x10^3$ (or $5x10^3$ for analysis at day 4 p.i.) of each memory and naïve P14 T cells were transferred 1 day pre-infection into mice for all co-transfer experiments. $1x10^6$ Smarta CD4 T cells were transferred after isolation from spleens of naïve Smarta TCR transgenic mice using a $CD4^+$ T cell isolation kit (Miltenyi biotech).

Lymphocyte isolation and flow cytometry

Lymphocytes were isolated from the spleen, liver, lung, lymph nodes, IEL, bone marrow and blood as previously described (60, 73). All antibodies were purchased from BD Biosciences (San Diego, CA) except CD44, Thy1.1 and Thy1.2 (Biolegend) and anti-PD-1, anti-Tim-3 (ebioscience). MHC Class I tetramers were prepared and used as previously described (4). Intracellular cytokine staining was performed as previously described (4). Cells were analyzed on a Canto or LSR II flow cytometer (BD Immunocytometry Systems). Dead cells were excluded by gating on Live/Dead NEAR IR (Invitrogen).

***In Vivo* Blockade and other *in vivo* Treatments**

For blockade of the PD-1 pathway, 200μg of rat anti-mouse PD-L1 antibody (10F.9G2 prepared in house) or rat IgG2b isotype control was administered intraperitoneally (ip) on day 0, 3 and 6. For blockade of the Lag-3 pathway, 200μg of rat anti-mouse Lag-3 (C9B7W from Biolegend) antibody was administered ip on day 0, 3 and 6. For blockade

of CD40L (CD154), 500ug of hamster anti-mouse CD154 (MR1 from BioXcell) antibody was administered ip on day -1, 0, 3 and 6.

Proliferation by *in vivo* BrdU incorporation

For assessment of proliferation by BrdU, mice were given 1mg of Brdu ip at day 6 and 8 p.i. and sacrificed 6 hours later. Brdu staining was carried out using the APC or FITC Brdu Kit (BD Biosciences) according to manufacturers instructions.

Microarray and analysis

Day 8 primary and secondary effectors from acute and chronic LCMV infected mice were FACS sorted based on $CD8^+$ and the congenic markers Thy1.1 and Thy1.2. RNA was isolated using a RNeasy kit (Qiagen) according to manufacturer's protocol. RNA was amplified, biotinylated and hybridized on mouse 430.2 Affymetrix microarray chips at the Functional Genomics Shared Resource (Vanderbilt University, TN, USA). Prior to analysis, microarray data were pre-processed and normalized using robust multi-chip averaging, as previously described (179). Genes that are differentially expressed between two classes were ranked using the GenePattern software package (180). The statistical significance of differentially expressed genes and hierarchical clustering was performed using GenePattern (180). Gene set enrichment analysis (GSEA) was performed as described previously (181).

Statistical Analysis

All data were analyzed using Prism 5.0 (GraphPad).

Acknowledgements:

We thank R. Karaffa and S. Durham for FACS sorting at the Emory University School of Medicine Flow Cytometry Core Facility, and we thank the members of the Ahmed Lab for helpful discussions. We also thank Rama Akondy for initial help with the microarray analysis. This work was supported by the National Institutes of Health (NIH) grant R01 AI030048 (to R.A.) and the American Cancer Society (ACS) postdoctoral fellowship PF-09-134-01-MPC (to B.Y.).

Accession numbers:
The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.hih.gov/gds) under the accession number GSE30962

Figures

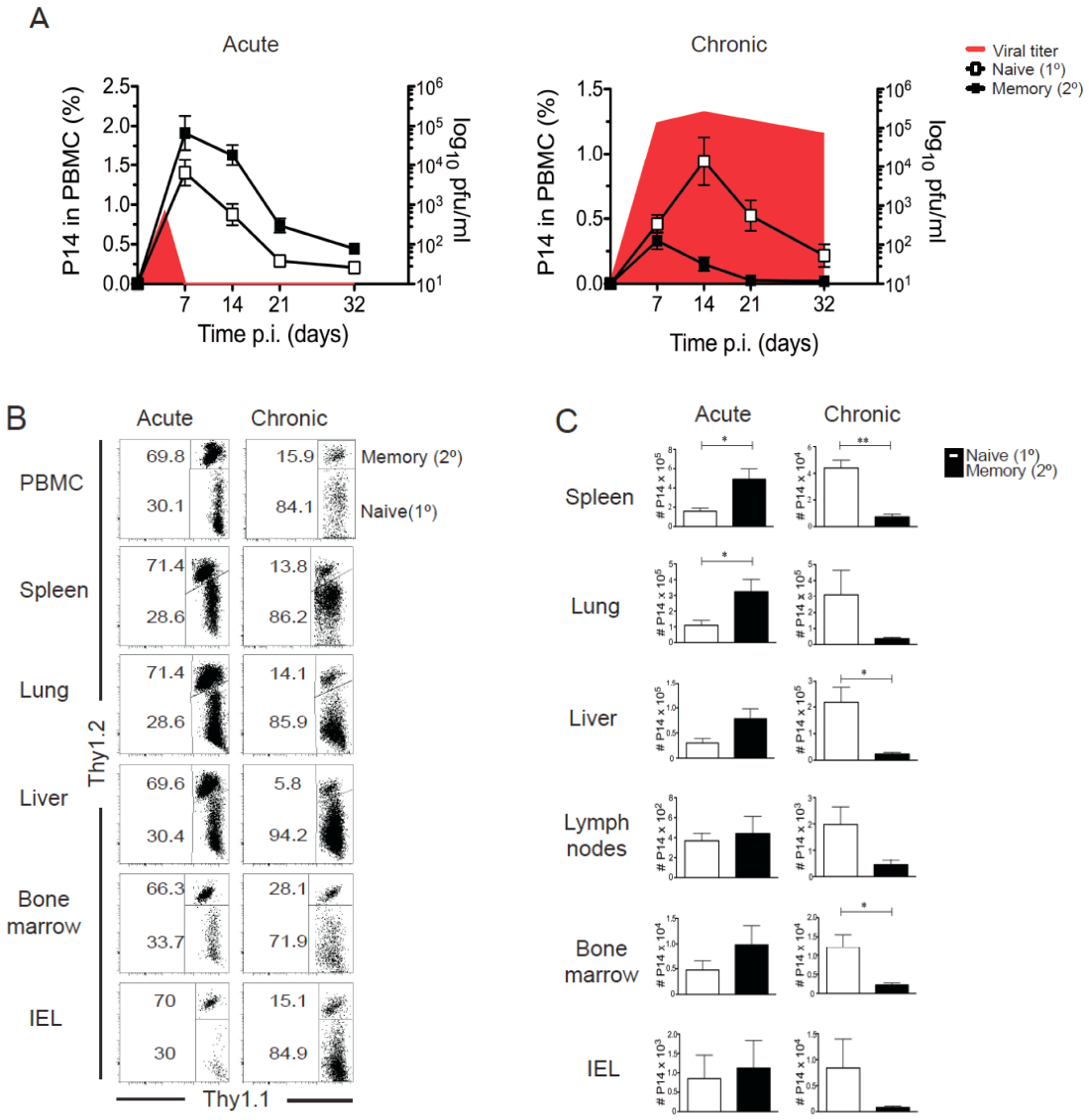

Figure 1: Naïve and memory CD8 T cell responses during acute versus chronic infection. $1x10^3$ of each memory ($Thy1.1^+1.2^+$) P14 and naïve P14 T cells ($Thy1.1^+1.2^-$) were co-transferred into naïve B6 mice. On the following day the mice were infected with either LCMV Arm (Acute) or LCMV cl-13 (chronic). Blood was taken at days 7, 14, 21 & 32 post-infection. (A) Percent of transferred memory and naïve P14 cells in the blood after infection. (B and C) Cells were co-transferred as above and mice were infected with either an acute ($2x10^2$ pfu) or chronic ($2X10^6$ pfu) dose of LCMV cl-13 (B) Representative dot plots and (C) numbers of transferred cells in the tissues day 14 p.i. Results representative of two to six independent experiments with 4-6 mice per group. Statistical comparisons were performed with the unpaired Students *t* test *p<0.05, **p< 0.01. Error bars represent SEM.

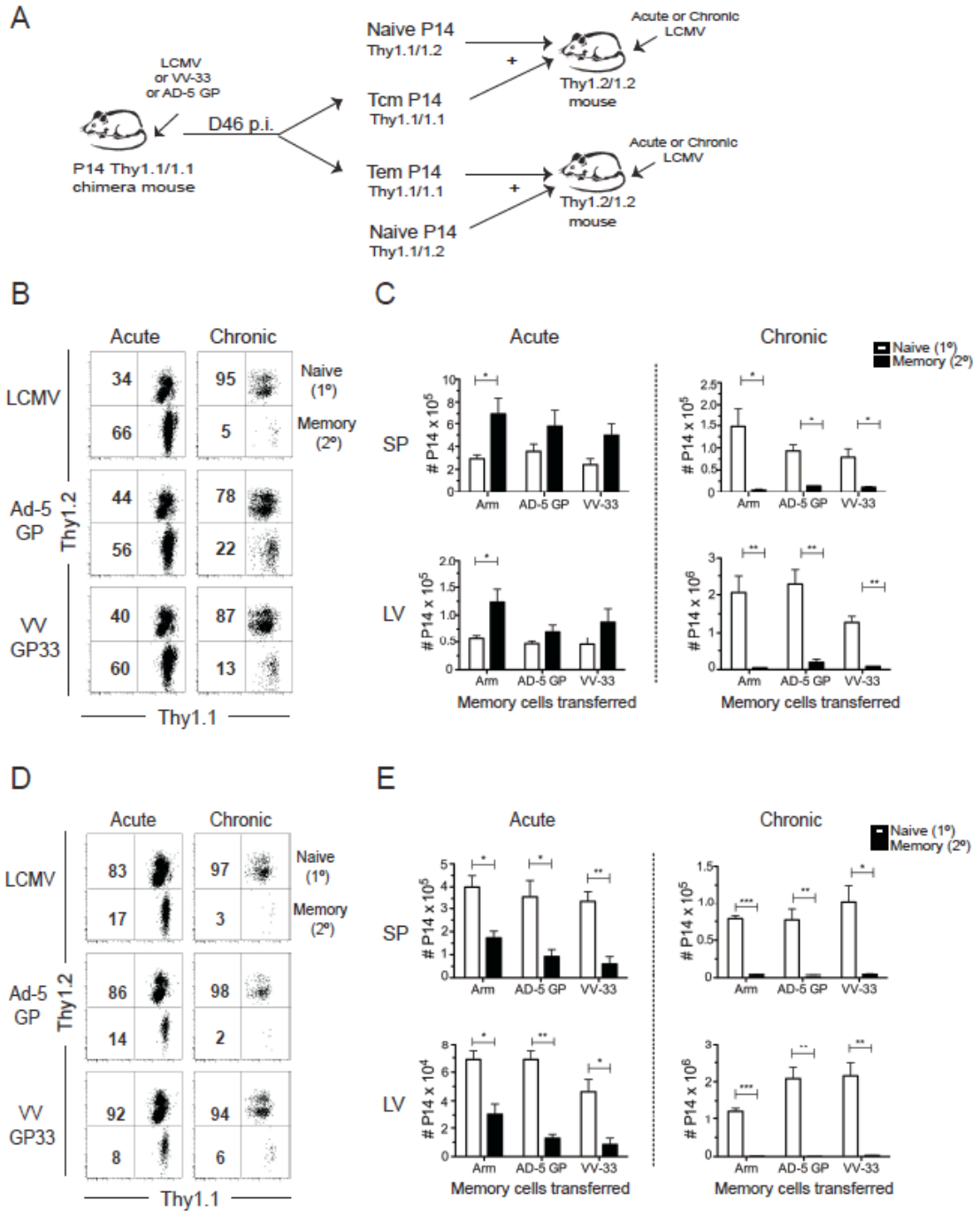

Figure 2: Memory cells are preferentially lost during chronic antigen exposure, regardless of the vaccine or virus used to generate the memory cells. Memory P14's (Thy1.1$^+$1.2$^-$) were generated by either Arm, Ad-5 GP or VV-33 infection. At day 46 p.i. memory cells were sorted based on CD62L expression. Tcm (CD62L$^+$) and Tem (CD62L$^-$) cells were each co-transferred with naïve P14's (Thy1.1$^+$1.2$^+$) and the mice were infected the following day with an acute (2X10^2 pfu) or chronic (2X10^6 pfu) dose of LCMV cl-13. (A) Experimental set-up. (B) Representative flow plots of Tcm memory and naïve P14 cells in the blood at day 14 p.i. and (C) numbers in the tissues at day 26 p.i. (D) Representative flow plots of Tem memory and naïve P14 cells in the blood at day 14 p.i. and (E) numbers in the tissues at day 26 p.i. Results representative of 6 mice per group. *p<0.05, **p< 0.01, ***p<0.005. Error bars represent SEM.

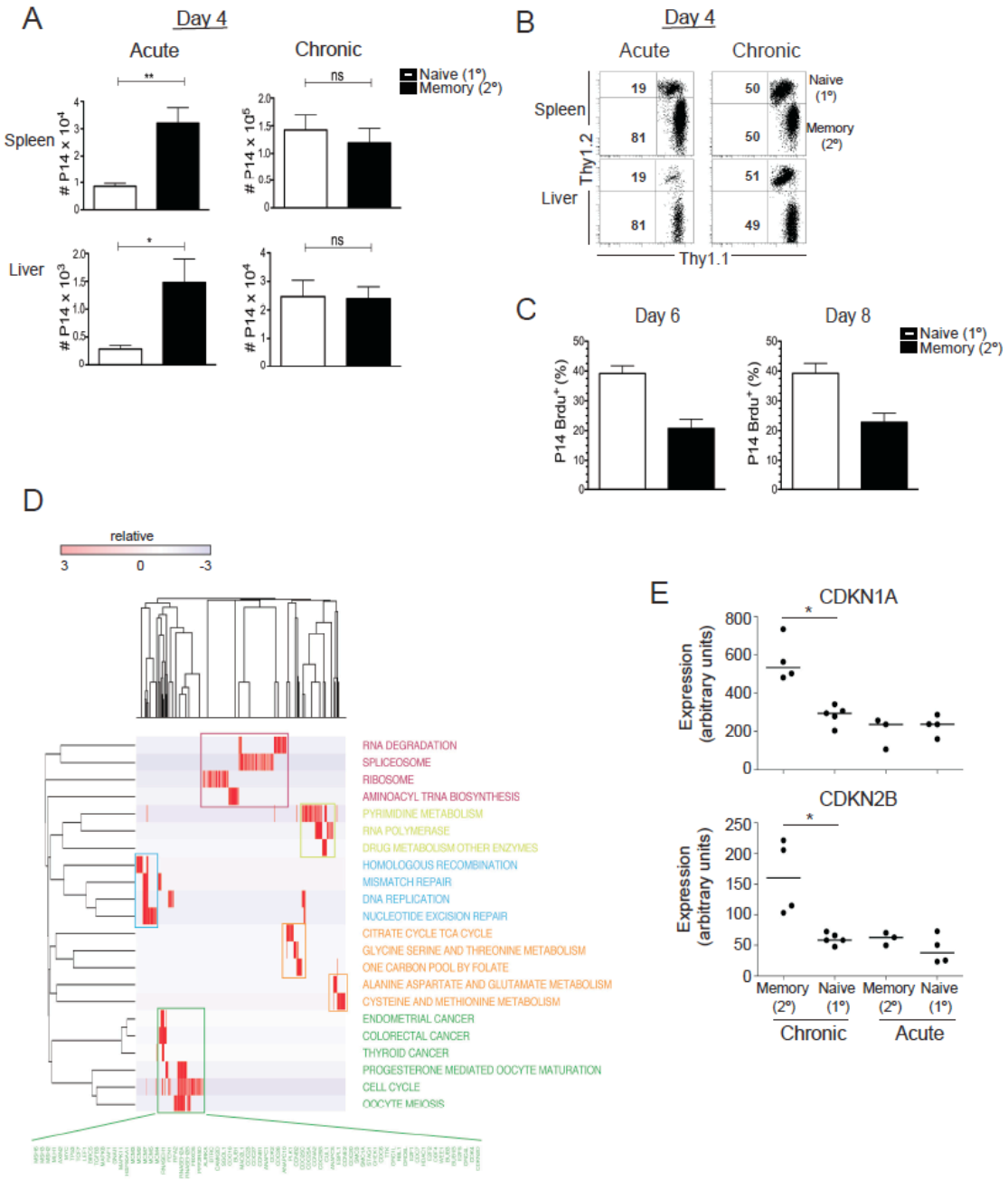

Figure 3: Initial recruitment of naïve and memory cells is similar during chronic infection, however memory cells have a block in sustaining cell proliferation. (A and B) $5X10^3$ of each memory ($Thy1.1^+1.2^-$) and naïve ($Thy1.1^+1.2^+$) P14 cells were transferred into B6 mice and the mice were infected the following day with either an acute ($2x10^2$ pfu) or chronic ($5X10^6$ pfu) dose of LCMV cl-13. (A) Numbers and (B) representative flow plots of naïve and memory P14's in the spleens and livers at day 4 p.i. (C) $1X10^3$ of each memory ($Thy1.1^+1.2^+$) and naïve ($Thy1.1^+1.2^-$) P14 cells were transferred into B6 mice and the mice were infected the following day with $2X10^6$ cl-13. Percent of P14 cells that are $Brdu^+$ 6 hours after Brdu i.p. injection at days 6 & 8 p.i. (D) Modular view of genes upregulated in primary vs secondary effectors at day 8 post-chronic infection. Genes upregulated in primary effectors were tested for enrichment with the KEGG collection of annotated gene-sets corresponding to major biological processes using Gene set enrichment analysis (GSEA). The expression of genes contained in the leading edges of gene-sets that were significantly enriched are displayed as a heatmap matrix, and clustered by gene (column) and row (gene-set). A cluster of gene sets related to RNA processing are colored purple; nucleotide synthesis, yellow; DNA replication, blue; metabolism, orange; and proliferation green. Genes contained in the proliferation module are listed in green. (E) Relative gene expression values of CDKNA1 (p21/Cip) (P=0.002) and CDKNB2 (p15) (P=0.002). Results representative of two or three independent experiments with 4-6 mice per group (A-C). *p<0.05, **p< 0.01, ns=p>0.05 (A-C). Error bars represent SEM.

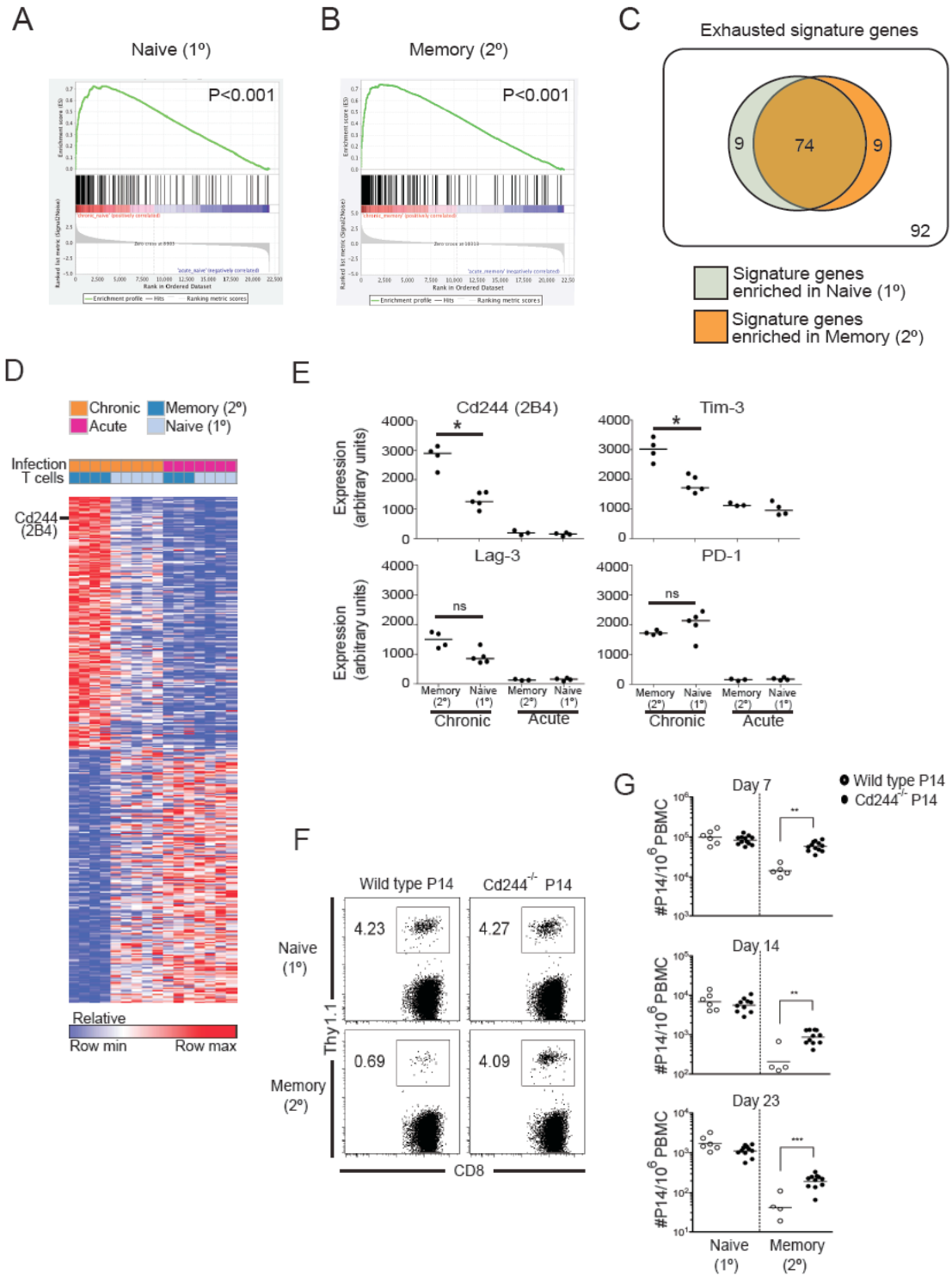

Figure 4 (legend on next page)

Figure 4: The role of 2B4 in regulating virus-specific memory CD8 T cells during chronic infection
(A and B) Gene set enrichment analysis (GSEA) of a signature of genes associated with exhausted CD8 T cells in the rank ordered list of genes differentially expressed in (A) primary effectors at day 8 in chronic vs. acute infection and (B) secondary effectors at day 8 in chronic vs. acute infection. (C) Genes at the leading edge of enrichment in primary effector GSEA (green set) and secondary effector GSEA (orange set) are largely overlapping. (D) Genes differentially expressed at day 8 in secondary effectors in chronic infection compared to secondary effectors in acute infection and primary effectors in either infection. Each column represents an individual sample, each row a gene and cells colored to indicate relative expression. Top 200 genes upregulated or downregulated are shown, ranked by the signal-to-noise metric, 2B4 (Cd244) is indicated. (E) Relative expression values of Cd244, Tim-3, Lag-3 and PD-1 (F and G) $2X10^3$ Wild-type (2B4 sufficient) or 2B4-deficient ($Cd244^{-/-}$) naïve and memory P14's (all $Thy1.1^+1.2^+$) were individually transferred into mice and on the following day the mice were infected with $2x10^6$ pfu cl-13 i.v. (F) Representative flow plots of P14's in the blood at day 7 p.i. and (G) number of P14 T cells per 10^6 PBMC at days 7,14 &23 post-infection. Results representative of 12 mice per group (F and G). *p<0.05, **p< 0.01, ***p<0.005 (F and G).

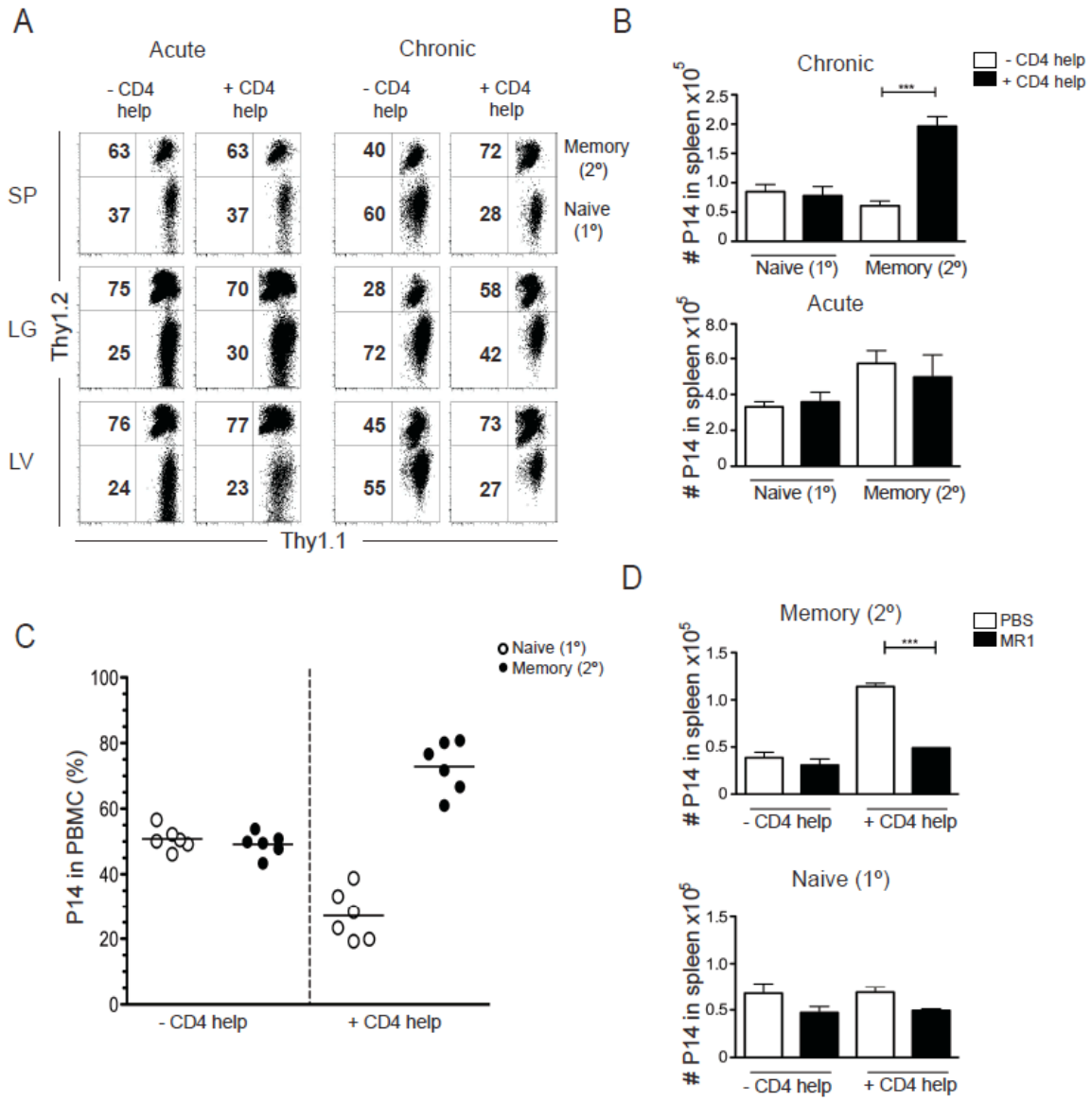

Figure 5: The role of CD4 T cell help in primary and secondary responses during acute versus chronic infection. $1x10^3$ of each memory ($Thy1.1^+1.2^+$) and naïve ($Thy1.1^+1.2^-$) P14's were co-transferred with or without $1X10^6$ LCMV-GP61 specific CD4 T cells (Smartas) into naïve mice. The next day mice were infected with either an acute ($2X10^2$) or chronic ($2X10^6$) dose of LCMV cl-13.(A) Representative dot plot of naïve and memory P14 T cells in the spleen, liver and lung at day 6.5 p.i. (B) Numbers of naïve and memory P14 T cells in the spleen at day 6.5 p.i. (C) Percent of naïve and memory P14 T cells in blood at day 6.5 p.i. (D) Mice were set-up as in A-D and were treated with MR1 antibody or PBS on day-1,0,3 and 6 p.i. Numbers of naïve and memory P14 T cells in spleen at day 6.5 p.i. In the figures and legends, +CD4 help indicates mice receiving Smarta cells, - CD4 help indicates mice that do not receive Smarta cells. Results are representative of two (D) or three (A-C) independent experiments with 4-6 mice per group. ***p<0.005. Error bars represent SEM.

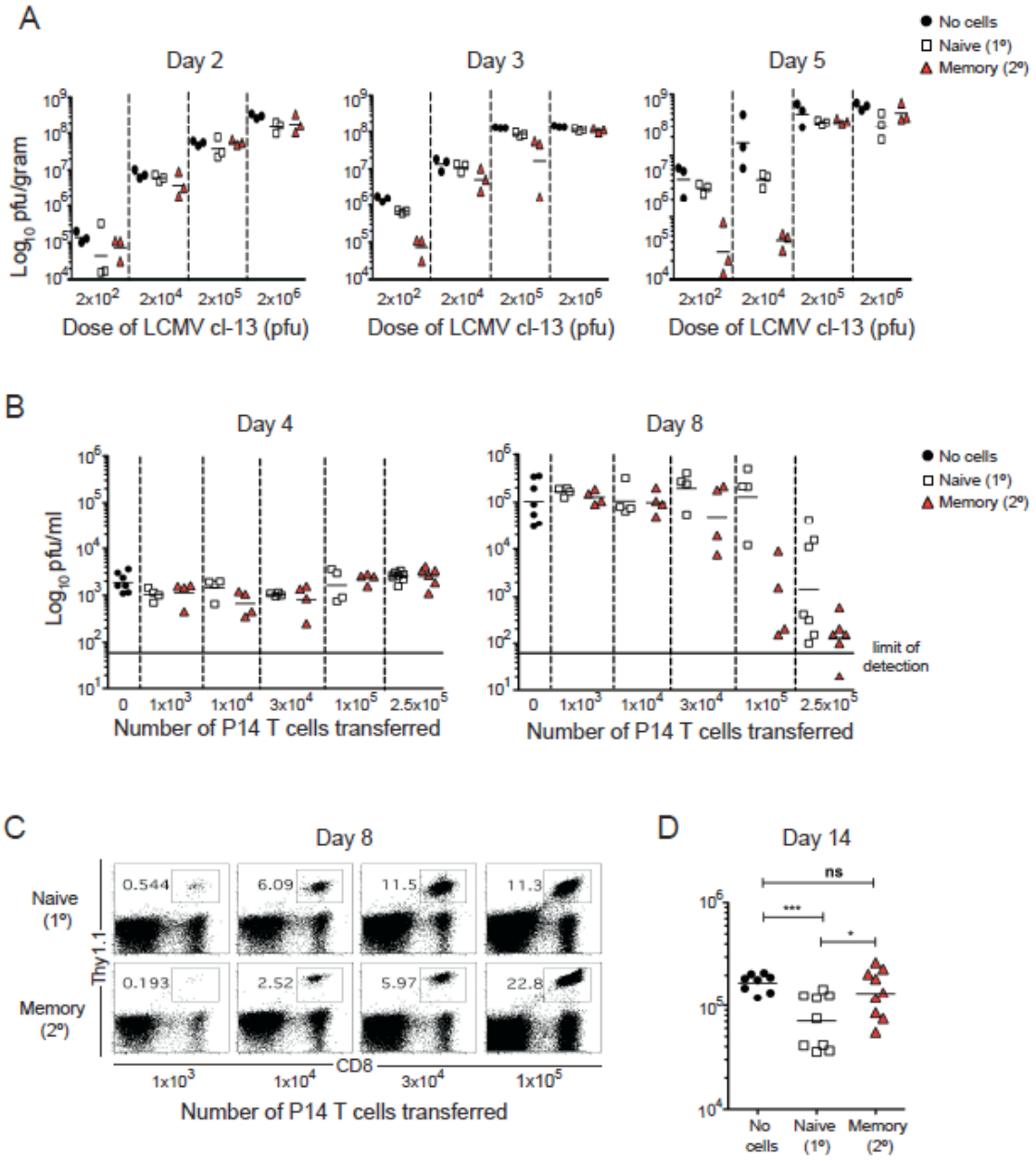

Figure 6: The ability of naïve and memory cells to control LCMV infection. (A and B) $3x10^4$ of memory or naïve P14 T cells were transferred into naïve B6 mice. The next day the mice were infected with $2x10^2$, $2x10^4$, $2x10^5$ or $2x10^6$ pfu of LCMV cl-13 iv. (A) Viral titer in the spleen on days 2, 3 and 5 p.i., as assayed by plaque assay on Vero E6 cells. (B-D) $1x10^3$, $1x10^4$, $3x10^4$ $1x10^5$ or $2.5x10^5$ naïve or memory Thy1.1$^+$ P14 T cells were transferred into naïve mice. The next day mice were infected with $2x10^6$ pfu of LCMV cl-13 iv. (B) Viral titer in the serum on days 4, and 8 p.i. (C) Representative dot plots of naïve and memory P14 T cells in the PBMC at day 8 p.i. (D) Viral titer in the serum in mice receiving either no cells, or $1x10^3$ memory or naïve P14 cells on day 14 p.i. Results are representative of 3-6 mice per group per time point. *p<0.05, ***p<.005, ns=p>0.05.

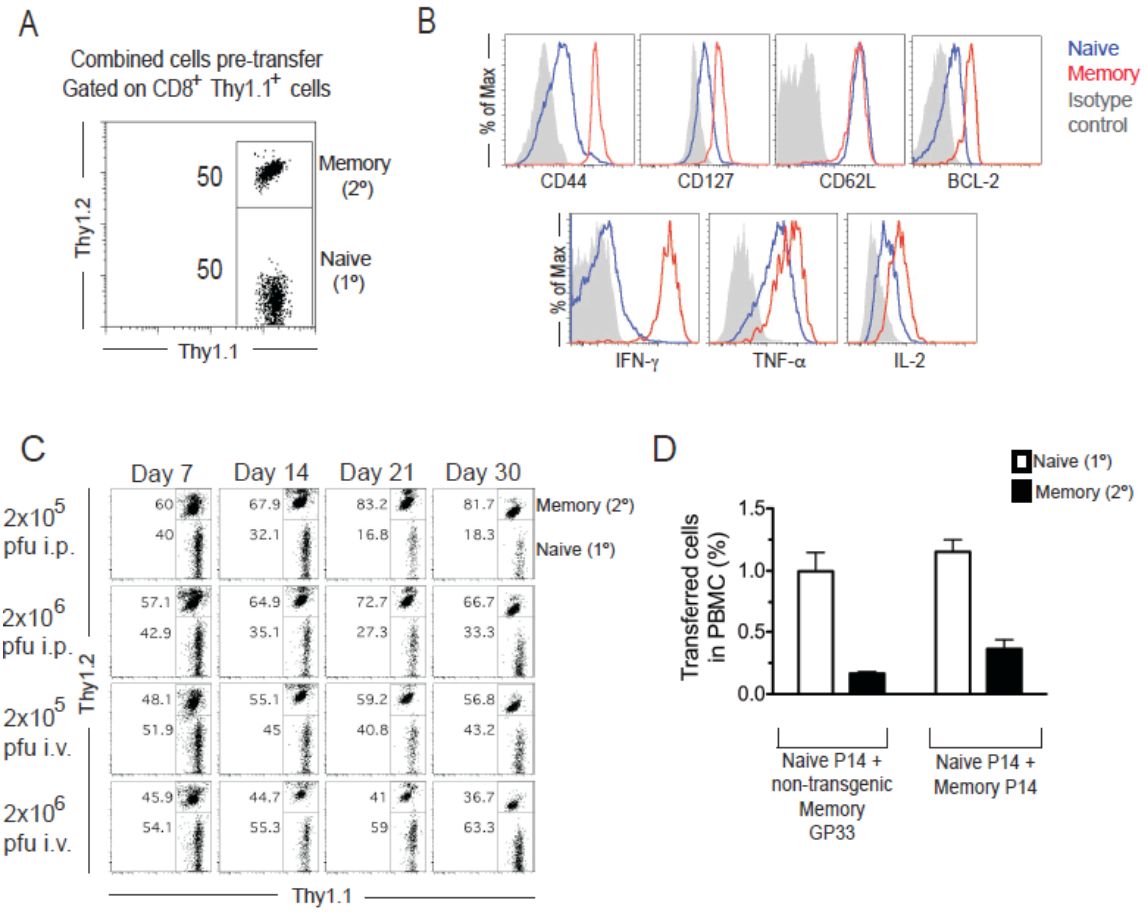

Figure S1: Increased amounts of antigen or antigen persistence negatively influences both TCR transgenic and non-transgenic memory CD8⁺ T cells. (A) Equal numbers of memory and naïve P14's were combined and the ratio of the two cell populations was verified by flow cytometry pre-transfer into the mice based on differential Thy1.1 and Thy1.2 expression (B) The expression of CD44, CD62L and CD127 on naïve and memory P14 cells pre-transfer into mice was assessed by multi-parameter flow cytometry. (C) $1X10^3$ of each memory ($Thy1.1^+1.2^+$) and naïve ($Thy1.1^+1.2^-$) P14 cells were co-transferred into B6 mice and the mice were infected the following day with $2x10^5$ or $2x10^6$ pfu Arm ip or iv. (C) Representative flow plots of naïve and memory P14's in the blood at day 7,14,21 and 30 p.i. (D) LCMV D^bGP33-41 specific memory cells were sorted from the spleens of LCMV immune mice at day 62 p.i. based on $CD8^+$ and D^bGP33^+ tetramer staining. Naive P14 cells were also sorted on $CD8^+$ and D^bGP33^+ tetramer staining. Memory P14 cells were sorted from the spleens of Armstrong immune chimeras at day 62 p.i based on $CD8^+$ and D^bGP33^+ tetramer staining and $Thy1.2^-$ staining. $1X10^3$ naïve P14 cells were co-transferred with $1x10^3$ of either LCMV D^bGP33-41 specific memory cells or P14 memory cells into B6 mice that were infected with $2x10^6$ LCMV cl-13 the following day. (D) Percent of transferred naïve P14's versus memory P14's or non-transgenic LCMV D^bGP33-41 specific memory cells in the blood at day 7 p.i. Results representative of (A and B) twenty independent experiments, (C and D) 4-6 mice per group. Error bars represent SEM.

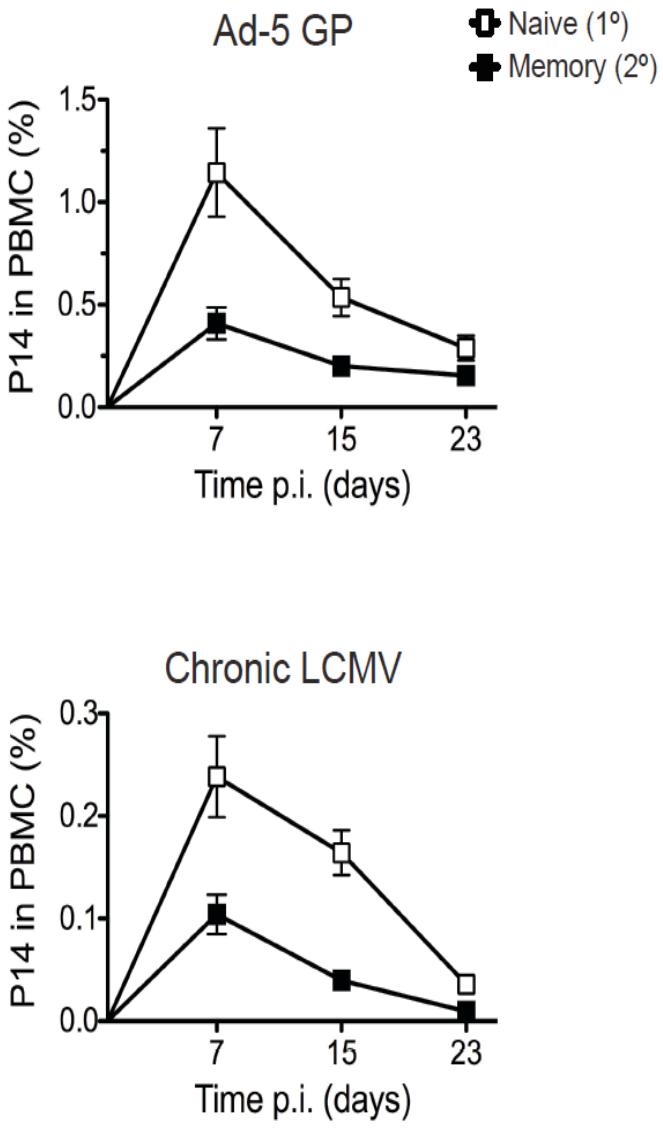

Figure S2: Loss of memory cells occurs during antigen persistence. $1X10^3$ of each memory ($Thy1.1^+1.2^+$) and naïve ($Thy1.1^+1.2^-$) P14 cells were co-transferred into B6 mice and the mice were infected the following day with either $2x10^6$ pfu of LCMV cl-13 or 10^{10} virus-particles of Ad-5 GP. Percent of naïve and memory P14 T cells in the blood at day 7,14 and 23 p.i. Results representative of two independent experiments of 4-6 mice per group. Error bars represent SEM.

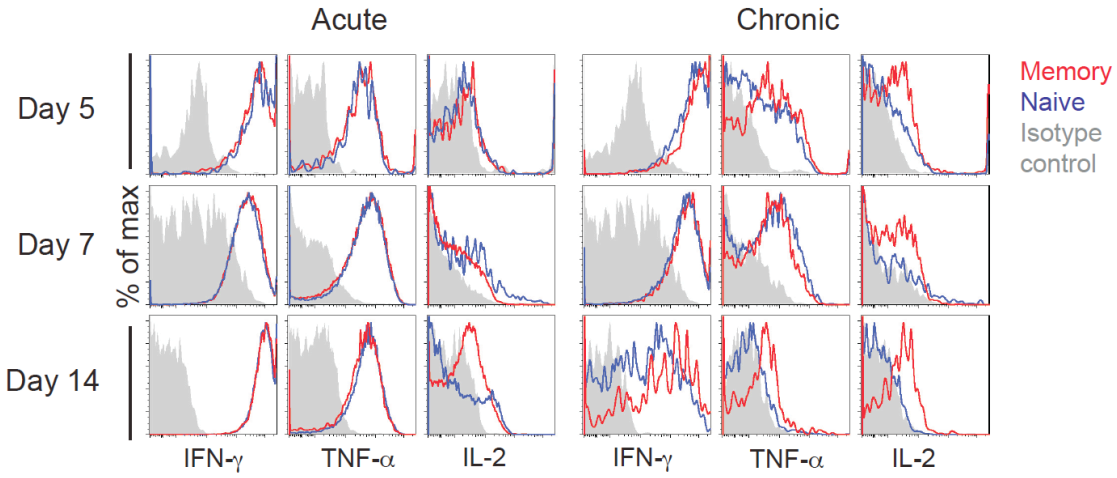

Figure S3: Functional exhaustion of secondary and primary P14 T cells post-chronic infection. IFN-γ, TNF-α and IL-2 production by primary and secondary effector P14 T cells after *ex-vivo* stimulation for 5 hours with GP33-41 peptide at day 5,7 and 14 after acute or chronic LCMV infection, as assayed by flow cytometry. Results representative of two to eight independent experiments, dependent upon time point.

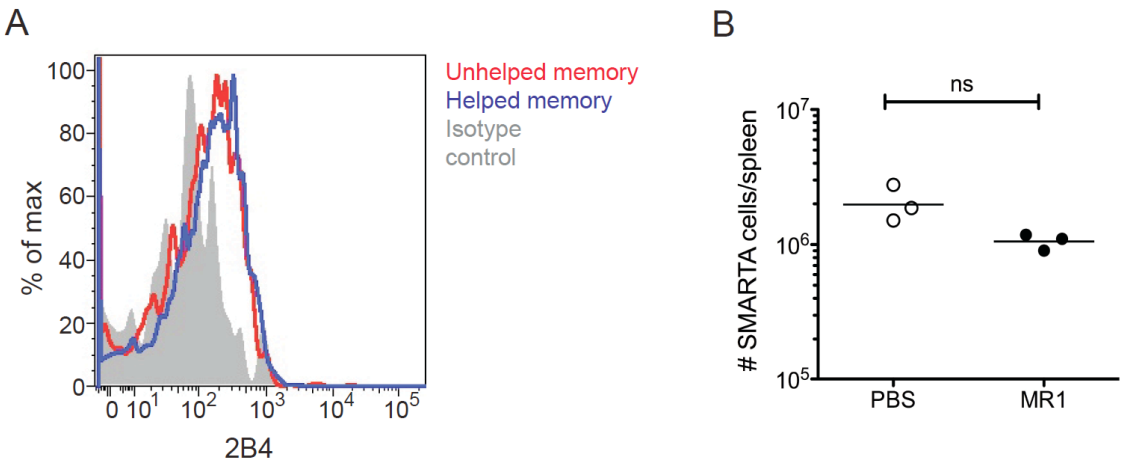

Figure S4: CD4 T cell help of memory CD8 T cells during chronic infection is unrelated to regulation by 2B4 and involves CD40:40L interactions. (A and B) $1x10^3$ memory and naïve P14 T cells were co-transferred with or without $1x10^6$ Smarta cells into naïve mice. The following day the mice were infected with $2x10^6$ pfu cl-13. (A) Representative histogram of 2B4 expression on transferred memory cells at day 6.5 p.i. from mice with smarta cell co-transfer (blue) or without smarta cell transfer (red). Gated on $Thy1.1^+$ $Thy1.2^-$ $CD8^+$ memory P14 T cells. (B) mice were treated with either PBS or MR1 on day -1,0,3 and 6 p.i. Number of Smarta cells in the spleen of mice receiving Smarta cell co-transfers along with naïve and memory P14 T cells. Gated on $Ly5.1^+$ $CD4^+$ $V\alpha2^+$ Smarta cells. Results representative of two independent experiments of 3-5 mice/group.

Transcript	Description	Fold Change (memory vs. naive)	P-value	FDR(BH)
PERP	PERP:PERP, TP53 apoptosis effector	2.14	0.02	0.08
ENPP1	ENPP1:ectonucleotide pyrophosphatase/phosphodiesterase 1	1.27	0.02	0.08
4631408O11RIK	na	1.38	0.04	0.13
GPR65	GPR65:G protein-coupled receptor 65	1.29	0.02	0.08
LITAF	LITAF:lipopolysaccharide-induced TNF factor	1.20	0.02	0.08
MDFIC	MDFIC:MyoD family inhibitor domain containing	1.59	0.03	0.12
ALCAM	ALCAM:activated leukocyte cell adhesion molecule	1.27	0.03	0.10
CCRL2	CCRL2:chemokine (C-C motif) receptor-like 2	1.74	0.07	0.18
RGS16	RGS16:regulator of G-protein signalling 16	1.46	0.05	0.17
CCL3	CCL3:chemokine (C-C motif) ligand 3	1.22	0.13	0.23
IRF8	IRF8:interferon regulatory factor 8	1.38	0.11	0.22
PBX3	PBX3:pre-B-cell leukemia transcription factor 3	1.53	0.09	0.21
ITGAV	ITGAV:integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	1.26	0.10	0.21
LAT2	LAT2:linker for activation of T cells family, member 2	1.55	0.11	0.22
PGLYRP1	PGLYRP1:peptidoglycan recognition protein 1	1.15	0.16	0.26
GPD2	GPD2:glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	1.11	0.16	0.26
LIMS1	LIMS1:LIM and senescent cell antigen-like domains 1	1.12	0.24	0.36
NAPSA	NAPSA:napsin A aspartic peptidase	1.20	0.29	0.40
SH2D2A	SH2D2A:SH2 domain protein 2A	1.15	0.32	0.43
IFIH1	IFIH1:interferon induced with helicase C domain 1	1.15	0.30	0.40
NFATC1	NFATC1:nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	1.23	0.52	0.62
BCL2A1A /// BC	na	1.04	0.49	0.61
IRF4	IRF4:interferon regulatory factor 4	1.11	0.66	0.73
CLIC4	CLIC4:chloride intracellular channel 4	1.04	0.68	0.74
NAB1	NAB1:NGFI-A binding protein 1 (EGR1 binding protein 1)	1.02	0.90	0.94
ANKRD28	ANKRD28:ankyrin repeat domain 28	1.01	0.96	0.97
IIGP1	na	1.01	0.91	0.94
LAMP2	LAMP2:lysosomal-associated membrane protein 2	1.00	0.99	0.99
PLSCR1	PLSCR1:phospholipid scramblase 1	0.97	0.77	0.81
STK39	STK39:serine threonine kinase 39 (STE20/SPS1 homolog, yeast)	0.96	0.69	0.74
AHR	AHR:aryl hydrocarbon receptor	0.92	0.62	0.70
LYCAT	LYCAT:lysocardiolipin acyltransferase	0.89	0.60	0.70
GJB2	GJB2:gap junction protein, beta 2, 26kDa (connexin 26)	0.89	0.51	0.62
GAS2	GAS2:growth arrest-specific 2	0.94	0.64	0.71
2610524H06RIK	na	0.94	0.46	0.58
BMP2K	BMP2K:BMP2 inducible kinase	0.94	0.56	0.65
ACADL	ACADL:acyl-Coenzyme A dehydrogenase, long chain	0.93	0.48	0.60
DNAJC13	DNAJC13:DnaJ (Hsp40) homolog, subfamily C, member 13	0.89	0.35	0.46
CASP3	CASP3:caspase 3, apoptosis-related cysteine peptidase	0.93	0.29	0.40
RYR1	RYR1:ryanodine receptor 1 (skeletal)	0.77	0.20	0.31
EGR2	EGR2:early growth response 2 (Krox-20 homolog, Drosophila)	0.85	0.34	0.45
ATPIF1	ATPIF1:ATPase inhibitory factor 1	0.93	0.25	0.36
HCCS	HCCS:holocytochrome c synthase (cytochrome c heme-lyase)	0.82	0.25	0.36
DDIT4	DDIT4:DNA-damage-inducible transcript 4	0.93	0.22	0.34
TANK	TANK:TRAF family member-associated NFKB activator	0.83	0.18	0.29
VAMP8	VAMP8:vesicle-associated membrane protein 8 (endobrevin)	0.83	0.14	0.24
AZIN1	AZIN1:antizyme inhibitor 1	0.76	0.14	0.24
PDCD1	PDCD1:programmed cell death 1	0.86	0.12	0.22
2010100O12RIK	na	0.79	0.10	0.21
EEA1	EEA1:early endosome antigen 1, 162kD	0.82	0.09	0.21
BRCA2	BRCA2:breast cancer 2, early onset	0.70	0.13	0.23
RFC3	RFC3:replication factor C (activator 1) 3, 38kDa	0.74	0.10	0.21
LOC14433 /// LO	na	0.84	0.09	0.21
NR4A2	NR4A2:nuclear receptor subfamily 4, group A, member 2	0.88	0.02	0.09
TNFRSF4	TNFRSF4:tumor necrosis factor receptor superfamily, member 4	0.61	0.09	0.21
IFIT1	IFIT1:interferon-induced protein with tetratricopeptide repeats 1	0.72	0.06	0.17
D17H6S56E-5	na	0.76	0.02	0.09
MYB	MYB:v-myb myeloblastosis viral oncogene homolog (avian)	0.68	0.01	0.08
IFI27	IFI27:interferon, alpha-inducible protein 27	0.84	0.09	0.21
MYO5A	MYO5A:myosin VA (heavy chain 12, myoxin)	0.72	0.05	0.15
SNX9	SNX9:sorting nexin 9	0.78	0.03	0.11
PSCD3	PSCD3:pleckstrin homology, Sec7 and coiled-coil domains 3	0.60	0.07	0.18
MYO1E	MYO1E:myosin IE	0.62	0.06	0.18
RSAD2	RSAD2:radical S-adenosyl methionine domain containing 2	0.58	0.02	0.09
TYKI	na	0.71	0.00	0.02
SMARCC1	SMARCC1:SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfa	0.67	0.00	0.02
CD9	CD9:CD9 molecule	0.58	0.02	0.08
HIF1A	HIF1A:hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	0.77	0.00	0.02
TCERG1	TCERG1:transcription elongation regulator 1	0.71	0.00	0.02
EZH2	EZH2:enhancer of zeste homolog 2 (Drosophila)	0.77	0.00	0.02
WEE1	WEE1:WEE1 homolog (S. pombe)	0.68	0.00	0.02
TACSTD1	TACSTD1:tumor-associated calcium signal transducer 1	0.65	0.00	0.02
CD200	CD200:CD200 molecule	0.20	0.00	0.02
RPA3	RPA3:replication protein A3, 14kDa	0.75	0.00	0.02

Table S1: Exhausted signature genes in primary and secondary CD8 T cells following chronic infection. Gene set enrichment analysis (GSEA) of a signature of genes associated with exhausted CD8 T cells expressed in primary and secondary effectors in chronic infection. List of genes at the leading edge of enrichment in primary and secondary effector GSEA. The magnitude of enrichment is similar among primary and secondary effectors.

Chapter 3: Distinct and Synergistic Effects of IL-2 Therapy and PD-1 blockade on T cell Exhaustion and Viral Control

Abstract

CD8 T cells play a vital role in controlling viral infections and cancers, however, during antigen persistence these cells become dysfunctional, or exhausted. Herein, we show that IL-2 therapy can act directly on exhausted CD8 T cells during chronic viral infection, increasing their numbers and function, while converting their phenotype towards one associated with decreased exhaustion, including decreased expression of multiple inhibitory receptors, increased expression of CD44 and T-bet, and upregulation of IL-7Rα (CD127), a marker of functional memory cells. Surprisingly, these enhancing effects of IL-2 therapy were not correlated with significantly decreased viral loads. However, combining IL-2 therapy with blockade of the inhibitory PD-1 pathway had striking synergistic effects, resulting in both enhanced T cell responses and decreased viral load. These results suggest that combined IL-2 therapy and PD-1 blockade may be a useful regimen for treating human chronic infections and cancer.

Introduction

CD8 T cells play a key role in providing protection against tumors and intracellular infections, such as viral infections. However, in the setting of chronic antigen stimulation, such as that seen in chronic infections and some tumors, CD8 T cells undergo exhaustion, causing them to become dysfunctional. This exhaustion is characterized by decreased proliferative capacity, loss of cytokine secretion, reduced cytotoxic killing abilities, and phenotypic changes, including low expression of canonical memory markers, such as the IL-7 receptor a chain (CD127), and also an increase in inhibitory receptors (4, 42).

Programmed death 1 (PD-1) is the best characterized inhibitory molecule upregulated during chronic infections and is associated with disease progression and immune dysfunction. *In vivo* or *in vitro* blockade of the PD-1 pathway, by use of blocking antibodies against PD-1 or its ligand PD-L1, has shown to be partially effective in restoring T cell function in both animal models and human chronic infections (42, 73, 99, 157). In addition, PD-1 has been shown to negatively regulate tumor-specific T cells (182-186) and blockade of PD-1 signaling, by use of a humanized anti-PD-1 antibody, has shown promising results in phase I clinical trials in patients with advanced solid tumors (187). This indicates that PD-1 may be an important immunotherapeutic for cancers and chronic infections. Multiple inhibitory mechanisms regulate exhausted CD8 T cells during chronic infections and cancers, thus, combining PD-1 blockade along with other therapies, such as simultaneous blockade of multiple inhibitory receptors or therapeutic vaccination, results in enhanced reduction of viral loads and increased CD8 T

cell responses during mouse chronic infections (74, 76, 116). This suggests that combining strategies or treatments to combat chronic infections and cancer can result in increased efficacy.

Interluekin-2 (IL-2) is a cytokine that has been used to augment T cell responses against virus or tumor antigens in HIV and metastatic cancer patients. While high dose intermittent IL-2 therapy has increased long-term survival for some metastatic renal cell carcinoma patients (138), and IL-2 therapy alone or in combination with a peptide vaccine has resulted in clinical improvement for metastatic melanoma patients (99, 139), it has shown very limited success when given during chronic human viral infections, such as when it is combined with anti-retroviral drugs during HIV (141-144, 146, 188). Greater improvement was seen in one trial with IL-2 administration combined with anti-retroviral drugs and therapeutic vaccination during HIV infection (147), although other small studies suggest that a long term effect is not seen after antiviral therapy is discontinued (148-150). However, continuous IL-2 administration along with therapeutic vaccination and anti-retroviral treatment in macaques infected with chronic SIV increases SIV-specific CD8 T cell responses and results in decreased viral burden (151, 152).

A major limitation of high dose intermittent IL-2 therapy is that it can result in severe toxicity issues, such as vascular leakage. By comparison, daily, much lower doses of IL-2 can ameliorate these toxicity issues (137). Our lab has previously shown that daily low dose IL-2 treatment during chronic mouse LCMV infection results in enhanced virus-specific CD8 T cell numbers and function, and slightly reduces viral burden, thus

indicating that daily low-dose IL-2 therapy may be beneficial during persistent infections (134).

While the clinical data on high dose intermittent IL-2 therapy during chronic viral infections has not been very promising, IL-2 therapy may be beneficial when combined with other immunomodulatory regimens, possibly allowing for the positive effects of IL-2 to be enhanced and diminishing the negative effects of IL-2 by increasing the effectiveness of treatment with a daily low dose of IL-2. Therefore, we wanted to determine whether combining IL-2 administration along with blockade of the inhibitory receptor PD-1 would be able to enhance T cell responses and decrease viral loads during chronic infection, while allowing the use of daily lower doses of IL-2 to decrease toxicity issues. To address this question we used the mouse LCMV model of chronic infection, and treated these chronically infected mice with combined daily IL-2 therapy and PD-1 blockade, while monitoring their CD8 T cell responses and viral burden.

Results

IL-2 therapy synergizes with PD-L1 blockade to enhance virus-specific CD8 T cell control of chronic LCMV infection

To determine whether combined IL-2 therapy and blockade of the inhibitory PD-1 pathway enhances virus-specific CD8 T cell numbers and function, and provides viral control during chronic viral infection, we infected mice with the clone-13 strain (cl-13) of LCMV which results in a protracted viral infection with >2 months of viremia (3, 4), and treated the mice with IL-2 therapy and PD-L1 blocking antibody (to block the PD-1 pathway) during established chronic infection. We administered anti-PD-L1 (every 3 days for 5 total treatments) beginning at day 23-27 post-infection and 15,000 IU of IL-2 was injected daily for the last 8 days of PD-L1 blockade **(Figure 1A).** This regimen was decided upon after consideration of previous dosing used during chronic LCMV infection (134), and some titration of the dose of IL-2, as similar LCMV-specific CD8 T cell responses and viral reduction were seen when two 15,000 IU of IL-2 was injected two times a day versus one daily injection of 15,000 IU **(Figure S1A-C).** While the administration of IL-2 or PD-L1 blockade alone increased LCMV-specific CD8 T cell responses, as determined by MHC class I tetramer staining, in the blood compared to treatment with PBS/isotype control (~ 3 fold each for the D^bGP33-41 epitope), as previously reported (73, 134), combined IL-2 therapy and PD-L1 blockade resulted in a far greater expansion (~4 fold for the D^bGP33-41 epitope) of both LCMV epitopes detected by tetramer staining **(Figure 1B and C).** Furthermore, a significant increase in

LCMV-specific CD8 T cells was seen in the tissues, such as the spleen and lung, after combined treatment **(Figure 1D).** In contrast, total CD4 T cell numbers and LCMV-specific antibody titers were not significantly influenced by any treatment **(Figure S2A and C)**. However, the number of FoxP3$^+$ CD4 T cells was increased by IL-2 or combined IL-2 therapy and PD-L1 blockade, but not by PD-L1 blockade alone **(Figure S2B),** yet, even with the increased numbers of FoxP3+ CD4 T cells, combined IL-2 therapy and PD-L1 blockade resulted in increased numbers of LCMV-specific CD8 T cells.

To characterize whether these LCMV-specific CD8 T cells regain their functional capabilities after combined IL-2 therapy and PD-L1 blockade, we re-stimulated the cells *ex-vivo* with LCMV-specific peptides covering both dominant (GP33 and GP276) and a epitope that is deleted during chronic infection (GP396). The GP396- specific cells are fully exhausted and are deleted to undetectable levels during chronic LCMV infection, unlike their dominance during acute infection (4, 68, 189). IL-2 treatment or PD-L1 blockade alone resulted in increased numbers of CD8 T cells producing IFN-γ and co-producing TNF-α and IFN-γ to GP33 and GP276 epitopes, however they had almost no effect on rescuing the GP396 response **(Figure 2A-C).** In contrast, after combined IL-2 therapy and PD-L1 blockade, CD8 T cells producing IFN-γ and those co-producing IFN-γ and TNF-α were greatly increased to all epitopes ($p<0.001$ for IFN-γ^+ and IFN-γ^+ TNF-α^+ to both GP33 and GP276), including the previously undetectable GP396 epitope **(Figure 2A-C).** Thus, combined IL-2 therapy and PD-L1 blockade results in a broader CD8 T cell response to multiple epitopes, which may help eliminate the selection of viral escape mutants.

Due to the large increase in virus-specific CD8 T cells and enhanced functionality of these cells after combined IL-2 therapy and PD-L1 blockade, we next tested the effect of treatment on viral control. While mice treated with IL-2 or anti-PD-L1 alone had decreased serum titers compared to PBS controls ($p<0.05$), all, except 1 outlier, treated with combined IL-2 therapy and PD-L1 blockade no longer had detectable virus in the serum, indicating faster viral control with the combined treatment **(Figure 2D).**

In the absence of CD4 T cell help, IL-2 therapy and PD-L1 blockade have distinct effects on virus-specific CD8 T cells and viral loads during chronic LCMV infection

Transient depletion of CD4 T cells before infecting mice with chronic LCMV, leads to extreme exhaustion (deletion) of LCMV-specific CD8 T cells and sustained viremia for the life of the mouse. We next tested the ability of combined IL-2 therapy and PD-L1 blockade to rescue LCMV-specific CD8 T cells in the absence of CD4 T cell help during chronic LCMV infection ("un-helped" chronic infection model). To address this, we transiently depleted mice of CD4 T cells, using an anti-CD4 depleting antibody, prior to infection with chronic LCMV cl-13. After 60 days post-infection we began treating the mice with either PBS/isotype control, IL-2 alone, PD-L1 blockade alone or combined IL-2 therapy and PD-L1 blockade. The appropriate groups were given PD-L1 blocking antibody once every 3 days for 5 total treatments and 15,000 IU of IL-2 was given every 12 hours i.p. continuously during the 12 days of PD-L1 blockade **(Figure 3A)**. This regimen of treatment was decided upon after determining that IL-2 administration given

continuously during PD-L1 blockade resulted in increased LCMV-specific CD8 T cells compared to IL-2 given early, in the middle, or late after the start of PD-L1 blockade **(Figure S3A-D)**. Furthermore, administration of IL-2 every 24 hours had less effect on the LCMV-specific CD8 T cells than giving IL-2 every 12 hours in this model of more severe CD8 T cell exhaustion (data not shown).

Combined IL-2 therapy and PD-L1 blockade lead to a huge expansion of LCMV-specific CD8 T cells even as early as day 8 post-beginning of treatment (in the middle of treatment), resulting in up to ~30% of the CD8 T cells in the blood of some mice being specific for one LCMV epitope two days after the last treatment **(Figure 3B),** and an overall increase in both GP33 and GP276-specific CD8 T cells in the blood **(Figure 3C)**. Furthermore, combined IL-2 therapy and PD-L1 blockade resulted in an large increase in the frequency and numbers of LCMV-specific CD8 T cells in the tissues at 2 days post-last treatment ($p<0.01$, $p<0.001$, $p<0.5$ for number of $GP33^+$ CD8 T cells in combined treated compared to PBS/isotype controls in the spleen, lung and liver respectively; $p<0.001$, $p<0.001$, $p<0.001$ and $p<0.05$ for number of $GP276^+$ CD8 T cells in the spleen, lung, liver and bone marrow) (**Figures 3D and E**). Furthermore, IL-2 treatment alone and PD-L1 blockade alone also resulted in an increase in the GP33 and GP276-specific cells in the blood and tissues, but to a much lesser extent than that seen in mice treated with the combination of IL-2 therapy and PD-L1 blockade (**Figures 3B-E**).

We next determined whether these increased CD8 T cells also regained function after combined IL-2 therapy and PD-L1 blockade. In order to address this question, we

assessed the ability of the CD8 T cells to produce cytokines post-treatment after *ex-vivo* re-stimulation with broad range of LCMV-specific peptides. While both IL-2 or PD-L1 blockade alone increased the number of CD8 T cells producing IFN-γ or co-producing IFN-γ and TNF-α to a broad range of LCMV epitopes, combined IL-2 therapy and PD-L1 blockade resulted in a massive increase in the frequency of CD8 T cells producing IFN-γ to a broad range of LCMV epitopes (**Figure 4A**). Interestingly the distribution of IFN-γ producing cells to different LCMV peptides, was altered in individual mice, with some mice making more of a response to the dominant epitopes, GP33 and GP276, (IL-2 + αPD-L1 #1 in **Figure 4A**), and other mice make a larger response to subdominant epitopes and the previously undetectable epitope NP396 (IL-2 + αPD-L1 #2 in **Figure 4A**). Furthermore, combined treatment resulted in a massive increase in the number of CD8 T cells producing IFN-γ, even up to an ~100 fold increase in cells responding to the GP33 epitope, and multi-functional cells co-producing IFN-γ and TNF-α to a broad range of dominant and subdominant LCMV epitopes (**Figures 4B and C**),

We next determined if combined IL-2 therapy and PD-L1 blockade was able reduce viral burden in these chronically infected mice. While IL-2 therapy alone resulted in a slight increase in the levels of virus after treatment, combined IL-2 therapy and PD-L1 therapy resulted in a more significant decrease in viral load than that provided by PD-L1 blockade alone (**Figure 4D**). Therefore, IL-2 therapy synergizes with PD-L1 blockade in this model of extreme exhaustion, resulting in a massive expansion of functional LCMV-specific CD8 T cells and a reduction of viral load greater than that seen with PD-L1 blockade alone.

IL-2 and combined therapy influences T regulatory cell numbers and activation markers

Thus far, we have focused on the LCMV-specific CD8 T cell responses after treatment, however, since T regulatory cells (Tregs) express constitutively high levels of the high affinity IL-2Ra chain and IL-2 is known to expand their numbers and increase their suppressive function (190, 191), we assessed the Treg compartment ($FoxP3^+$ CD4 T cells) after administration of IL-2 or PD-L1 blockade alone, and after combined IL-2 therapy and PD-L1 blockade during chronic LCMV infection. IL-2 administration alone resulted in an increase in the frequency of $FoxP3^+$ CD4 T cells in the blood in the middle (Day 8 post-start of treatment) and after the end of treatment (Day 14 post-start of treatment) (**Figure 5A and B)** and also in the tissues after the end of treatment ($p<0.01$ for spleen and liver, $p< 0.05$ for lung compared to PBS/isotype control group) (**Figure 5C**). In contrast, PD-L1 blockade alone did not significantly affect the frequency of $FoxP3^+$ CD4 T cells in the blood, nor their numbers in the tissues (**Figure 5A-C**). Combined IL-2 therapy and PD-L1 blockade resulted in an increase in the frequency of $FoxP3^+$ CD4 T cells in the blood (**Figure 5A and B**) and increased numbers in the tissues ($p<0.01$ for spleen and $p< 0.05$ in liver compared to PBS/isotype control), however this increase was not larger than that seen after administration of IL-2 therapy alone (**Figure 5C**). This indicates that IL-2 expands the $FoxP3^+$ CD4 T cell population, but PD-1 blockade does not. To determine whether these $FoxP3^+$ CD4 T cells had a more activated phenotype after treatment, we assessed CD44, CD25, CD103 and GITR expression on these cells after treatment by measuring the mean florescence intensity (MFI) of these

markers using flow cytometry. CD44, CD25, CD103 and GITR expression were all increased on the FoxP3$^+$ CD4 T cells after IL-2 or combined IL-2 + PD-L1 blockade, as assessed by MFI, indicating that these FoxP3$^+$ CD4 T cells had a more activated phenotype (**Figure 5D**). In contrast, PD-L1 blockade alone only very slightly affected the expression of these markers (**Figure 5D**). These data indicate that IL-2 therapy increases the number of "activated" Foxp3+ CD4 T cells during chronic infection, unlike PD-1 blockade. Therefore, combined IL-2 therapy and PD-Ll blockade results in increased FoxP3$^+$ CD4 T cells.

To better determine whether the increased numbers of FoxP3$^+$ CD4 T cells were dampening the LCMV-specific CD8 T cell responses after combined IL-2 therapy and PD-L1 blockade, we used our "un-helped" LCMV chronic infection model. After the mice were infected for 60 days, we re-depleted all CD4 T cells prior to combined IL-2 administration and PD-L1 blockade (combo treatment) (**Figure S4A**). Mice in which CD4 T cells were re-depleted before combo treatment, had increased numbers of LCMV-specific GP33 and GP276 CD8 T cells in the tissues, as measured by tetramer, compared to treated mice without CD4 re-depletion (**Figure S4B**). Furthermore, the LCMV-specific CD8 T cells were more functional when CD4 T cells were re-depleted before combo treatment, as more made IFN-γ and TNF-α after ex-vivo stimulation with LCMV peptides covering both dominant and subdominant epitopes (**Figure S4C**). Lastly, viral titers were more reduced in the spleen and kidneys of mice that received combo treatment after CD4 re-depletion (**Figure S4D**). It is important to note that in this experiment the mice were only given IL-2 for 8 days, instead of the normal 13 day treatment, which is

why the combined IL-2 therapy and PD-L1 blockade treatment in mice not re-depleted of CD4 T cells has less effect on viral titer than that seen in Figure 4. This experiment suggests that CD4 T cells, and thus perhaps the expanded FoxP3$^+$ CD4 T cell population, may negatively influence the LCMV-specific CD8 T cells during combined IL-2 therapy and PD-L1 blockade, but whether this is a direct effect is unknown, and is of interest for future studies. However, even with the expansion of Tregs after combined therapy, the number and function of LCMV-specific CD8 T cells increases dramatically and viral loads are reduced (**Figure 3 and Figure 4**). Therefore, this indicates that treatment may be successful even in the face of increased Treg cell numbers.

IL-2 can directly act on exhausted virus-specific CD8 T cells

Since exhausted CD8 T cells have low surface expression of the high affinity IL-2Rα chain (62), it is unclear whether IL-2 is acting directly on these CD8 T cells. To better understand whether IL-2 can directly act on exhausted virus-specific CD8 T cells, we transferred Thy1.1$^+$ LCMV-specific D^{b}GP33-41 transgenic TCR (P14) T cells into Thy1.2$^+$ B6 mice, infected them with LCMV cl-13 and then assessed the P14 T cell numbers and IL-2 signal transduction in the Thy1.1$^+$ P14 cells after establishment of chronic infection (day 23-27) by measuring the level of STAT-5 phosphorylation after *in vivo* treatment with IL-2 (**Figure 6A**). Foremost, after 6 days of daily IL-2 administration the frequency of P14 T cells in the blood was increased from ~0.3% of CD8 T cells in the blood pre-treatment to ~13% post-treatment, indicating a large expansion of the P14 T cells in the blood after IL-2 therapy **(Figure 6B).** In addition,

there was ~a 4 fold increase in the number of P14 T cells in the spleens of mice treated with IL-2 for 6 days compared to those treated with PBS **(Figure 6C).** This indicates that IL-2 causes the expansion of existing exhausted CD8 T cells, and does not just act on CD8 T cells that are newly recruited during the ongoing infection. Secondly, to address whether IL-2 acts directly on exhausted LCMV-specific CD8 T cells, we assessed phospho-STAT-5 staining after IL-2 administration. Notably, STAT-5 remained unphosphorylated 60 minutes after one injection of IL-2 (data not shown). However, when 15,000 IU of IL-2 was administered i.p. every 24 hours for 7 days and then on the 7^{th} day the mice were sacrificed either 0 or 30 minutes post-IL-2 injection, some P14 T cells were phospho-STAT-5^{+} indicating that they had received the IL-2 signal directly (~40% are pSTAT-5^{+} after 30 minutes), and mice given PBS on the 7^{th} day instead of IL-2 were mostly pSTAT-5 negative (~10% were pSTAT-5^{+}) **(Figure 6D).** Taken together these data indicate that IL-2 can act directly on exhausted CD8 T cells.

IL-2 decreases inhibitory receptors and increases CD127 expression on previously exhausted CD8 T cells during chronic LCMV infection

We have shown that IL-2 can directly act on exhausted CD8 T cells, so we next wanted to determine whether IL-2 changes the expression of markers known to be important in the exhaustion process. Multiple inhibitory receptors are expressed on CD8 T cells during chronic infection and have been shown to play a central role in inhibiting their function (42, 64), so to begin we first assessed the expression of the inhibitory receptors, PD-1, 2B4, Tim-3 and Lag-3 on exhausted CD8 T cells after IL-2 therapy. LCMV-specific P14 TCR transgenic cells congenically marked with Thy1.1 were transferred into

mice that were subsequently infected with LCMV cl-13. 27 days post-infection the mice were treated with either PBS or 15,000 IU of IL-2 every 24 hours for 8 days, and the following day the expression of an activation marker, CD44, and the inhibitory receptors on the P14 T cells was assessed by flow cytometry. The P14 transgenic system was used to show how IL-2 acts on pre-existing exhausted cells (eliminating the newly primed CD8 T cells), however similar results are seen with endogenous (tetramer^{+}) LCMV-specific CD8 T cells. Furthermore, IL-2 treatment was given alone, not in combination with anti-PD-L1, so that way virus loads would still be high, helping to eliminate the impact of viral load on inhibitory receptor expression. The activation molecule CD44 was substantially increased on the LCMV-specific CD8 T cells after IL-2 treatment (~two fold increase in mean fluorescence intensity (MFI)) (**Figure 7A**). Furthermore there was a large decrease in the inhibitory receptors, PD-1, 2B4 and Tim-3, as measured by MFI, after IL-2 treatment. In contrast, Lag-3 was not altered by IL-2 treatment (**Figure 7A**). In addition to this decrease in expression of inhibitory receptors on P14 T cells after IL-2 therapy, we found that these cells expressed higher levels of the transcription factor T-bet (**Figure 7B**). A recent paper by Kao et. al, showed that LCMV-specific CD8 T cells that expressed high levels of the transcription factor T-bet, had decreased expression of inhibitory receptors and increased functional capabilities during chronic infection(89). These data indicate that IL-2 can influence inhibitory receptor expression on exhausted CD8 T cells during chronic LCMV infection and thus, this may help lead to decreased inhibition of these cells.

Secondly, we used these same mice to assess two other markers, Bcl-2 and granzyme B, that have been shown to be increased in CD8 T cells after *in vivo* or *in vitro* IL-2 administration(192-196). Bcl-2 is a known negative regulator of apoptosis and is important for T cell survival, and while IL-2 has been shown to increase Bcl-2 expression in CD8 T cells (194-196), we found that Bcl-2 expression was similar on LCMV-specific CD8 T cells after IL-2 and PBS treatment during chronic infection (**Figure 7C**). In contrast, granzyme B expression was increased in the LCMV-specific CD8 T cells after IL-2 therapy during chronic infection (**Figure 7C**), indicating that these cells may have increased cytolytic potential.

Lastly, we looked at the expression of the IL-7 receptor α chain (CD127) on exhausted CD8 T cells after treatment with IL-2 or PD-L1 blockade or combined IL-2 therapy and PD-L1 blockade. CD127 is a CD8 T cell marker that helps define functional memory cells in acute infection, as the responding CD8 T cells that re-express CD127 are preferentially destined to become memory CD8 T cells (57). However, in contrast, exhausted CD8 T cells do not re-express this memory marker during chronic infection (69). Interestingly, we found that CD127 was up-regulated on the LCMV-specific CD8 T cells after IL-2 therapy alone or with combined IL-2 and PD-L1 blockade treatment during chronic LCMV infection (**Figure 7D**). This increase in CD127 expression was not due to viral clearance, as mice treated with only IL-2 (which does not result in viral clearance), also resulted in increased CD127 expression (**Figure 7D**). In addition, in the "unhelped" model of chronic LCMV infection, where viremia is maintained at high levels for the life of the animal, only combined IL-2 therapy and PD-L1 blockade

resulted in increased CD127 expression. This increased expression of CD127 may indicate a re-programming away from the exhausted state, and may lead to increased survivability of the cells, which is the focus of future studies.

Discussion

Herein we show that combined daily low dose IL-2 therapy and PD-L1 blockade enhances CD8 T cell responses and function during chronic LCMV infection and results in decreased viral burden. The effects of the combined therapy was greater than that of either treatment given alone. To better understand the way in which IL-2 affected the T cell responses, we treated mice solely with IL-2 therapy. IL-2 administration alone was shown to act directly on CD8 T cells, causing the phosphorylation of STAT-5. Furthermore, low dose IL-2 therapy resulted in a decrease in the expression of the inhibitory receptors Tim-3, PD-1 and 2B4 (CD244). This reduction in inhibitory receptor expression was seen even though IL-2 treatment alone does not clear virus, indicating that IL-2 therapy can reduce their expression even in the persistence of virus. In addition to a reduced expression of inhibitory receptors, we found that the activation molecule CD44 was upregulated after Il-2 therapy. CD8 T cells that have increased CD44 expression and intermediate PD-1 expression, have been shown to have greater proliferative potential, increased ability to control infections, and are more responsive to PD-1 blockade than their CD44 intermediate and PD-1 high expressing counterparts (197). This suggests that IL-2 administration may result in the generation of a pool of CD8 T cells that are more responsive to PD-L1 blockade. Furthermore, a recent report showed that antigen-specific CD8 T cells expressing high levels of T-bet were more functional during chronic LCMV infection and had reduced inhibitory receptor expression (89), and our data show that IL-2 treatment increases T-bet expression.

Lastly, IL-2 increased granzyme B expression, consistent with enhanced cytolytic potential.

Importantly, IL-2 treatment alone increased IL-7 receptor α expression (CD127), which is a marker of good memory CD8 T cells that has not previously been shown to be upregulated on CD8 T cells during chronic infection. This increase in CD127 expression was seen even in cases where antigen persisted, such as in the IL-2 only treated mice, therefore it is re-expressed even in the presence of high viral loads. Additionally, during normal chronic LCMV infection, CD127 was induced by IL-2 alone or by combined IL-2 therapy and PD-L1 blockade, however in the more severe "unhelped" chronic LCMV infection only the combined IL-2 therapy and PD-L1 blockade was able to induce CD127 expression. This discrepancy between the two models may be due to the fact that the CD8 T cells are more extremely exhausted during "unhelped" LCMV infection and therefore need more immunomodulatory stimulus to "revive" them, and allow for re-expression of CD127. Expression of CD127 may indicate that these cells are more functional and have increased survival capacity than normal CD8 T cells during chronic infection

Our result of IL-2 increasing CD127 expression on exhausted CD8 T cells during chronic infection is the opposite result seen when early effector CD8 T cells are given IL-2 either *in vitro* or *in vivo* during an acute infection, where IL-2 sways the cells toward a CD127 negative and more terminally differentiated state (132, 133, 198). This distinct effect of IL-2 on CD127 expression in cases of limited antigen stimulation versus persistent

stimulation during chronic infection may be due to the different programming/state of exhausted cells versus normal CD8 T cells in acute infection. It has been well documented that exhausted cells are molecularly distinct from naïve, effector or memory CD8 T cells (42, 62). Moreover, IL-2 has been shown to have differential effects on CD8 T cells when administered in the presence of inflammatory signals (133). Therefore, IL-2 may act differently on these exhausted CD8 T cells during a chronic infection that has a distinct inflammatory milieu compared to that seen by CD8 T cells during a quickly resolved acute infection. Overall, IL-2 appears to play a unique role on exhausted CD8 T cells, compared to CD8 T cells during acute infection.

Surprisingly, even though IL-2 treatment alone resulted in a large expansion of LCMV-specific CD8 T cells, it did not result in significant viral reduction during chronic LCMV infection. One possible explanation for the inability of these cells to reduce viral loads is that while IL-2 therapy alone greatly expands LCMV-specific CD8 T cells that are capable of cytokine secretion, these cells still express PD-1, albeit at lower levels than CD8 T cells from untreated mice. Therefore, while IL-2 therapy effectively increases number of functional CD8 T cells, these cells may still be unable to kill their target cells due to inhibition by PD-1 binding to it's widely expressed ligand, PD-L1, on target cells. Accordingly, PD-L1 expression on non-hematopoietic cells has been shown to impair the ability of CD8 T cells to clear virus during chronic LCMV infection (199). Importantly, when IL-2 therapy and PD-L1 blockade are combined, LCMV-specific CD8 T cells are expanded, thereby increasing the effector to infected target ratio, and blockade of the inhibitory signal PD-1 now

results in increased ability of the cells to effectively reduce viral burden. This implies a distinct and synergistic effect of IL-2 therapy and PD-L1 blockade on CD8 T cell responses and viral control.

While IL-2 therapy, PD-L1 blockade or IL-2 therapy combined with PD-L1 blockade greatly increased antigen-specific CD8 T cell numbers and increased their function, none had much effect on overall CD4 T cell numbers or LCMV-antibody production. However, IL2 therapy alone or combined therapy resulted in an increase of $FoxP3^+$ CD4 T cells (Tregs). This data is consistent with previous work, which has shown that IL-2 is important for Treg development and function and IL-2 can increase FoxP3 expression(200, 201). Notably, combined treatment did not enhance Treg numbers above those seem with IL-2 therapy alone and PD-1 blockade alone did not increase Treg numbers, thus indicating that IL-2 itself is responsible for increasing Tregs in this system. Furthermore, increased effectiveness of treatment was seen when CD4 T cells were depleted immediately before combined IL-2 therapy and PD-L1 blockade treatment, as this increased the number of LCMV-specific CD8 T cells, increased their function, and lead to a decrease of viral loads with a shorter IL-2 regimen. This indicates that CD4 T cells may negatively regulate the LCMV-specific CD8 T cells during combined treatment, and this could be due to greater regulation, direct or indirect, by the increased $FoxP3^+$ CD4 T cell population, or may also be due to the fact that these $CD25^+$ CD4 T cells have been depleted, thus eliminating an IL-2 "sink", allowing the CD8 T cells to receive more IL-2 signal themselves. However, even in mice where Tregs were increased with IL-2 or combined treatment, LCMV-specific CD8 T cells were greatly expanded,

regained function and were able to reduce viral loads, showing that the CD8 T cells are still functional even in the presence of increased Tregs. Combining IL-2 therapy with PD-1 blockade and another therapy to reduce T regulatory cells, when one exists, may increase the effectiveness of the treatment however care would need to be taken to avoid the induction of autoimmune complications.

Overall these data suggest that combined IL-2 and PD-1 blockade therapy may be a promising therapy for increasing CD8 T cell function and reducing viral loads during chronic infections. Combining these two therapies together may allow for titration of the dose of IL-2, allowing for the positive effects of IL-2 to be gained while using a low enough dose to minimize toxicity issues. Furthermore, more studies will need to be done to determine if IL-2 or combined IL-2 therapy helps "re-program" exhausted CD8 T cells, making them more like the functional memory CD8 T cells that express high levels of CD127 found after acute infections. Lastly, since IL-2 therapy or combined IL-2 therapy and PD-L1 blockade results in increases in the IL-7 receptor alpha chain (CD127), it may render these cells more responsive to IL-7 therapy. While IL-7 therapy has recently been shown to enhance CD8 T cell responses and decrease viral loads when given for long periods of time in the less stringent model of chronic LCMV infection (103, 125), in our hands we have found little effect of IL-7 therapy in the more stringent model of chronic LCMV infection (where CD4 T cell help is absent) (data not shown). However, since IL-2 therapy increases expression of the IL-7 receptor alpha chain on CD8 T cells during chronic infection, combining IL-2 therapy (plus or minus PD-L1 blockade) along with IL-7 therapy may have enhanced therapeutic benefit during chronic

infection, especially in cases of very high viral loads and extreme exhaustion. In conclusion, combining daily low dose IL-2 therapy along with blocking the inhibitory receptor PD-1 may be a useful strategy for reversing CD8 T cell exhaustion during chronic infections, leading to an enhanced reduction in viral burden or viral control. Furthermore, this work may help us to design rational strategies for developing immunotherapies for chronic infections and cancer.

Materials and Methods

Mice, Infections and Cell transfers

Six-week old female C57BL/6 mice were purchased from Jackson Laboratory. P14 TCR transgenic mice were bred in-house. Mice were infected with $2x10^6$ pfu of LCMV cl-13 i.v. by tail vein. Viral titers were determined by plaque assay on Vero E6 cells as described previously (4). For chronic infection in an "un-helped" environment, mice were given 500μg of the CD4 depleting antibody GK1.5 i.p. (BioXcell) one day prior to infection and again on the day of infection. For experiments using P14 T cells, $2x10^3$ $Thy1.1^+$ P14 T cells were transferred into mice i.v. one day prior to infection.

Lymphocyte Isolation and Flow Cytometry

Lymphocytes were isolated from the blood, spleen, liver, lungs and bone marrow as previous described (60, 73)**.** All antibodies were purchased from BD except CD44, Thy1.1 and Thy1.2 (Biolegend) and anti-PD-1 and anti-FoxP3(ebioscience) and anti-Tim-3 (R&D systems). MHC class I tetramers were prepared and used as previously described (4). Intracellualar cytokine staining was performed as previously described (4). Phospho-STAT-5 staining was done following the manufacturer's protocol (BD Biosciences). Cells were analyzed on a LSR II or Canto flow cytometer (BD Immunocytometry Systems). Dead cells were excluded by gating on Live/Dead NEAR IR (Invitrogen).

In vivo blockade and IL-2 therapy

For blockade of the PD-1 pathway, 200μg of rat anti-mouse PD-L1 antibody (10F.9G2 prepared in house) or rat IgG2b isotype control was administered i.p. every 3 days for 5 total treatments beginning on the day after infection indicated in the appropriate figure/figure legend. For IL-2 therapy, 15,000 IU of recombinant human IL-2 (Amgen) diluted in PBS with 0.1% normal mouse serum was given i.p. to the mice either every 12 or 24 hours for 8-12 consecutive days (as indicated in the appropriate figure legend) beginning after chronic infection was established (at the time point indicated in the appropriate figure legend).

Statistical analysis

All data were analyzed using Prism 5.0 (GraphPad).

Figures

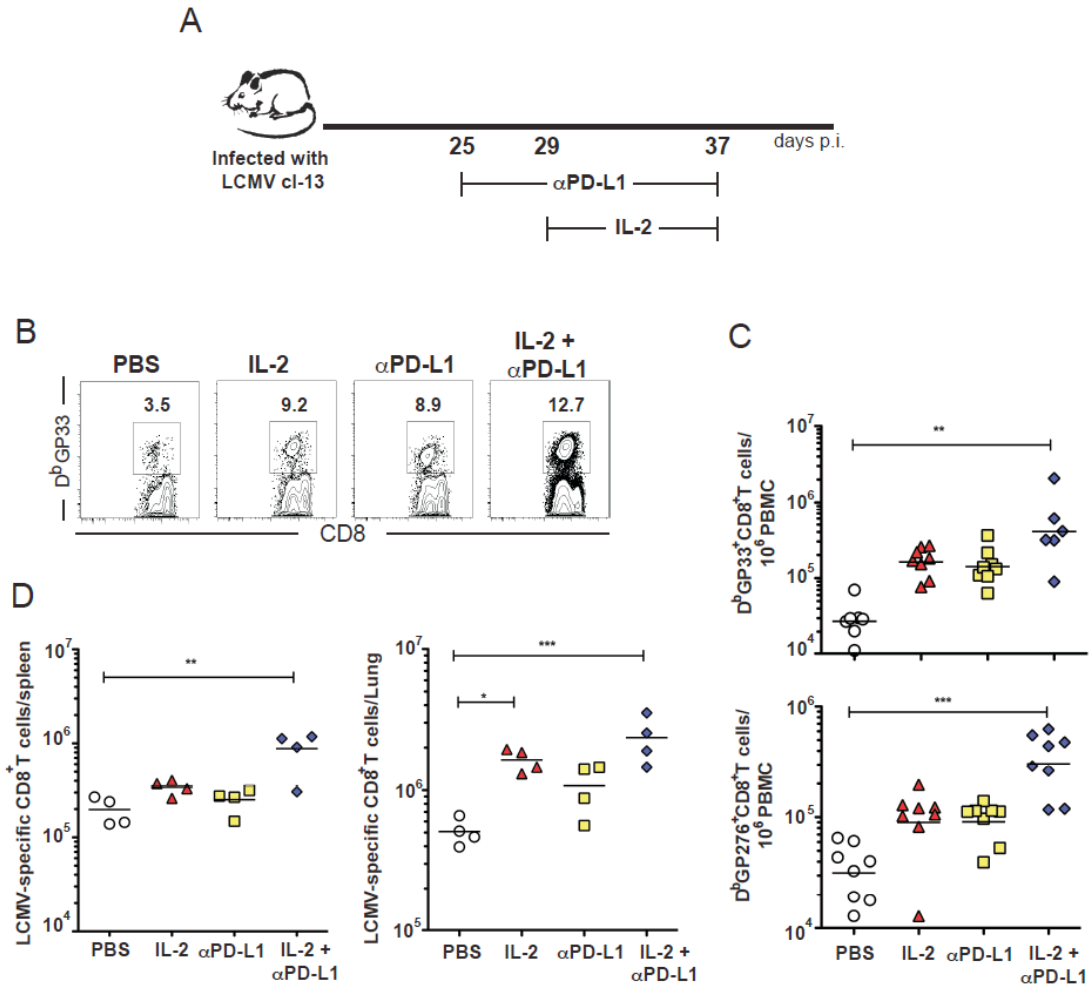

Figure 1. IL-2 therapy combined with PD-L1 blockade enhances antiviral CD8 T cell responses during chronic LCMV infection. C57BL/6 mice were infected with LCMV clone 13 and beginning on day 23-27 p.i. the mice were treated with 200μg of anti-PD-L1 antibody every three days for 12 days (5 total treatments). All IL-2 treated groups were given $1.5X10^4$ IU of IL-2 (i.p.) once a day for the last 8 days of anti-PD-L1 treatment. (A) Experimental design (B) Frequency of H-$2D^b$ GP33 specific CD8 T cells in the blood 1 day post-last treatment (gated on CD8 cells). (C) Number of H-$2D^b$ GP33 & GP276 specific CD8 T cells in the blood 1 day post-last treatment (D) Number of LCMV-specific CD8 T cells (GP33 and GP276 combined) in the spleen and lung 1 day post-last treatment. Representative results of 3 separate experiments.

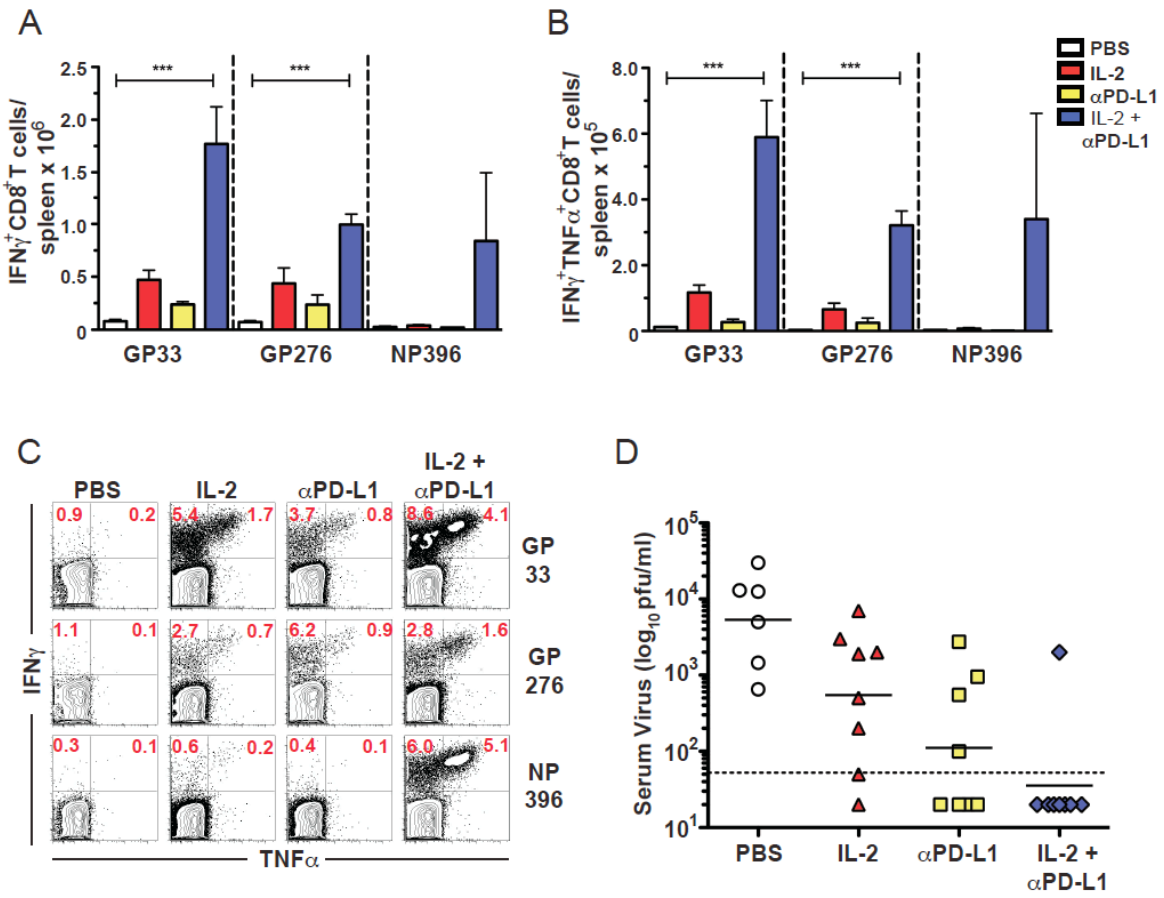

Figure 2. IL-2 therapy combined with PD-L1 blockade enhances antiviral CD8 T cell cytokine production and decreases viral load during chronic LCMV infection. C57BL/6 mice were infected with LCMV clone 13 and beginning on day 23-27 p.i. the mice were treated with 200μg of anti-PD-L1 antibody every three days for 12 days (5 total treatments). All IL-2 treated groups were given $1.5X10^4$ IU of IL-2 (i.p.) once a day for the last 8 days of anti-PD-L1 treatment. (A) Number of IFN-γ producing and (B) simultaneous IFN-γ and TNF-α producing CD8 T cells in the spleen after ex-vivo re-stimulation with the indicated peptides. (C) Representative dot plots of IFN-γ and TNF-α producing CD8 T cells in the spleen after ex-vivo re-stimulation with the indicated peptides (D) Viral titer in the serum 1 day post-last treatment as quantified by plaque assays using Vero E6 cells. Representative results of 3 separate experiments.

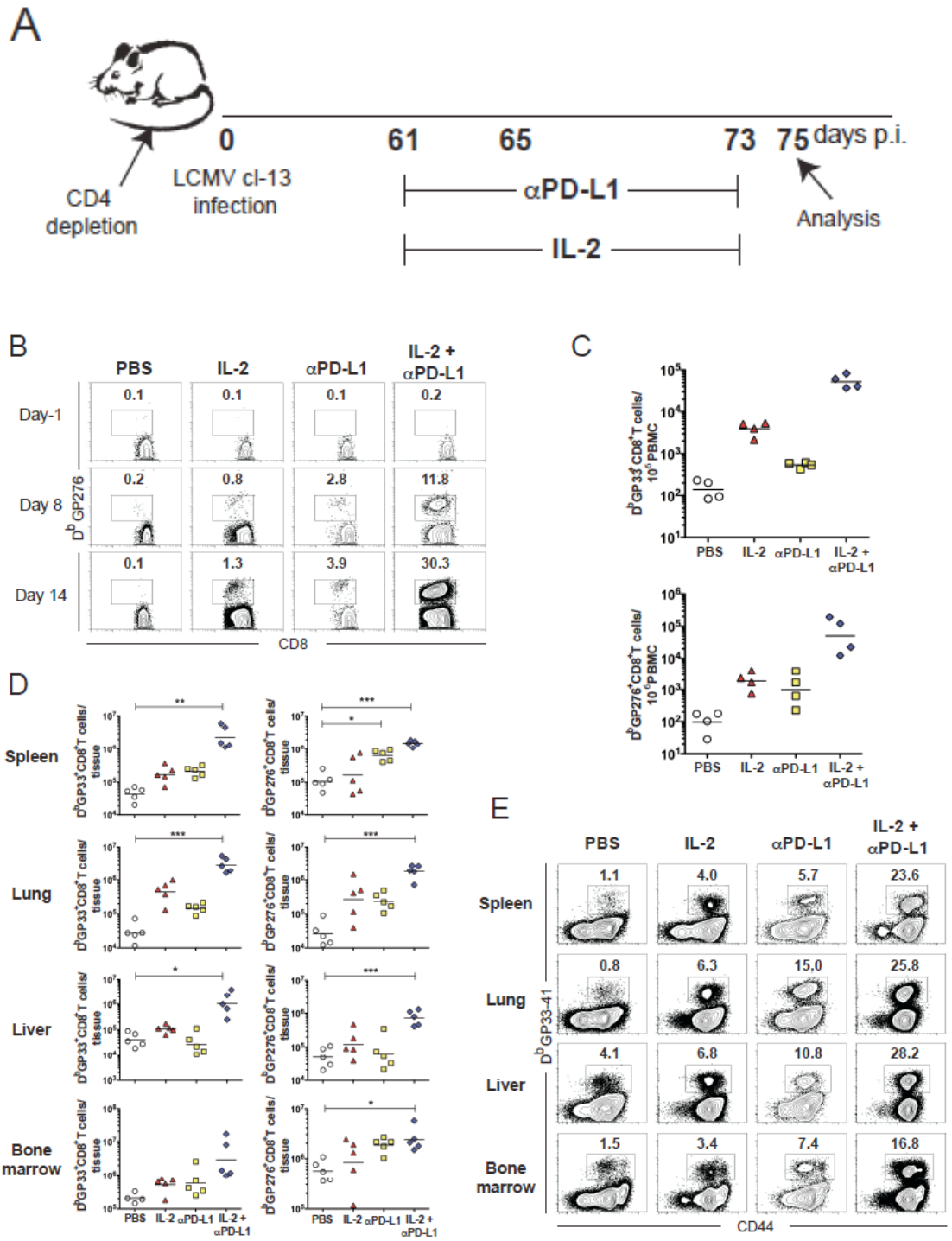

Figure 3. IL-2 therapy combined with PD-L1 blockade enhances antiviral CD8 T cell responses during "unhelped" chronic LCMV infection. C57BL/6 mice were depleted of CD4 T cells and infected with LCMV clone 13. After day 60 post-infection, appropriate groups of mice were treated with either PBS/isotype antibody, 200μg of anti-PD-L1 antibody every three days for 12 days (5 total treatments), and/or IL-2 (i.p.). IL-2 treated groups were given $1.5X10^4$ IU of IL-2 (i.p.) twice a day for the duration of the anti-PD-L1 treatment. (A) Experimental set-up (B) Frequency of H-$2D^b$ GP276 specific CD8 T cells in the blood pre (day-1), mid (day 8), and post- (day 14) treatment (gated on CD8 cells). (C) Number of H-$2D^b$ GP33 & GP276 specific CD8 T cells in the blood 1 day post-last treatment (D) Number and (E) frequency of $GP33^+$ and $GP276^+$ CD8 T cells tissues 2 days post-last treatment. Representative results of 3 separate experiments.

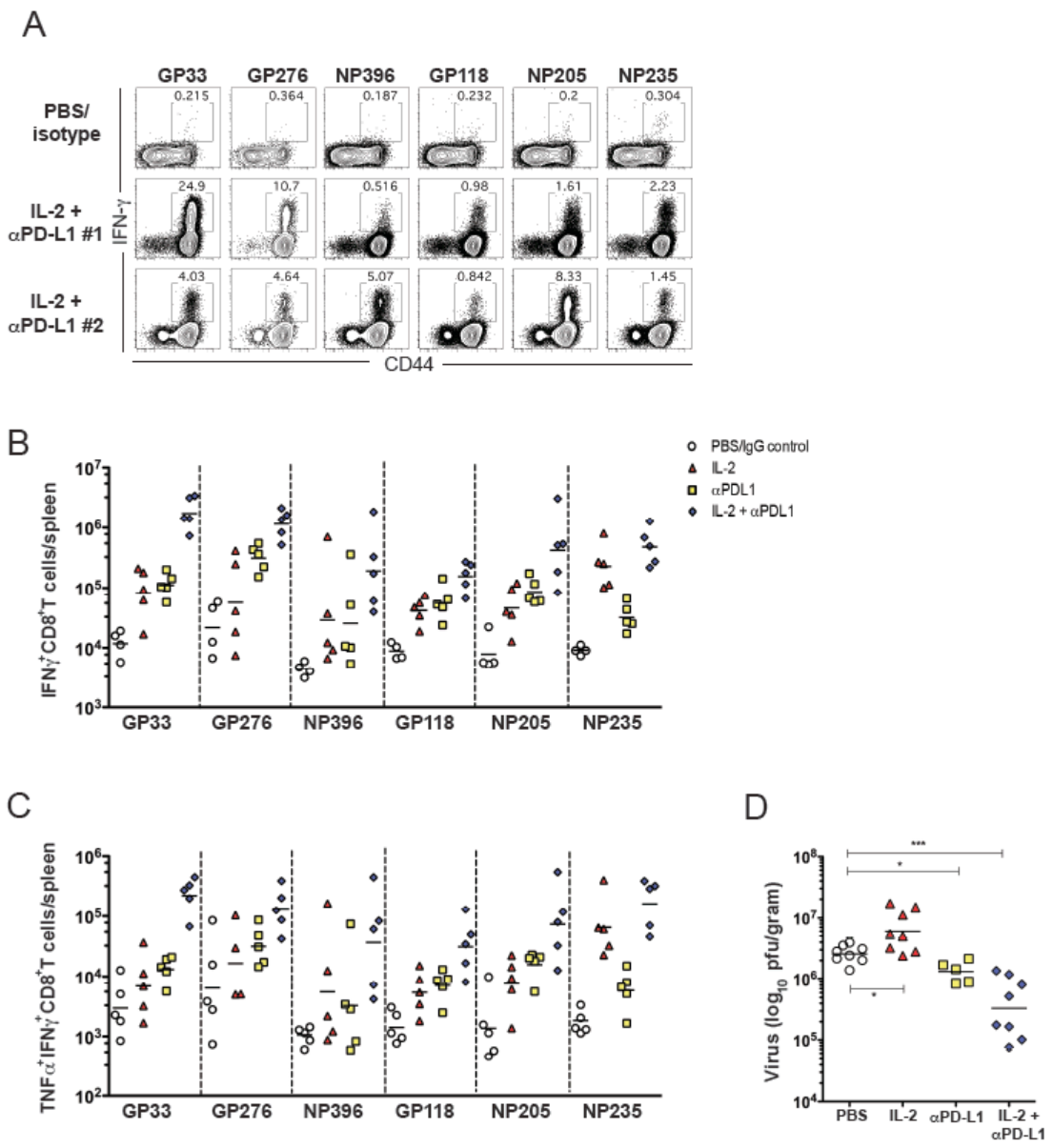

Figure 4. IL-2 therapy combined with PD-L1 blockade enhances antiviral CD8 T cell cytokine production and decreases viral load during "un-helped" chronic LCMV infection. C57BL/6 mice were depleted of CD4 T cells and infected with LCMV clone 13. After day 60 post-infection, appropriate groups of mice were treated with either PBS/isotype antibody, 200μg of anti-PD-L1 antibody every three days for 12 days (5 total treatments), and/or IL-2 (i.p.).IL-2 treated groups were given $1.5X10^4$ IU of IL-2 (i.p.) twice a day for the duration of the anti-PD-L1 treatment. (A) Frequency and (B) number of IFN-γ producing CD8 T cells in the spleen after ex-vivo re-stimulation with the indicated peptides. (C) Number of simultaneous IFN-γ and TNF-α producing CD8 T cells in the spleen. (D) Viral titer in the spleen 2 days post-last treatment as quantified by plaque assays using Vero E6 cells. Representative results of 3 separate experiments.

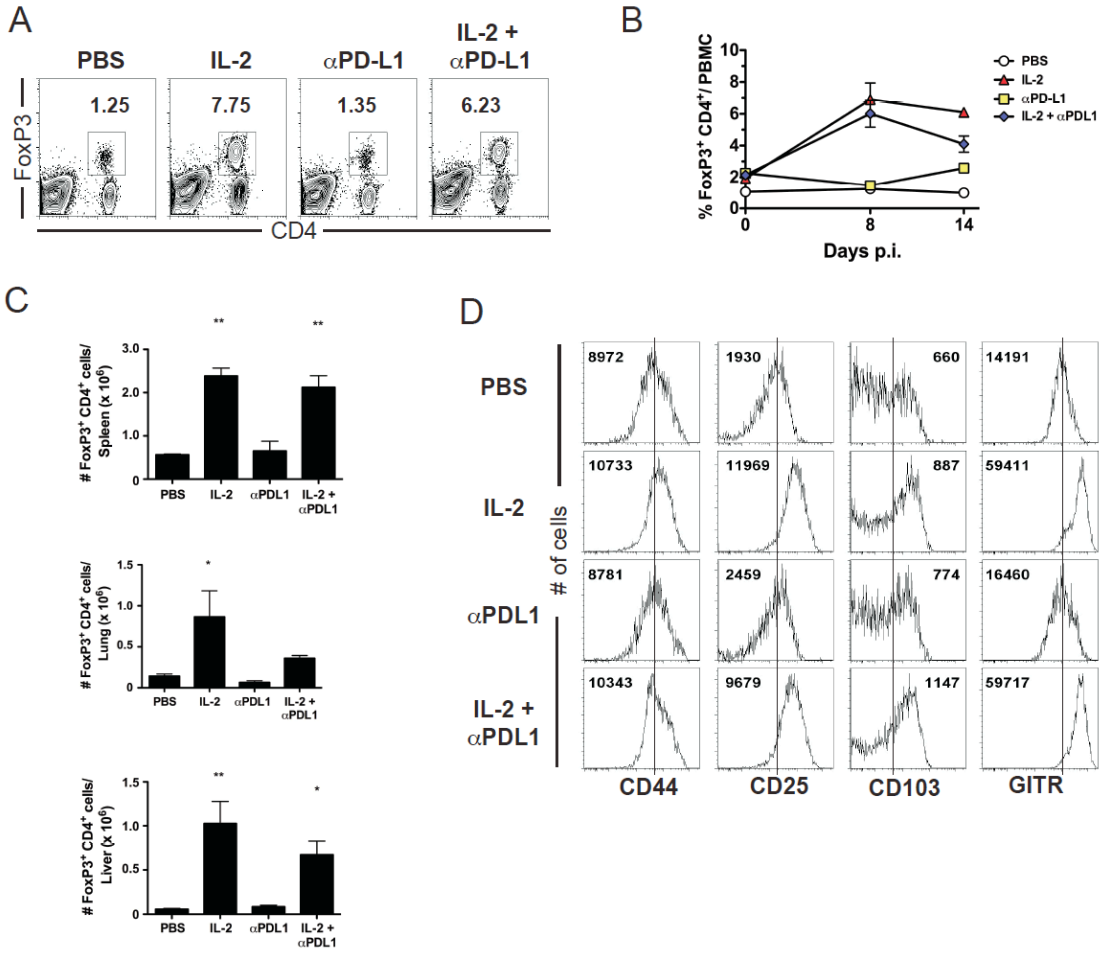

Figure 5. IL-2 therapy and T regulatory cells during "un-helped" chronic LCMV infection. C57BL/6 mice were depleted of CD4 T cells and infected with LCMV clone 13**.** After day 60 post-infection, appropriate groups of mice were treated with either PBS/isotype antibody, 200mg of anti-PD-L1 antibody every three days for 12 days (5 total treatments), and/or IL-2 (i.p.). IL-2 treated groups were given $1.5X10^4$ IU of IL-2 (i.p.) twice a day for the duration of the anti-PD-L1 treatment. (A) Representative flow plots showing the frequency of $FoxP3^+$ CD4 T cells in the blood in the middle of treatment (Day 8 post-start of treatment). (B) Frequency of $FoxP3^+$ CD4 T cells in the blood at pre- (Day0), mid- (day 8) and post- (day14) treatment. (C) Number of $FoxP3^+$ CD4 T cells in the tissues 2 days post- final treatment. (D) Representative histograms showing the expression of CD44, CD25, CD103 and GITR on $FoxP3^+$ CD4 T cells in the spleen post-treatment.

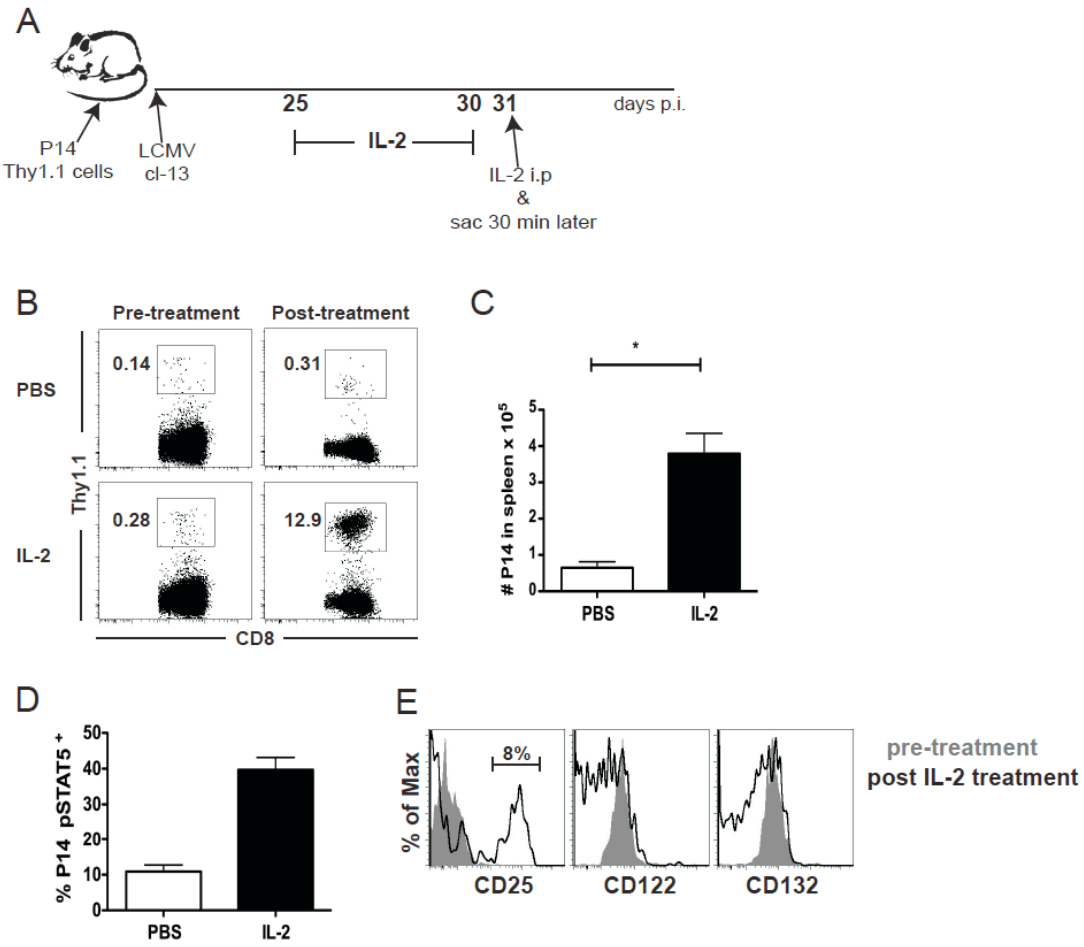

Figure 6. IL-2 therapy acts directly on exhausted antiviral CD8 T cells. $2x10^3$ Thy1.1$^+$ D^b GP33$^+$ LCMV-specific CD8 T cells (P14 transgenic) were transferred iv into C57/BL6 mice (Thy1.2$^+$) that were subsequently infected with LCMV clone 13 and starting on day 23-27 p.i., the mice were treated with $1.5x10^4$ IU of IL-2 (i.p.) every 24 hours for 5 days. On the 6th day, the mice were either treated with PBS or $1.5x10^4$ IU of IL-2 and mice were sacrificed 30 minutes after IL-2 treatment. Splenocytes were removed and stained with Thy1.1, CD8 and phospho-STAT-5 antibodies. (A) Experimental set-up. (B) Frequency of P14 T cells in the blood (gated on CD8) and (C) number in the spleen pre and post- 6 days of IL-2 treatment. (D) Percent of P14 T cells in the spleen that are phospho-STAT-5$^+$. (E) Representative histograms of CD25, CD122 and CD132 expression on P14 T cells in the blood pre and post-IL-2 treatment. Results representative of 2 independent experiments.

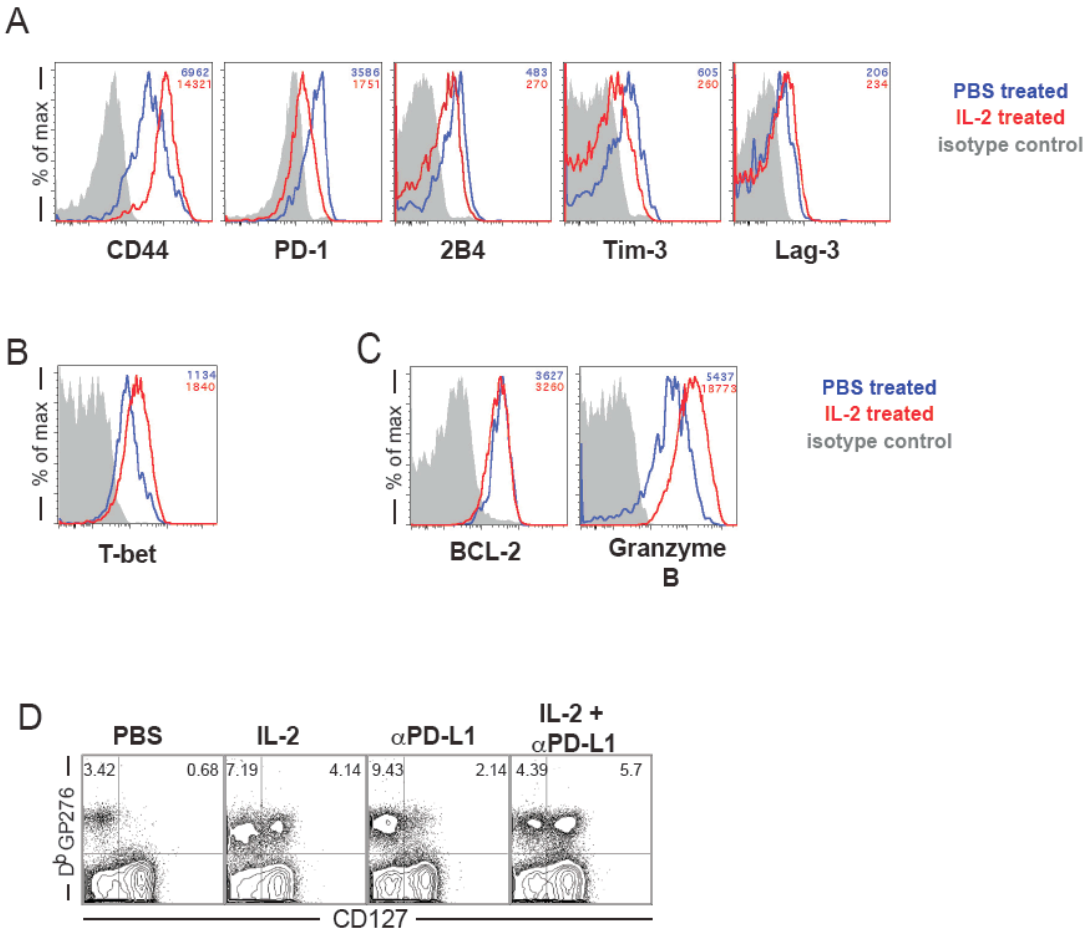

Figure 7. The effects of IL-2 therapy on inhibitory receptor expression and phenotype of anti-viral CD8 T cells during chronic LCMV infection. $2x10^3$ Thy1.1$^+$ D^b GP33$^+$ LCMV-specific CD8 T cells (P14 transgenic) were transferred iv into C57/BL6 mice (Thy1.2$^+$) that were subsequently infected with LCMV clone 13 and beginning on day 23-27 p.i., the mice were treated with either PBS of $1.5x10^4$ IU of IL-2 (i.p.) every 24 hours for 8 days. After the end of treatment, the splenocytes were removed and stained with Thy1.1, CD8, CD44 and the indicated antibodies. The mean fluorescence intensity (MFI) of the markers is indicated by numbers in the histograms. Representative histograms showing the expression of (A) CD44 and the inhibitory receptors (B) intracellular T-bet expression and (C) intracellular Bcl-2 and granzyme B expression 1 day post-end of PBS or IL-2 treatment. (D) C57/BL6 mice were infected with LCMV cl-13, 23-27 days post-infection the appropriate mice were treated with either isotype control antibody or anti-PDL1 (every 3 days for 5 total treatments), and $1.5X10^4$ IU of IL-2 was given to the appropriate groups every 24 hours for the last 8 days of anti-PD-L1 treatment. Representative dot plots showing CD127 expression on D^b GP276 tetramer$^+$ CD8 T cells at 3 weeks post-end of anti-PD-L1 and IL-2 treatment.

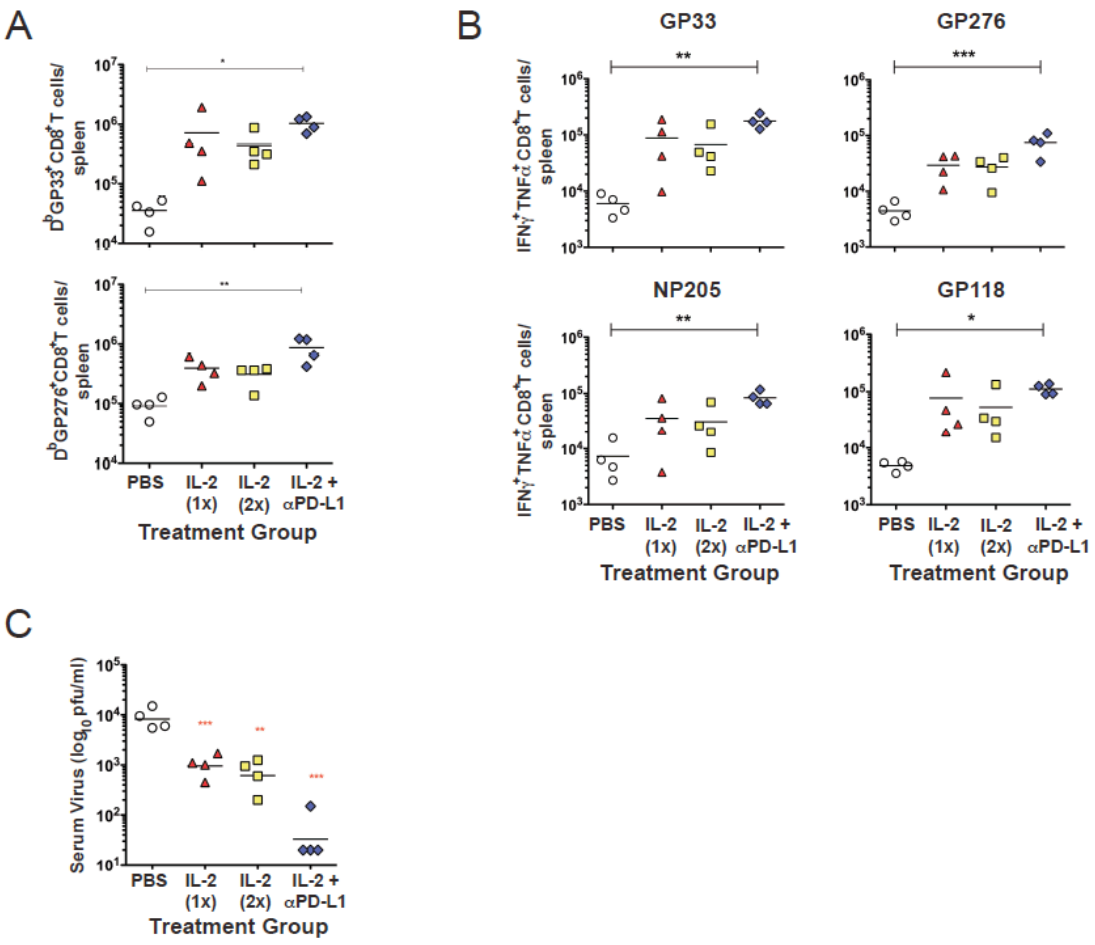

Figure S1. Titration of IL-2 injections during chronic LCMV infection. C57BL/6 mice were infected with LCMV clone 13 and beginning on day 23-27 p.i. the mice were treated with 200μg of anti-PD-L1 antibody every three days for 12 days (5 total treatments). All IL-2 treated groups were given $1.5X10^4$ IU of IL-2 (i.p.) once (1X) or twice (2X) a day for the last 8 days of anti-PD-L1 treatment. (A) Number of H-$2D^b$ GP33 & GP276 specific CD8 T cells in the blood 1 day post-last treatment. (B) Number of CD8 T cells simultaneously producing IFN-γ and TNF-α in the spleen after ex-vivo re-stimulation with the indicated peptides. (C) Viral titer in the serum 1 day post-last treatment as quantified by plaque assays using Vero E6 cells.

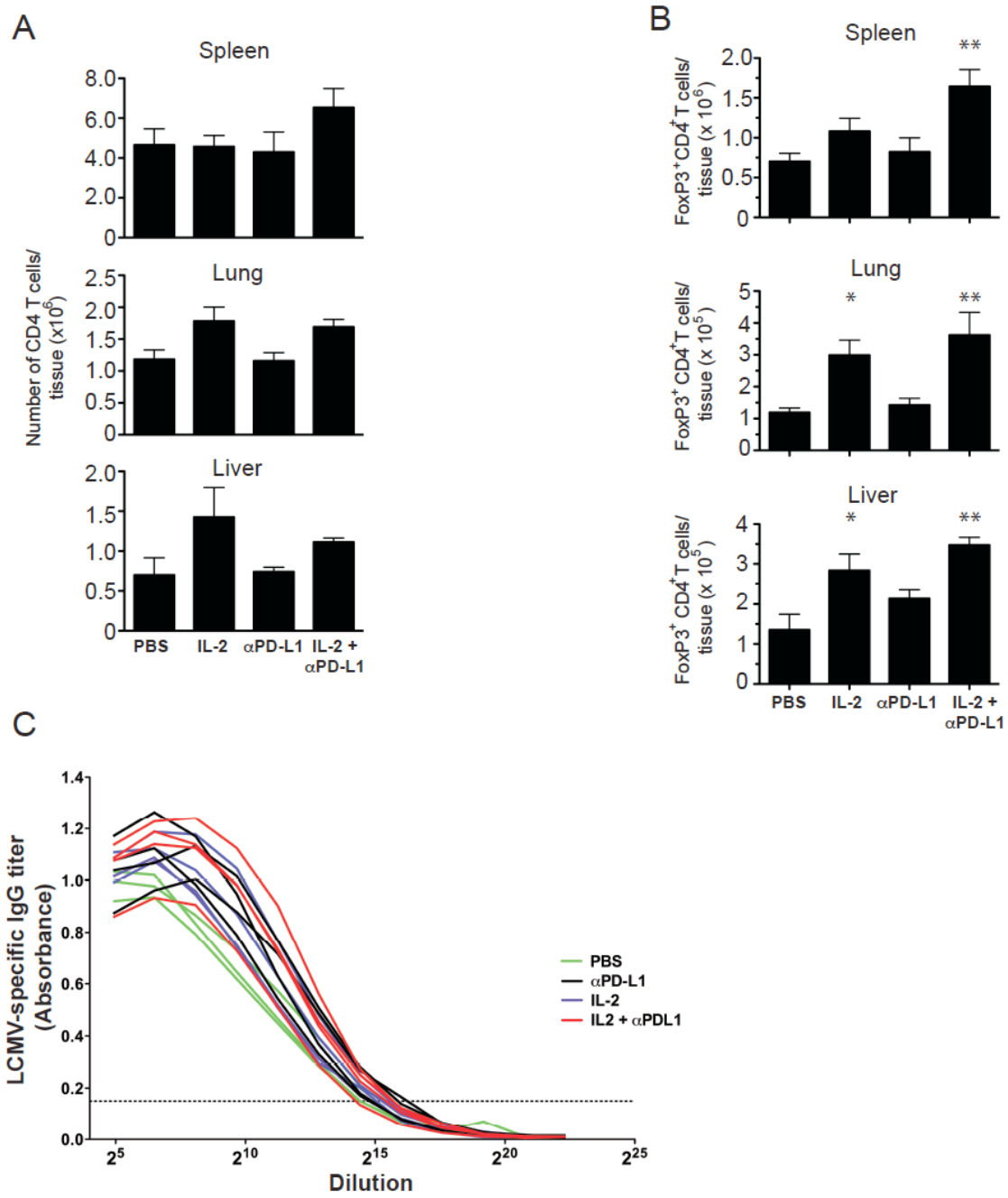

Figure S2. CD4 T cells and antibody responses after IL-2 + αPD-L1 therapy during chronic LCMV infection. C57BL/6 mice were infected with LCMV clone 13 and beginning on day 23-27 p.i. the mice were treated with 200μg of anti-PD-L1 antibody every three days for 12 days (5 total treatments). All IL-2 treated groups were given $1.5X10^4$ IU of IL-2 (i.p.) once a day for the last 8 days of αPD-L1 treatment. (A) Number of CD4 T cells in the tissues 1 day post-last treatment. (B) Number of $FoxP3^+$ CD4 T cells in the indicated tissues 1 day after the end of treatment. * indicates $p<0.05$, **is $p< 0.01$ compared to PBS/isotype control. (C) LCMV-specific IgG antibody titers as measured by ELISA 46 days after the last treatment.

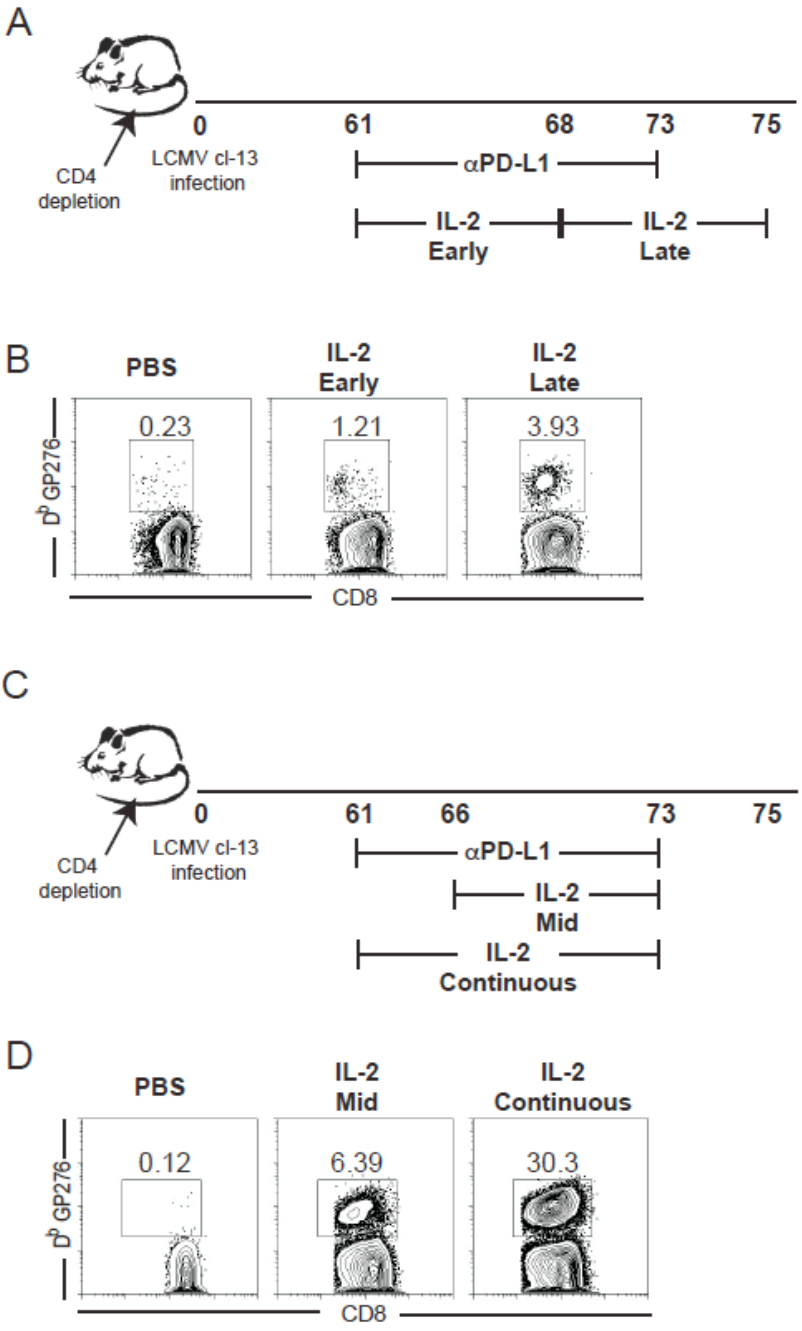

Figure S3. Timing of IL-2 therapy and PD-L1 blockade during chronic LCMV infection. C57BL/6 mice were depleted of CD4 T cells and infected with LCMV clone 13. After day 60 post-infection, the appropriate groups of mice were treated with either PBS/isotype antibody, 200μg of anti-PD-L1 antibody every three days for 12 days (5 total treatments), and/or IL-2 (i.p.). IL-2 treated groups were given $1.5X10^4$ IU of IL-2 (i.p.) twice a day for either 8 days or the duration of the anti-PD-L1 treatment (as indicated in (A) and (C)). (A) Experimental set-up for part B (B) Frequency of H-$2D^b$ GP276 specific CD8 T cells in the blood 3 days after the last IL-2 treatment (gated on CD8 cells). (C) Experimental set-up for part D. (D) Frequency of H-$2D^b$ GP276 specific CD8 T cells in the blood 2 days after the last treatment (gated on CD8 cells).

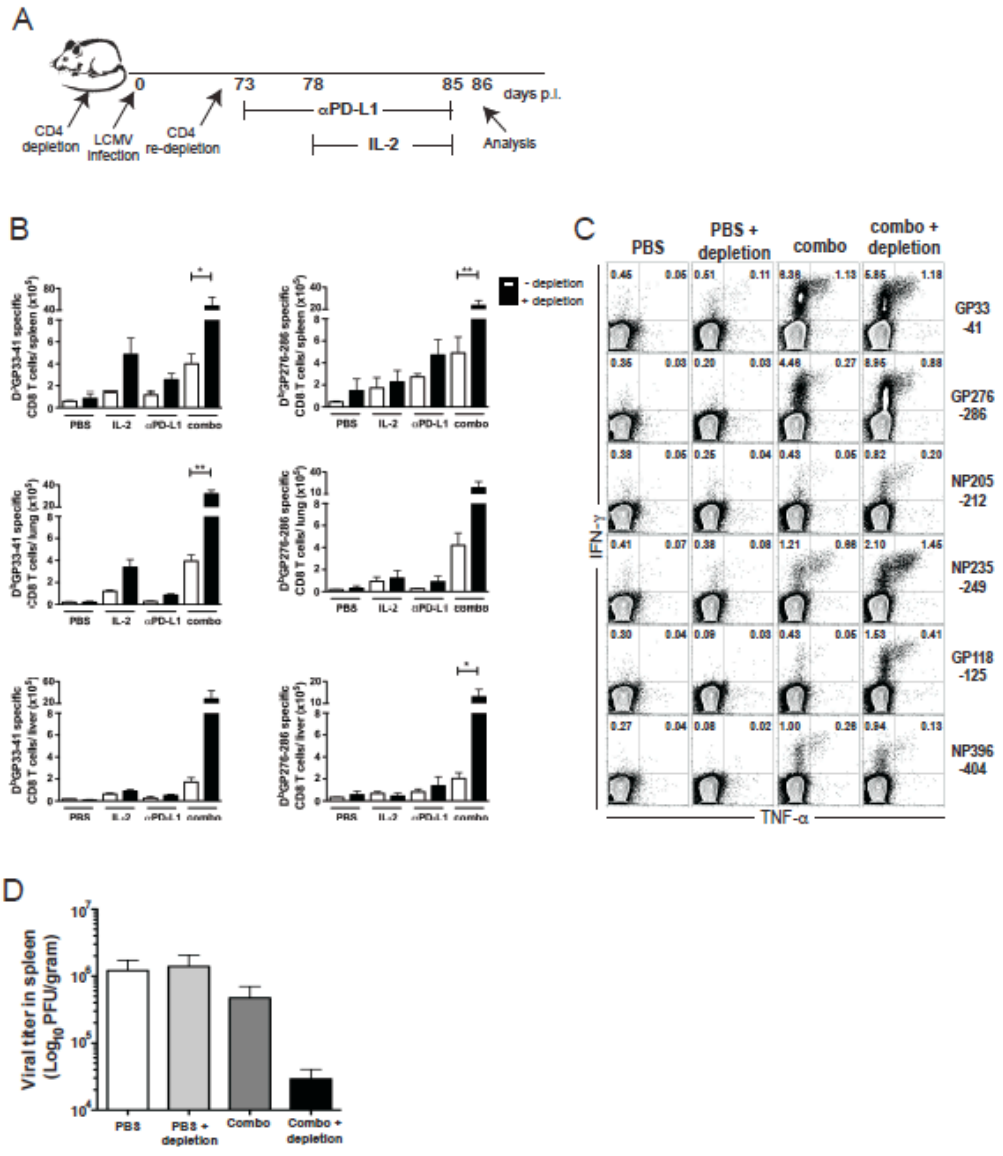

Figure S4. Re-depletion of CD4 T cells before administration of IL-2 + αPD-L1 during chronic LCMV infection further enhances CD8 T cell responses. C57BL/6 mice were depleted of CD4 T cells and infected with LCMV clone 13. After day 60 post-infection, half of the mice were re-depleted of CD4 T cells. The appropriate groups of mice were treated with either PBS/isotype antibody, 200μg of anti-PD-L1 antibody every three days for 12 days (5 total treatments), and/or IL-2 (i.p.).IL-2 treated groups were given $1.5X10^4$ IU of IL-2 (i.p.) twice a day for the duration of the αPD-L1 treatment. Combo indicates the IL-2 + αPD-L1 treated group. (A) Experimental design (B) Total number of GP33 and GP276-specfic CD8 T cells in the tissues post-treatment. (C) Frequency of IFN-γ and TNF-α producing CD8 T cells after 5 hour ex-vivo re-stimulation with the indicated peptides. (D) Viral titers in the spleen post-treatment, as measured by plaque assay.

Chapter 4: Discussion

Regulation of Memory CD8 T Cells during Chronic Infection: Implications for Vaccine Design

The body of work presented in chapter 2 shows that memory CD8 T cells are differentially regulated than naïve CD8 T cells during situations of high viral load or viral persistence. Previous work by others and in chapter 2, has shown that on a per-cell basis memory CD8 T cells undergo less contraction than naïve CD8 T cells during a rapidly cleared acute infection, and thus persist in greater numbers than naïve cells (28). Furthermore in chapter 2, we show that on a per-cell basis, memory CD8 T cells are better able to control or reduce viral loads during a low dose or acute infection. However, in stark contrast, memory CD8 T cells are rapidly lost during situations of high viral load or viral persistence, while naïve CD8 T cells are better able to persist in these situations (Chapter 2). Some question asked by this study are: Why are memory CD8 T cells unable to persist during high viral load or viral persistence? How are they regulated? And what are the implications for vaccine design for chronic infections?

One reason for the loss of memory CD8 T cells during chronic infection is that after similar recruitment of memory (secondary effectors) and naïve (primary effectors) CD8 T cells, secondary effectors have reduced proliferative capacity during chronic infection as evidenced by the their reduced incorporation of BrdU and downregulation of genes related to cell cycle, proliferation, RNA processing, amino acid synthesis, mitochondrial metabolism and DNA repair compared to primary effectors (naïve CD8 T

cells) during chronic infection (Chapter 2). Furthermore, during chronic infection secondary effectors also have increased expression of two cell cycle inhibitors, CDKN1A (p21) and CDKN2B (p15) (Chapter 2), which block transition from G1 to the S phase of the cell cycle (202). These data suggest that memory CD8 T cells (secondary effectors) are unable to proliferate as well as naïve CD8 T cells during a challenge with a chronic viral infection, and this may be one mechanism in which memory CD8 T cells are not maintained in these situations of high antigen persistence.

Another explanation is that memory CD8 T cells reach senescence more rapidly than naïve CD8 T cells during chronic infection. Through microarray analysis we found that markers of senescence, such as KLRG-1, are upregulated on secondary effectors during chronic infection. These data correspond well with two recently published studies from Dr. Restifo's laboratory. First, effectors derived from *in vitro* stimulated memory CD8 T cells were less effective at reducing tumor burden in a mouse model(171) and, secondly, *in vitro* stimulated human memory CD8 T cells had increased markers of senescence and less proliferative ability than effectors derived from naïve CD8 T cells (172). These studies suggest that memory CD8 T cells undergo senescence faster than naïve CD8 T cells. However, we do not think that the loss of memory CD8 T cells during chronic infection in our experiments is due to memory CD8 T cells reaching hayflick's limit (the maximum number of possible divisions a cell can divide)(203) more quickly than the naïve cells. While the memory CD8 T cells have proliferated more than the naïve CD8 T cells before challenge with the chronic viral infection, e.g. during their development, we purposely developed our memory CD8 T cells in situations with high precursor frequency in order to limit the number of divisions that they go through before

their co-transfer with naïve CD8 T cells. Therefore, these memory CD8 T cells have not undergone extensive divisions pre-transfer, and thus have not divided enough to have reached the hayflick limit. However, it is important to note that we see the same results when memory CD8 T cells were made in mice that were seeded with a number of LCMV-specific TCR transgenic CD8 T cells that is equivalent to the natural precursor frequency of this epitope in mice (Chapter 2). Overall, it appears that memory CD8 T cells may be more prone increased senescence than naïve CD8 T cells after re-challenge with a chronic viral infection, which corresponds well with data from other labs showing that after repeated rounds of antigenic stimulation, such as a prime-boost vaccine regimen, CD8 T cells express more markers of senescence (204).

Interestingly, we found that during chronic infection memory CD8 T cells appear to be differentially regulated by inhibitory receptors than naïve CD8 T cells. Co-expression of multiple inhibitory receptors is a feature of dysfunctional (exhausted) CD8 T cells during chronic infection (42). The final, most extreme, stage of exhaustion of CD8 T cells is cellular deletion (4, 67, 68), therefore a potential hypothesis to explain the loss of memory CD8 T cells during sustained antigen loads could be that memory CD8 T cells become more exhausted (or more quickly exhausted) than naïve CD8 T cells. However, we found that both memory and naïve CD8 T cells appear to have similar exhaustion during chronic LCMV infection, as evidenced by their equal enrichment for the "molecular profile of exhaustion" described by Wherry et. al (62) and by their similar progressive loss of cytokine production (Chapter 2). Therefore, memory CD8 T cells are not lost in chronic infection due to an increase in "exhaustion" compared to naïve CD8 T cells.

However, while both memory and naïve CD8 T cells express high levels multiple inhibitory receptors (such as PD-1, Lag-3, 2B4 and Tim-3) during chronic infection compared to acute infection, memory CD8 T cell express higher levels of the genes encoding the inhibitory receptors 2B4 and Tim-3, than naïve cells during antigen persistence (Chapter 2). We found a key role for the inhibitory receptor 2B4 in differentially regulating memory compared to naïve CD8 T cells in chronic infection. Using LCMV-specific CD8 T cells lacking expression of 2B4, we found that memory CD8 T cells deficient in 2B4 were able to persist better than wildtype memory CD8 T cells (2B4 sufficient). In contrast, deletion of 2B4 in naïve CD8 T cells did not alter the number of naïve cells, indicating that 2B4 selectively regulates memory CD8 T cells during chronic infection (Chapter 2). These data imply that a better understanding of the immunomodulatory receptor 2B4 is needed. The current data concerning the role of 2B4 in regulating CD8 T cell responses is confusing. 2B4 is a complicated molecule, as it has been shown to have both activating and inhibitory functions (82). Furthermore, in mice there are two isoforms of 2B4 that arise from alternative splicing, a short and a long form, that differ only in their cytoplasmic tail region (205). In natural killer cells the expression of the long form of 2B4 may be associated with inhibition, while the short form may result in an activation signal (206). In humans only one isoform is expressed that more resembles the long form found in the mouse(207, 208). While 2B4 has been studied in more detail in natural killer cells, less is understood about how 2B4 influences CD8 T cells. A recent study has shown that *in vitro* blockade of the ligand of 2B4, CD48, improves cytokine production by exhausted CD8 T cells expressing high levels of 2B4 (74). Furthermore, studies in human chronic infections, such as HCV(83) and HBV(86),

have indicated an inhibitory role of 2B4 on CD8 T cells. Lastly, recent data in mice has shown that the inhibitory function of 2B4 is related to increased receptor cross-linking of 2B4 and decreased levels of SAP (signaling lymphocyte activation molecule-associated protein)(82). During chronic LCMV infection, secondary effectors express higher levels of 2B4 than primary effectors CD8 T cells, while the levels of SAP during chronic and acute LCMV is similar in both primary and secondary effector CD8 T cells, suggesting that 2B4 may be acting in an inhibitory manner during chronic LCMV infection (Chapter 2). Overall, more work is needed to understand the role of 2B4 on CD8 T cells during antigen persistence. Some pertinent questions that remain are: How does 2B4 regulate CD8 T cells? Is the short or long form of 2B4 expressed on mouse CD8 T cells during chronic infection? Since the receptor for 2B4 (CD48) can also bind to CD2 in mice (209), how does CD2 –CD48 binding influence CD8 T cells? For example, does blocking 2B4 result in increased CD2-CD48 interactions, which are lower affinity reactions than 2B4-CD48 interactions and may be stimulatory(210), thus affecting the survival of memory CD8 T cells during chronic infection due to a decrease in inhibition by 2B4 plus an increase in CD2-CD48 interactions? Additionally, *in vivo* work in other animal models of chronic infection and *in vitro* work in human chronic infections, may help elucidate the role of 2B4 on CD8 T cells. Perhaps blockade of 2B4 may be a therapeutic option during chronic infection, but more detailed studies are need. Lastly, since the gene encoding the inhibitory receptor Tim-3 was also seen to be upregulated on secondary effectors during chronic LCMV infection, although to a lesser extent than 2B4, it would also be of interest to determine whether Tim-3 regulates memory CD8 T cell responses during chronic (42) infections. However, preliminary studies indicate that blockade of

Tim-3 does not increase the ability of memory CD8 T cells to persist during chronic LCMV infection (West EE, unpublished data).

Besides increased regulation of memory CD8 T cells by the inhibitory receptor 2B4, we also found that memory CD8 T cells are more dependent on CD4 T cell help than naïve CD8 T cells during chronic infection. While CD4 T cell help has been well documented to be important for CD8 T cell responses during chronic infection (16, 17), previous reports in acute infection imply that naïve CD8 T cells are more reliant on CD4 T cell help than memory CD8 T cells (22, 161, 162). Thus, it was surprising that we found that memory CD8 T cells require more help than naïve cells. However, a report by Garidou et. al shows that in a therapeutic model of adoptive transfer of memory CD8 T cells during chronic infection, the memory CD8 T cells require CD80/86 costimulation for effective clearance of virus (211), indicating that the requirements for memory CD8 T cell maintenance/function is very different during chronic infections compared to acute infections (where memory CD8 T cells generally do not need co-stimulation). Recently, multiple reports have shown that the cytokine IL-21 is important for increasing CD8 T cell responses during chronic LCMV infection (96-98). The major producers of IL-21 are CD4 T cells, therefore one mechanism in which CD4 T cells may help CD8 T cells during persistent infection is through IL-21 production. However, in a preliminary study, we found that LCMV-specific CD4 T cells that are unable to produce IL-21 (the IL-21 gene is knocked out) are still capable of rescuing memory CD8 T cells during chronic LCMV infection (West EE, unpublished data), indicating that IL-21 may not play a role in the rescue of these memory CD8 T cells. In contrast, we did find that rescue of memory CD8 T cells by CD4 T cell help was greatly diminished when CD40:CD40L

interactions were blocked (Chapter 2). However, more detailed studies need to be performed to better understand the increased dependence of memory CD8 T cells on CD4 T cell help, and the direct or indirect mechanism of this help.

Overall, we found that memory CD8 T cells are more tightly regulated than naïve CD8 T cells during chronic infection; through increased inhibition of proliferation, regulation by the inhibitory receptor 2B4, and increased reliance on CD4 T cell help. The question is why are memory CD8 T cells more tightly regulated than naïve CD8 T cells? Memory CD8 T cells sit poised for action, and kill infected cells and secrete inflammatory cytokines faster than naïve CD8 T cells upon re-encounter with their cognate antigen (20, 61, 170). One possible explanation for the increased regulation of memory compared to naïve CD8 T cells during high antigen loads or antigen persistence is to limit excessive immunopathology. If memory CD8 T cells are not tightly regulated in situations where they are constantly bombarded by their cognate antigen they may cause over abundant inflammation, possibly resulting in death of the host before achieving clearance of the antigen/pathogen.

Looking at the big picture, this work may have implications for vaccine design for human chronic infections. We found that on a per cell basis, memory CD8 T cells are better at controlling low dose acute infection than naïve CD8 T cells. However, as antigen dose (and thus persistence) increased, low numbers (~60,000 or less of transferred cells, which equals 6,000 cells after accounting for the 10% take) of memory or naïve CD8 T cells were unable to control virus, although there were some instances where there was a slight hint that memory cells may reduce viral loads slightly more than naïve cells (Chapter 2). In contrast, when larger numbers of memory cells were

transferred (~100,000 or greater), then memory CD8 T cells were better able to control or reduce viral loads than an equal number of transferred naïve CD8 T cells during high dose chronic LCMV infection (Chapter 2). This data fits well with previous studies, such as study by Kaech et. al which showed that transfer of 500,000 memory CD8 T cells into mice congenically infected with LCMV (a high viral load chronic infection), were better at reducing viral loads than transfer of an even greater number of effector CD8 T cells taken at day 8 post-acute LCMV infection (24). The bottom line is that on a per-cell basis memory CD8 T cells are much more efficient than naïve cells at controlling low dose or acute infection, and when a large enough number of memory CD8 T cells are present to rapidly reduce viral loads then memory cells are again better than naïve CD8 T cells at controlling a high dose persistent infection (Chapter 2). This suggests that memory CD8 T cells do not undergo this extra negative regulation (as discussed in Chapter 2) in situations where antigen levels/virus is quickly reduced or controlled. Therefore, when designing a CD8 T cell based vaccine for chronic infections it appears to be important to elicit enough functional CD8 T cells to quickly reduce antigen/viral loads, an idea supported by previous studies in SIV and LCMV (174, 175). In further support, a recent vaccine study in SIV, showed efficacy when CD8 T cells were able to rapidly reduce viral loads (178). Moreover, while little efficacy was seen in the recent human HIV clinical trials, the Merck STEP trial and the RV144 Thai trial, in reducing the viral set-point during chronic infection, in contrast, some efficacy was seen in decreased acquisition of infection in the RV144 Trail (176, 177). These studies and our own indicate that rapid reduction of viral loads may be vastly important for an effective vaccine to combat infections that can become persistent.

Furthermore, these studies bring up another important consideration in designing a CD8 T cell based vaccine, determining the type of memory CD8 T cells best able to provide protection. Memory CD8 T cells can be divided into two main groups, central memory cells ($CD62L^{hi}$ $CCR7^{hi}$) and effector memory cells ($CD62L^{lo}$ $CCR7^{lo}$)(58). Central memory CD8 T cells (Tcm) are generally confined to lymphoid tissues and have increased proliferative potential than effector memory CD8 T cells (Tem), however in contrast, Tem can be found distributed in tissues throughout the body(58, 60). We found that even during acute LCMV infection only Tcm (not Tem) were better than naïve CD8 T cells (at persisting) (Chapter 2). Furthermore, multiple groups have shown that Tcm are better able to reduce or control a high dose chronic LCMV infection than Tem (59, 212). In contrast, Hansen et. al. showed that a vaccine which elicited Tem was effective at reducing viral loads after SIV challenge (178). This discrepancy between the LCMV and SIV data may be inherent in the differing nature of the two infections. The studies in LCMV were done during systemic chronic LCMV infection (or after iv. infection of LCMV, allowing rapid dissemination of the virus), while in the SIV study the viral challenge was given intrarectally. Therefore Tcm may be more protective for a systemic LCMV infection, while a vaccine that elicits Tem cells in the gut, that can rapidly eliminate virus at the site of entry, may be more protective during a rectal SIV challenge. This suggests that the route of infection and perhaps the nature of infection (the virus/pathogen) may determine what type of memory CD8 T cells may be most protective.

Lastly, since memory CD8 T cells are more reliant on CD4 T cells during chronic infection it also is important for vaccines to elicit enough CD4 T cell help to "sustain"

these memory CD8 T cells upon encounter with high amounts of antigen/virus. Exactly how these CD4 T cells help memory CD8 T cells during chronic infection is a project worth developing further. Overall, it appears that in order for a CD8 T cell based vaccine to be effective in combating a high dose infection (or one that can become chronic), it is of vital importance that the vaccine results in a CD8 T cell response that rapidly reduces viral load. Some factors regulating the ability of the CD8 T cells to reduce viral load quickly are the number of CD8 T cells, the amount of CD4 T cell help, and the location of the memory cells (e.g. at the site of pathogen entry). If memory CD8 T cells are unable to rapidly reduce or control infection, they are tightly regulated by multiple mechanisms and are quickly lost during high viral loads or high antigen persistence.

Regulation of CD8 T Cells during Chronic Infection: Implications for Therapeutic Strategies

The body of work detailed in chapter 3 of this dissertation indicates that combined IL-2 therapy and PD-L1 blockade may be an attractive strategy for treating chronic infections. *In vivo* PD-L1 blockade alone has been shown to successfully increase virus-specific CD8 T cell responses and decrease viral loads in LCMV(73) and SIV(157) infection, however this effect is only partial in situations of high viral persistence. Therefore, it has been of interest to combined strategies to increase the effectiveness of therapy. Many of these strategies have been outlined in chapter 1 (the introduction). It is important to note that in general combined therapies, such as blocking multiple inhibitory receptors at the same time or combining therapeutic vaccination with blockade of inhibitory receptors or regulatory cytokines, has resulted in increased efficacy in chronic

infection models (42, 99). The strategy that we used in our work presented in chapter 3 is to combine an immunostimulatory signal for CD8 T cells (IL-2 therapy) along with blockade of an inhibitory receptor pathway (PD-1). IL-2 therapy alone has been shown to increase CD8 T cell responses during LCMV and SIV infection (134, 151, 152), however IL-2 therapy alone or combined with anti-retroviral drugs in HIV patients has shown little success (141-146). In contrast, there has been some clinical efficacy of IL-2 therapy, alone or combined with vaccines, in the treatment of metastatic cancers (138-140). Overall, the existing data on IL-2 therapy during chronic infections is relatively conflicting and another major limitation for IL-2 therapy is that it can have toxic side effects, such as vascular leakage, however, using lower doses of IL-2 can help minimize these effects (137). We were interested in combining IL-2 therapy and PD-Ll blockade as a therapy for treating chronic infections, in the hopes that combing these two therapies would allow for use of a low dose of IL-2, to increase the positive effects of IL-2 therapy that may be able to synergize with the blockade of PD-1 signaling to further efficacy, while limiting the negative side effects that can occur with IL-2 treatment, such as toxicity or activation induced cell death (AICD).

We show that in the chronic LCMV infection model, IL-2 therapy synergizes with PD-L1 blockade to massively increase CD8 T cell numbers and function, and decrease viral burden (Chapter 3). CD8 T cells numbers and function are enhanced even though IL-2 treatment or combined IL-2 therapy and PD-L1 blockade (combined treatment) increases T regulatory ($CD4^+$ $FoxP3^+$) numbers. It is important to note that IL-2 therapy itself is the cause of increase T regulatory (Treg) numbers, as PD-L1 blockade alone does not seem to increase Treg numbers, and combined therapy does not increase Treg

numbers over those seen after IL-2 therapy alone (Chapter 3). However, depletion of total CD4 T cells before combined treatment does further increase the effectiveness of treatment, indicating that CD4 T cells (or most possibly the expanded Treg population) is somehow dampening the effect of combined treatment (Chapter 3). The mechanism of the "suppression" of the effect of combined therapy by CD4 T cells is not known, it may be direct, possibly be the result of depletion of an IL-2 sink (since the expanded Tregs express high levels of the high affinity IL-2 receptor alpha chain) (213, 214), thus allowing the CD8 T cells to receive more IL-2 stimulus. How these CD4 T cells are negatively regulating the effects of combined therapy is an avenue of future interest. However, it is important to note that we still saw a huge expansion of CD8 T cell numbers, increased function of these cells and decreased viral loads even in the presence of expanded Treg numbers, thus combined therapy may be efficacious even in the presence of increased Treg numbers.

IL-2 has been documented as having contrasting effects on CD8 T cells, as it can increase pro-survival molecules, such as Bcl-2, or lead to negative effects, such as AICD (129-131). Also recent work of IL-2 administration done *in vitro* or *in vivo* early during acute infection, has shown that increased IL-2 signals can induce terminal differentiation of CD8 T cells (132, 133). However, it is important to note that inflammation can alter the effects of IL-2 on CD8 T cells (133). Therefore, we explored the effects of IL-2 therapy on CD8 T cells during chronic infection (a highly inflammatory environment), to help us understand how IL-2 influences exhausted CD8 T cells. We show in chapter 3 that IL-2 therapy can act directly on exhausted CD8 T cells, and leads to a decrease in the expression of multiple inhibitory receptors (PD-1, Tim-3 and 2B4), while increasing the

expression of the transcription factor T-bet. A recent report by Kao et al. showed that CD8 T cells that expressed high levels of T-bet during chronic infection had reduced inhibitory receptor expression and increased function (89). Therefore, one way that IL-2 may increase the function of exhausted CD8 T cells during chronic infection, is possibly by increasing T-bet levels in the cells or alternatively, IL-2 may cause the selective expansion of the T-bet high expressing cells.

In addition, we found that IL-2 administration or combined therapy increases IL-7 receptor alpha (CD127) expression on CD8 T cells during chronic infection. CD127 is a phenotypic marker that identifies the subset of CD8 T cell effectors that differentiate into memory CD8 T cells after an acute infection, therefore, CD127 is a canonical memory marker (40, 56, 57). Exhausted CD8 T cells have low surface expression of CD127, even at late time points during chronic LCMV infection (69). Therefore it is interesting to speculate that increased CD127 expression on CD8 T cells after IL-2 or combined therapy during chronic infection indicates a "re-programming" of the exhausted CD8 T cells back to a more "memory-like" state. Further experiments may elucidate what this increase in CD127 means for CD8 T cells during chronic infection, whether it infact indicates reprogramming of the cells or increased function, or if it has no significance for the differentiation state or properties of these CD8 T cells and is only a marker that is increased with IL-2 therapy. Forced expression of CD127 has been shown to be insufficient for inducing CD8 T cells to differentiate into memory CD8 T cells during acute infections (215, 216), therefore it is necessary to further explore the significance of CD127 upregulation on exhausted CD8 T cells during chronic infection. It is also important to note that this increase in CD127 is not just a function of reduced viral loads,

as we see increased CD127 expression even in cases where high levels of virus are maintained, such as in chronically infected mice given IL-2 therapy alone (Chapter 3). Furthermore, the fact that we see increased CD127 expression on exhausted CD8 T cells after IL-2 therapy is in contrast with studies showing that *in vivo* or *in vitro* administration of IL-2 during situations of limited antigen duration (ie. acute infection), results in a decrease in CD127 expression (132, 133, 198). The discrepancy between the effect of IL-2 on CD8 T cells during situations of limited antigen duration and that seen during antigen persistence may be reflective of a difference in the programming/state of the cells, difference in environmental signals, or a combination of both. Exhausted CD8 T cells have been shown to have a distinct molecular and phenotypic profile from naïve, effector or memory CD8 T cells during acute infection (62), therefore exhausted CD8 T cells may respond differently to IL-2 stimulus than these cells. Furthermore, pipkin et. al showed that in the presence of inflammation, IL-2 has altered effects on CD8 T cells, thus, the inflammatory environment of chronic infection may influence how IL-2 affects exhausted CD8 T cells (133). These are some important distinctions that need to be better understood by future work.

Overall, the work presented in chapter 3 indicates that combined IL-2 therapy and PD-L1 blockade may be a promising therapy for increasing CD8 T cell function and reducing viral loads during chronic infections. IL-2 itself may have very different effects on exhausted CD8 T cells in the chronic environment than on CD8 T cells in situations of limited antigenic duration, as IL-2 increases CD127 expression on CD8 T cells during chronic infection. This increase in IL-7 receptor alpha (CD127) expression may increase the sensitivity of these cells to IL-7 therapy. Recently, multiple reports in SIV (122-124)

and LCMV (103, 125) have indicated that IL-7 therapy results in increased T cell responses (LCMV and SIV) and decreased viral load (LCMV), therefore IL-2 therapy combined with IL-7 therapy may increase effectiveness. Moreover, combining IL-2 therapy and PD-L1 blockade may allow for the use of a lower dose of IL-2, allowing us to reap the positive benefits of IL-2 therapy while decreasing the negative effects, such as toxicity. Lastly, instead of using recombinant IL-2 alone for IL-2 therapy, it may be beneficial to administer it in the form of IL-2 plus anti-IL-2 antibody complexes that more specifically target binding to the IL-2 receptor β chain (CD122). Exhausted CD8 T cells express low levels of the high affinity IL-2 receptor α chain (CD25), however T regulatory cells express high levels of CD25 and other cells like pulmonary endothelial cells express some CD25. IL-2 administration affects these $CD25^{+}$ cells leading to Treg expansion and can also cause pulmonary edema due to it's binding to pulmonary endothelial cells (190, 191, 217). Recent reports have shown that administration of IL-2 plus some anti-IL-2 antibody complexes that target IL-2Rβ can lead to increased immune responses, while decreasing the negative side effects of IL-2 administration alone, such as Treg expansion and pulmonary edema (217-219). Therefore combining IL-2 or IL-2 + IL-2 antibody complexes and PD-1 blockade may be an attractive strategy to pursue in further investigations for the treatment of human chronic infections and cancer.

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