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Regulation of Cd8 T Cell Dysfunction During a Chronic Viral infection

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Abstract
After an acute infection or vaccination, antigen-specific CD8 T cells undergo memory differentiation once the pathogen has been completely cleared. Memory CD8 T cells acquire cardinal properties that allow them to confer long-term protection, including antigen-independent homeostasis and self-renewal, rapid reacquisition of effector functions and the ability to mount a rapid, potent secondary response. During a chronic viral infection, however, the pathogen is not cleared, and this appears to drive antigen-specific CD8 T cells down an altered path of differentiation. During chronic viral infection, antigen-specific CD8 T cells become functionally exhausted, in which they progressively lose effector function and upregulate the expression of multiple inhibitory receptors. Specific memory defects also occur, as exhausted CD8 T cells do not use the IL-7/IL-15 pathway efficiently. Antigen load and lack of CD4 help correlate to the severity of dysfunction, and gene expression studies show that the differentiation of exhausted CD8 T cells may be regulated by a unique transcriptional program. However, the exact pathways and mechanisms that directly regulate the differentiation of dysfunctional CD8 T cells during chronic viral infection are not clear. In this work, we examine transcriptional, homeostatic and 'inflammatory' vs. antigenic regulation of functional exhaustion. Through a system of partial and total conditional deletion, we identify the transcriptional repressor Blimp-1 as an important regulator of functional exhaustion and repressor of memory differentiation. We also describe a key memory property defect and the mechanism by which exhausted antigen-specific CD8 T cells are maintained during chronic infection. Lastly, we show that prolonged, pathogen-induced 'inflammation' alone can alter memory CD8 T cell differentiation, while other signals such as antigen may be necessary to lead to the loss of effector function and high expression of inhibitory receptors that are hallmarks of functional exhaustion. Together, we have identified multiple pathways at different levels of regulation that further our understanding of how functional exhaustion may occur during chronic viral infection.

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ABSTRACT

REGULATION OF CD8 T CELL DYSFUNCTION DURING A CHRONIC VIRAL INFECTION

Haina Shin

E. John Wherry, Ph.D.

After an acute infection or vaccination, antigen-specific CD8 T cells undergo memory differentiation once the pathogen has been completely cleared. Memory CD8 T cells acquire cardinal properties that allow them to confer long-term protection, including antigen-independent homeostasis and self-renewal, rapid reacquisition of effector functions and the ability to mount a rapid, potent secondary response. During a chronic viral infection, however, the pathogen is not cleared, and this appears to drive antigen-specific CD8 T cells down an altered path of differentiation. During chronic viral infection, antigen-specific CD8 T cells become functionally exhausted, in which they progressively lose effector function and upregulate the expression of multiple inhibitory receptors. Specific memory defects also occur, as exhausted CD8 T cells do not use the IL-7/IL-15 pathway efficiently. Antigen load and lack of CD4 help correlate to the severity of dysfunction, and gene expression studies show that the differentiation of exhausted CD8 T cells may be regulated by a unique transcriptional program. However, the exact pathways and mechanisms that directly regulate the differentiation of dysfunctional CD8 T cells during chronic viral infection are not

clear. In this work, we examine transcriptional, homeostatic and 'inflammatory' vs. antigenic regulation of functional exhaustion. Through a system of partial and total conditional deletion, we identify the transcriptional repressor Blimp-1 as an important regulator of functional exhaustion and repressor of memory differentiation. We also describe a key memory property defect and the mechanism by which exhausted antigen-specific CD8 T cells are maintained during chronic infection. Lastly, we show that prolonged, pathogen-induced 'inflammation' alone can alter memory CD8 T cell differentiation, while other signals such as antigen may be necessary to lead to the loss of effector functon and high expression of inhibitory receptors that are hallmarks of functional exhaustion. Together, we have identified multiple pathways at different levels of regulation that further our understanding of how functional exhaustion may occur during chronic viral infection.

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Chapter 1

Introduction

Immunological memory: A (brief) historical perspective.

The concept of immunological memory has existed for a long time, stretching back over two thousand years to the plague of Athens during the Peloponnesian War. The historian Thucydides noted that in the time of this terrible disease, the most effective caregivers were those that had been previously infected and recovered, “for the same man was never attacked twice – at least not fatally” (1). About a millennia later, the Chinese and Indians first put this concept to practice through a crude form of vaccination known as variolation (2). In an attempt to protect against the scourge of smallpox, material from the pustules of infected patients was introduced into healthy individuals, who were subsequently protected from disease (2). Despite the significant morbidity and mortality associated with variolation and the occasional epidemics that the practice caused, it remained popular until the observations of Edward Jenner (3). The birth of modern vaccinology is credited to Jenner, based on experiments that showed that innoculation of a milder disease (cowpox) would protect from a more severe but related infection (smallpox) (3).

Despite this breakthrough in disease prevention, the immunological and microbiological basis behind vaccination was unknown. It was not until the establishment of the germ theory of disease and the discovery of multiple

microbes that the cause of infection was known (2, 4). Later, the observation that immunity could be passively transferred through sera (antibodies) laid the immunological groundwork for the study of vaccines (2). Although recognition of cellular immunity did not occur until well after the discovery of humoral immunity (5), the field of immunology was rapidly becoming very effective at combating many major public health threats.

By 1962, progress in the study of immunology, the ability to develop new vaccines and emergence of antibiotics caused the famed virologist and immunologist Sir McFarlane Burnet to declare that "…almost all the major practical problems of dealing with infectious disease had been solved" and that "The late 20th century will be the witness to the virtual elimination of infectious disease as a significant factor in social life" (6). This, however, has not proven to be the case. Rather than seeing the end of infectious disease, the late 20th century has been witness to the rise of enormous new problems, including super-resistant pathogens and the HIV/AIDS pandemic.

At present, almost thirty years after the onset of the pandemic, over 33 million people suffer from HIV/AIDS, with millions more infected each year (7). Hundreds of millions suffer from other chronic viral infections such as HCV and HBV (8). While vaccines for HBV exist (8) and antiviral medications for these infections are available, their limited efficacy, uneven distribution and expense make it imperative to discover vaccination strategies and immunotherapies that will help relieve disease burden, particularly in the developing world. Although great advances have been made in understanding the biology and pathogenesis

of infections such as HIV (9), all of the immunological determinants that are required in order to successfully control these infections have not yet been defined. One parameter that appears to correlate with good viral control is potent cytotoxic T cell responses (10-13) . However, during many human chronic viral infections, as well as animal models of chronic viral infection, virus-specific CD8 T cells often lose their effector function and become functionally exhausted (14). The mechanisms that regulate functional exhaustion are not well understood, and the studies that lie herein will attempt to elucidate some of the the cell-intrinsic and –extrinsic factors that regulate the differentiation of dysfunctional T cells during chronic viral infection.

An overview of the adaptive immune system.

The immune system consists of two major arms – the innate immune system, and the adaptive immune system. Lymphocytes within the adaptive immune system each express a unique receptor, and all together they create a broad repertoire of specificities (15, 16). The defining characteristic of the adaptive immune system is its ability to use this broad repertoire to 'adapt' immune responses to individual pathogens by the process of clonal selection (15, 16). Another key characteristic of the adaptive immune system is immunological memory – the ability to 'remember' previous encounters with a particular pathogen (17). The adaptive immune system is primarily composed of two cell types: T cells and B cells. While B cells provide the humoral component of

adaptive immunity and are critical for most current vaccines, this study will focus on T cells, and specifically, CD8 T cells.

T cells, like all leukocytes, are generated from hematopoeitic stem cells (HSCs). HSCs give rise to a number of progenitor cells, including a thymic settling progenitor that seeds the thymus and gives rise to an early thymic progenitor (18). Within the thymus, thymocytes go through three distinct phases: double negative (CD4-CD8-, DN), double positive (CD4+CD8+, DP) and single positive (CD4+CD8- or CD4-CD8+, SP) (18). Expression of a functional pre-T cell receptor (TCR) by DN thymocytes leads to the DP stage (19), where successful V(D)J recombination of the TCRα chain leads to the expression of a TCR by DP thymocytes (20). To pass from the DP to the SP stage, thymocytes must successfully go through positive selection, which determines the functionality of the TCR by its ability to recognize self-peptide bound to MHC class I (CD8 SP) or class II (CD4 SP), and the lineage of the future T cell is established (20). DP thymocytes must also undergo negative selection, a process that mediates central tolerance and removes most self-reactive T cells from the repertoire (21).

Once in the periphery, naïve T cells are maintained by the cytokine IL-7 and contact with the appropriate self-peptide/MHC complex (22). Upon infection or vaccination, T cells become activated when they encounter a mature antigen-presenting cell (APC) that carries a pathogen-derived peptide bound to an MHC molecule that is recognized by their specific TCR (signal 1). Costimulatory signals (signal 2), such as those received from molecules like CD28, CD27,

OX40 and others (23), and a ‘signal 3’ provided by inflammation generally leads to a productive T cell response (24-26). Once activated, the inflammatory environment can reinforce the type of response that will occur (27, 28). Other signals, such as those received through toll-like receptors (TLRs) have been shown to play a role in the activation and proliferation of T cells (29, 30).

CD4 and CD8 T cells perform distinct functions during immune responses. CD4 T cells, or T helper (Th) cells can determine the type of response that will be made to a pathogen. The heterogeneity within the CD4 T cell population was first defined by the cytokines secreted by Th1 (IFNγ and IL-2) and Th2 cells (IL-4 and IL-5) (31). Ensuing work with *Leishmania major* infection showed that the T helper subsets could be further distinguished by the ability to protect against or exacerbate disease (32). Subsequently, Th1 responses were designated as protective against intracellular pathogens, while Th2 responses were shown to be protective against helminths and extracellular pathogens (27). More recently, a novel lineage has joined the T helper paradigm (33-35). Called Th17 for the production of IL-17, this subset plays a an important role in host defense and promotes inflammation during autoimmune disorders (36). These three subsets, along with regulatory T cells (Treg), were thought to be distinct, mutually exclusive lineages (28). Th1, Th2, Th17 and Treg cells all express definitive transcription factors, and interactions between these transcription factors can lead to the repression of other lineages (28). Furthermore, fully differentiated subsets of T helper cells can undergo chromatin remodeling to reinforce gene expression and silence genes of other lineages (37). However, recent studies

with Tregs and Th17 cells show that the CD4 T cell lineages may be more plastic than originally thought (38). In addition to the direct response of CD4 T cells against pathogens, T helper cells can condition DCs (39-41) and also provide help to both B cells (42) and CD8 T cells (43).

CD8 T cells are cytotoxic lymphocytes (CTLs) that kill infected target cells. CD8 T cells can respond to both intracellular and extracellular pathogens as well as cancers (44). CD8 T cells, like CD4 T cells, can differentiate into T cytotoxic type I (Tc1) cells, which secrete IFNγ and TNFα, and T cytotoxic type II (Tc2) cells, which produce IL-4, IL-5 and IL-10 (44, 45). Although Tc2 cells have been found in situations of chronic infections as well as cancer (46-48), and can be generated *in vitro* (49), their role in the immune response has not been as well explored as Tc1. CTLs are capable of directly lysing infected target cells using the cytotoxic granules granzyme and perforin (50), although in some murine cancer models Tc2 cells have been shown to be less effective at utilizing this pathway (51). CTLs may also induce apoptosis of target cells through Fas/FasL interactions (50). More recently, it has been shown that CD8 T cells can also produce IL-17, with or without genetic manipulation, although some Tc17 cells do not appear to have any cytolytic capacity (52-54).

Once activated, CD8 T cells undergo a massive clonal burst during which naïve precursors differentiate into effector CD8 T cells and increase in number by several orders of magnitude (55-57). For CTLs, the size of the clonal burst can be programmed within a very short priming phase (58, 59). Within the effector population, there is heterogeneity that can broadly be defined as cells that will

eventually die, and those that will not. These two subsets are terminally differentiated effector CD8 T cells and memory precursors (60, 61). Both subsets are equally cytotoxic have similar effector gene expression patterns, but terminally differentiated effector CD8 T cells are characterized by their high expression of KLRG-1, low expression of the IL-7Rα chain (CD127), and high levels of the transcription factor Tbet, while memory precursors are KLRG-$1^{lo/int}$,$CD127^{hi}$, $Tbet^{lo}$ and can produce high levels of IL-2 (60-63). Memory precursor cells survive and differentiate into a population of long-lived memory CD8 T cells (64). Unlike memory precursors, many terminally differentiated effector CD8 T cells are destined to die during or shortly after the contraction phase (60, 61), although a small percentage does survive, most likely by IL-15 signaling (60, 65). These cells appear form a small pool of 'terminal' effector memory CD8 T cells with memory properties that are less developed than effector memory CD8 T cells that arise from memory precursors (60, 65, 66).

It is not yet clear how the CD8 T cell effector subsets are generated, and there is also some contention as to whether there is a linear differentiation relationship between effector and memory CD8 T cells (66-69). Several models have been set forth to explain the how these two subsets are formed, and thus far a model of progressive differentiation appears to encompass the current data most effectively (66). In this model, two lineages are formed upon activation through differences in TCR signal strength and length of stimulation (60, 61, 70-72). Asymmetric division has been proposed as a mechanism to initiate heterogeneity at the activation stage, where one single cell can give rise to two

daughter cells that are of different fates (72). Although the subsets are formed early, they remain plastic as KLRG-1^{lo} cells retain the ability to give rise to KLRG-1^{hi} cells (60, 61). The size of each subset is likely determined not only by TCR signals, but also by the strength and length of exposure to inflammation (60, 61). While this model appears to best describe the events after an acute infection, it is possible that other models are more relevant in other situations, such as chronic infection or non-infectious immunizations (66, 73). After a non-virulent immunization with DCs pulsed with an *L. monocytogenes* (LM)-derived peptide, the presence of memory-phenotype antigen-specific CD8 T cells have been observed at the peak of the CD8 T cell response (68). Furthermore, these memory-phenotype antigen-specific CD8 T cells provide comparable protective immunity to antigen-specific memory CD8 T cells generated after acute LM infection (68). It has also been shown that CD8 T cells treated *in vitro* with IL-15 or low doses of IL-2 acquire a memory phenotype, suggesting that these cells do not need to bypass the effector stage to become memory (67). Thus, while memory CD8 T cells may be generated through mutliple different pathways, this work will focus on the linear model in which memory CD8 T cells differentiate from effector CD8 T cells.

Lymphocytic choriomeningitis virus model of infection.

Lymphocytic choriomeningitis virus, or LCMV, is an arenavirus whose natural reservoir is *Mus musculus* (74). Although a natural mouse pathogen, LCMV is known to cause disease in humans, mostly from exposure to infected

rodents (75). LCMV is a non-cytopathic virus that causes a systemic infection, and almost all of the tissue damage associated with the infection is due to immunopathology (76). Although many genetic variants of this virus exist, the strains that are used for this study are LCMV Armstrong (Arm) and LCMV clone 13.

Infection of H-2(b) restricted C57BL/6 mice with LCMV induces a strong Tc1 response, and immunity to LCMV is mediated almost entirely by CD8 T cells (77-80). Accordingly, LCMV Arm infection elicits an enormous CD8 T cell response which peaks around day 8 post infection (55, 56). Of the CD8 T cells, 85-95% are $CD44^{hi}$, and almost all of the $CD44^{hi}$ CD8 T cells are LCMV-specific (81). Two immunodominant epitopes, DbNP396 and DbGP33, as well as other epitopes including KbGP34, DbGP276 and KbNP205, comprise approximately 80% of the LCMV-specific response, and are derived from the glycoprotein (GP) and nucleoprotein (NP) of the virus, which are products of the S-RNA segment of LCMV (81-83). Recently, epitopes derived from the L-RNA segment of the LCMV genome were identified, and these L epitopes roughly account for the remaining 20% of the LCMV-specific CD8 T cell response (82). Infection with LCMV Arm lasts for 8-10 days, after which the virus is completely cleared.

LCMV clone 13 is a genetic variant of LCMV Arm which was isolated from the spleens of carrier mice that were neonatally infected with LCMV Arm (84). While both LCMV Arm and clone 13 infect fibroblasts and macrophages, neither infects lymphocytes well (85). Two amino acid changes in LCMV clone 13 account for its persistence after infection. One mutation in the glycoprotein (F-

>L, position 260) provides LCMV clone 13 with greater affinity to its receptor, α-dystroglycan, which allows the virus to infect a greater number of macrophages as well as infect DCs, something LCMV Arm is unable to do (85-88). Another amino acid change in the viral polymerase (K->Q, position 1079) increases the viral replication rate, leading to greater virus yield after infection (85). These two mutations lead to different patterns of dissemination, as LCMV Arm remains in the red pulp of the spleen, while LCMV clone 13 also invades the white pulp (87).

Infection of C57BL/6 mice with LCMV clone 13 leads to a chronic infection in which multiple tissues become infected. LCMV clone 13 virus can be found in the spleen and liver for approximately one month after infection, in the serum for two to three months and in the kidney and brain for the life of the animal (89). LCMV clone 13 infection can also invade the bone marrow, lymph nodes and thymus, which leads to central tolerance early during chronic LCMV infection and in neonatally infected carrier mice (89). LCMV clone 13 also elicits a strong CD8 T cell response with kinetics that are similar to that of LCMV Arm (89). Both LCMV Arm and clone 13 present the same CD8 T cell epitopes, which allows a direct comparison of CD8 T cell responses during infection (85, 86). However, the overall CD8 T cell response during LCMV clone 13 is diminished compared to LCMV Arm due to the deletion of certain epitope-specific populations, resulting in mild lymphopenia (89-92). Regardless, the availability and similarity of these two strains make LCMV a powerful tool in the study of CD8 T cell respones to chronic viral infections.

Antigen-specfic memory CD8 T cell differentiation after acute infection or vaccination.

I. Generation of memory CD8 T cells.

After an acute infection or vaccination, antigen-specific CD8 T cells become activated and differentiate into a heterogeneous pool of effector CTLs, as described above. During the contraction phase, 90-95% of the effector population dies, and the remaining 5-10% survive to differentiate into memory CD8 T cells (66). Apoptosis of CD8 T cells during this phase is mediated by the molecules such as Fas and Bim (93, 94). After LCMV Arm infection, although memory precursors express high levels of CD127, expression of this marker is independent of IL-7 and does not confer any selective advantages (63, 95). Furthermore, memory precursors that are present within the effector population (d8 p.i.) are not yet able to respond to IL-7 and IL-15 signals *in vivo* (64). It is important to note that memory differentiation of CTLs after acute infection occurs only after the infection has been completely cleared.

As they differentiate, memory CD8 T cells acquire properties that allow them to confer long-term protection. Memory CD8 T cells have a resting phenotype and downregulate the expression of effector molecules, but upon restimulaton, can rapidly reacquire their effector function, including production of antiviral cytokines and cytolysis (43, 96-99). In natural settings, the immune system is called upon to respond to a number of different infections. It was originally thought that the size of the total memory CD8 T cell pool was constrained, and that with each new, heterologous infection, previously existing

memory populations would undergo attrition to make ‘room’ for new specificities (100, 101). However, recent work by Vezys et al suggests that rather than undergo attrition, the memory CD8 T cell pool appears to increase in size to accommodate new specificities, and that previously existing populations remain intact (102). It is not clear why this discrepancy has been observed, but could have important implications for vaccination strategies.

Various components are required to generate functional memory CD8 T cells. One key factor that is required for the proper differentiation of memory CD8 T cells is CD4 T cell help. CD8 T cell responses to primary challenges are independent of CD4 T cell help, provided that sufficient inflammation is available to induce the maturation of DCs (43). However, in the absence of CD4 T cells, memory CD8 T cells are deficient in secondary responses and do not appear to mature over time (103-105). While the necessity of CD4 T cells in establishing a functional memory CD8 T cell population has been well described, the exact mechanism by which CD4 T cells provide help is still unclear. Suggested mechanisms include CD40/CD40L interactions, via DCs or directly between CD4 and CD8 T cells (39-41, 106), and the regulation of TRAIL expression on CD8 T cells (107). IL-2 is required during the priming of CD8 T cells, so it is also possible that CD4 T cells may provide some help through early IL-2 production (108). However, depending on the model of used to generate memory CD8 T cells, CD40/CD40L interactions and absence of TRAIL have been shown to be dispensible (109-112). Furthermore, it is unclear as to when CD4 T cells provide help. While originally thought to be necessary for the early ‘programming’ of

memory CD8 T cells (104, 113), it seems that CD4 T cells may be required for the mainenance, rather than the 'programming' of memory CD8 T cells (105). Also, while it is not clear what CD4 help imparts to a differentiating CTL, studies have shown that CD4 help may help 'imprint' a memory program through chromatin remodeling (114, 115). Aside from CD4 help, certain inflammatory signals have been shown to be important for the generation of memory CD8 T cells. Along with IL-2, depending on the priming agent, both IL-12 and type I interferons can support the differentiation of memory CD8 T cells (116, 117).

II. Maintenance of memory CD8 T cells.

A major function of memory CD8 T cells is to provide protection against reinfection long after the primary challenge has been been encountered. In order to do so, a memory CD8 T cell population must be stably maintained long-term. Original observations suggested that persistent antigen or interaction with MHC was necessary in order to maintain protective immunity (17, 118), but experiments in MHC class I knockout animals and adoptive transfer models have shown that antigen or interaction with MHC is not required (119, 120). Upon the observation that adjuvants alone could drive transient, bystander proliferation of memory CD8 T cells (121), it was discovered that type I interferons induced the production of IL-15, which in turn acted directly on memory CD8 T cells (122). Memory CD8 T cells rely on the homeostatic cytokines IL-7 and IL-15 for their survival and self-renewal (73, 123, 124). IL-7 appears to provide a survival signal, most likely through the upregulation of pro-survival molecules such as

Bcl-2 (125, 126). IL-15 mediates the slow, steady homeostatic proliferation of memory CD8 T cells (127, 128) which preferentially occurs in the bone marrow (129).

Since the self-renewal program that memory CD8 T cells undergo is reminiscent of stem cells, there has been interest in determining whether memory CD8 T cells possess any other stem-cell like qualities. Unique populations of memory CD8 "stem cell" populations have been identified (130, 131) in different inflammatory settings. Comparisons of transcriptional profiles show that memory CD8 T cells are enriched for molecules that are also expressed by HSCs (132). Expression of Bmi-1, a transcriptional repressor expressed by HSCs to prevent senescence, is upregulated in KLRG-1^{lo} but not KLRG-1^{hi} activated CTLs (133). While intriguing, further studies are necessary to determine whether the 'memory stem cell' actually exists in memory populations generated after infection.

III. Heterogeneity in the memory CD8 T cell population.

Memory CD8 T cells continue to differentiate over an extended period of time, as a pool of less mature effector memory CD8 T cells (CD62L^{lo}, CCR7^{lo}; T_{EM}) gradually converts to a population of central memory CD8 T cells (CD62L^{hi}, CCR7^{hi}; T_{CM}) (134, 135), although not all T_{EM} may differentiate to T_{CM}. Some evidence suggest that a more terminally differentiated population of T_{EM} may arise, perhaps from short-lived effector CD8 T cells (60, 65, 66), and that these T_{EM} may never convert to T_{CM} (136). The lifespan of these 'terminally

differentiated' T_{EM}, the role they may play in providing protective immunity and whether they are generated after different types of acute infections is not yet clear.

As the conversion from T_{EM} to T_{CM} occurs, memory CD8 T cells also become CXCR3^{hi}, CD43^{lo} and CD27^{hi}, indicating a more mature and less activated state (137). Due to their differences in the expression of lymphoid homing markers, T_{EM} and T_{CM} also occupy different anatomical niches, with T_{EM} localizing primarily in non-lymphoid tissues and T_{CM} localizing in lymphoid organs (134, 138). Functionally, T_{CM} produce higher levels of IL-2 than T_{EM}, have a higher proliferative capacity, may produce more antiviral cytokines such as TNFα and undergo more efficient homeostatic turnover (135, 139). Upon secondary challenge, the protective capacity of T_{EM} vs. T_{CM} appears to depend on the route of infection and dissemination of the pathogen (i.e. systemic vs. local) (140, 141). Interestingly, it has also been shown that regardless of the subset, memory CD8 T cells that have been 'rested' for a longer period of time after a primary challenge respond better than memory CD8 T cells that have been 'rested' for a short period of time (137, 142), indicating that maturation of the memory CD8 T cell population does not necessarily have to be accompanied by a phenotypic shift.

While it is clear that the memory CD8 T cell population is dynamic, there is some controversy over the origins of the central and effector memory subsets. While considerable evidence suggests that the conversion from T_{EM} to T_{CM} is a linear path of differentiation (68, 135, 143, 144), some have suggested that the

two lineages may arise separately depending on the frequency of naïve precursors (136, 145, 146). However, it has also been shown that many factors, including the strength of stimulation, clonal competition (143) and length of infection (68, 147) can affect the differentiation of T_{EM} to T_{CM}, and thus differences in precursor frequency may impact the rate of conversion (i.e. increasing precursor frequency leads to faster conversion).

IV. Transcriptional control of memory differentiation.

The differentiation of CTLs from effector to memory CD8 T cells is accompanied by changes in gene expression (64). Several transcription factors have been shown to play key roles in regulating this process. The T-box factors Tbet and Eomesodermin (Eomes) have been shown to play critical roles in regulating CD122 expression and thus, IL-15 responsiveness in memory CD8 T cells (148). Deletion of Tbet also corrects the defective T_{CM} differentiation observed in memory CTLs that have not received CD4 help (149). Blimp-1, a zinc-finger containing repressor, has been shown to play an important role in the activation and differentiation of CTLs. Inactivation of Blimp-1 resulted in the improper activation of naïve T cells, which led to the accumulation of effector and memory-like populations (150, 151). Both Tbet and Blimp-1 have been implicated in the fate decision between terminally differentiated effector and memory precursor after acute infection (Rutishauser et al, Immunity, in press; Kallies et al, Immunity, in press). Other transcription factors such as Bcl6 and its homologue Bcl6b (152, 153), Id2 (154) and Bmi-1 (133) have also been shown to

play a role in the differentiation of memory CD8 T cells. As more transcription factors are identified, it will be important to establish a molecular identity for memory CD8 T cells in order to better understand what happens when differentiation occurs improperly, as during chronic infections.

CD8 T cell exhaustion during chronic infections.

I. Properties of functionally exhausted CD8 T cells.

Proper memory differentiation occurs after acute infection or vaccination in the complete absence of antigen. During chronic infection, however, the antigen persists, and this appears to have a profound impact on the differentiation of antigen-specific CD8 T cells. First observed in the LCMV system, it was noted that 'functionally exhausted' antigen-specific CD8 T cells during chronic infection persisted but lost the ability to produce effector cytokines, and that the phenotype was more profound in the absence of CD4 help (90). Subsequently, it was found that chronic infection could impact multiple properties of antigen-specific CTLs (89, 155), and that the loss of effector function occurred in a progressive manner (89, 155, 156). IL-2 production, cytotoxicity and a high proliferative capacity are lost early during functional exhaustion, followed by TNFα production and finally, IFNγ production (89, 155, 156). In extreme cases where antigen burden is particularly high, some antigen-specific CD8 T cells are physically deleted from the response (156, 157). Functional exhaustion of CD8 T cells is not limited to chronic LCMV, as it has been described in other murine models of chronic infection such as polyoma virus, Friend's leukemia virus, adenovirus and mouse

hepatitis virus (14). Observations also extend to human chronic infections, including HIV, HCV and HBV and human T lymphotrophic virus (14). It should be noted, however, that not all persistent infections lead to functional exhaustion. Persistent but latent infections such as CMV, EBV in humans and murine γHV leads to CD8 T cells that are dysfunctional but have intact effector functions (14).

One key characteristic of functionally exhausted antigen-specific CD8 T cells is their high expression of multiple inhibitory receptors (158-161). A strong correlation has been shown between the amount and number of inhibitory receptors co-expressed per cell and the severity of dysfunction (160). One inhibitory pathway that has received considerable attention is the PD-1/PD-L pathway. First described within the LCMV system, PD-1 is highly expressed on antigen-specific CD8 T cells during chronic viral infection (162), and expression can be divided into two subsets of PD-1^{int} and PD-1^{hi} cells (163). *In vivo* antibody blockade of this pathway led to selective re-invigoration of the PD-1^{int} subset of CD8 T cells (163), resulting in vigorous proliferation and enhanced viral control (160, 162, 163). It should be noted that all activated CD8 T cells upregulate PD-1, but expression levels never reach that of exhausted CD8 T cells (162, 163). Since the initial observation, PD-1 expression on antigen-specific CD8 T cells has been noted during HIV (164, 165), HCV (166), and HBV (167), as well as during SIV (168). PD-1 expression correlated directly with viral load and inversely with CD4 counts in HIV patients, and PD-1 levels declined in patients treated with HAART (164). During HCV infection, CD8 T cells in the blood expressed less PD-1 than those found in the liver, suggesting that tissue-specific

expression of the inhibitory receptor may be dependent on viral load (169, 170). *In vivo* blockade in an SIV model led to increased proliferation and improved effector function of virus-specific CD8 T cells (162, 171). *In vitro* blockades show that PD-1 directly mediates the survival of exhausted CD8 T cells, and that increased proliferation and improved effector function are likely secondary effects (163, 172). Although the role inhibitory receptors play in the initiation of functional exhaustion is uncertain, it is clear that they regulate multiple, distinct aspects of T cell dysfunction. Other inhibitory receptors expressed by exhausted CD8 T cells include LAG-3, CD160, 2B4, Tim-3 and CTLA-4 (158, 160, 161, 173). Along with PD-1, inhibitory receptors such as LAG-3 and CD160 regulate proliferation and cytotoxicity, respectively (160). In order to further understand the biology of functionally exhausted CD8 T cells and the factors that influence their differentiation, this thesis will examine, in three chapters, the cell-intrinsic and extrinsic mechanisms regulating T cell dysfunction during chronic viral infection.

II. Transcriptional control of functional exhaustion.

While significant work has been done with the role of inhibitory receptors during chronic infection, very little is known about the transcriptional control of functional exhaustion. Gene expression studies show that functionally exhausted CD8 T cells differentially express over three hundred genes as compared to effector and memory CD8 T cells (158), suggesting that functional exhaustion is a unique differentiation state. Many of these genes included transcription factors,

including Blimp-1, Tbet, and Eomes (158). It is interesting to note these transcription factors play an important role in regulating the fate decision between terminally differentiated effector CD8 T cells and memory precursors after an acute infection (Rutishauser et al, Immunity, in press; Kallies et al, Immunity, in press)(60). Chapter 2 of this thesis will explore the role of Blimp-1 in regulating functional exhaustion during chronic viral infection. Our data show that Blimp-1 plays a role in regulating key aspects of functional exhaustion, including the upregulation of inhibitory receptors. With this work, we have identified a molecular mechanism underlying functional exhaustion, and have provided a novel potential target for future therapeutic strategies.

III. Memory defects in functionally exhausted CD8 T cells.

Despite their loss of effector function, exhausted antigen-specific CD8 T cells persist indefinitely in chronically infected hosts (90, 174). Although the exhausted CD8 T cell population survives long-term like memory CD8 T cells, there are clear alterations in the memory differentiation program during chronic viral infection. Unlike memory CD8 T cells which gradually convert from T_{EM} to T_{CM} over time, exhausted CD8 T cells do not adopt the $CD62L^{hi}$ $CCR7^{hi}$ phenotype associated with T_{CM} (174-176). While effector functions that are lost during functional exhaustion can, to some extent, be recovered after viremia is controlled (155), the memory defects described here remain even after viral burden begins to decrease (174, 177).

After acute infection, memory CD8 T cells are maintained by the homeostatic cytokines IL-7 and IL-15, a mechanism which is facilitated by the high expression of CD127 and CD122 (43). However, during many chronic infections, including LCMV, γHV, HIV, HCV, HBV and others, virus-specific CD8 T cells fail to upregulate these receptors (14). Exhausted CD8 T cells also do not respond to IL-7 and IL-15 *in vitro*, and do not use these cytokines efficiently *in vivo* (174, 178-180). The persistence of the exhausted CTLs *in vivo* and their inability to make use of the IL-7/IL-15 pathways raises the question as to how these cells are maintained long-term during chronic infection. Two studies have explored the possibility that recent thymic emigrants (RTEs) may join the existing pool of virus-specific exhausted CD8 T cells. One study infected thymectomized mice with LCMV clone 13 and found that the antigen-specific CD8 T cell population could be stably maintained over a long period of time without input from the thymus (181). Another study, however, showed that congenically marked CD8 T cells specific for polyoma virus could not be maintained after adoptive transfer to a new, infection-matched host (182). Furthermore, it was shown that RTEs could be primed by persistent antigen, and that these RTEs contributed to the dynamic phenotype of the antigen-specific CD8 T cell population (182). Aside from RTEs, other possible maintenance signals include persistent antigen, an unidentified cytokine or growth factor or some unknown cell-cell contact. In the third chapter, we investigated these possibilities and found that persistent antigen is the required signal for the maintenance of the exhausted CD8 T cell population, and that these cells are maintained by a unique

proliferative mechanism. We believe that our findings have important implications for the treatment of chronic infections in patients, where viral clearance or enhanced viral control could have an impact on the maintenance of protective immunity.

IV. Extrinsic regulation of the differentiation of functional exhaustion.

The cellular changes that occur in functionally exhausted CD8 T cells are numerous. Recently, several cell-extrinsic pathways have been identified that have an important impact on both the pathogenesis of infection as well as the differentiation of antigen-specific CD8 T cells. During chronic LCMV infection, the immunosuppressive cytokine IL-10 was shown to play a role in suppressing T cell function (183, 184). Genetic ablation of IL-10 production or treatment with αIL-10R antibody at the onset of infection prevented the infection from persisting and improved T cell responses (183, 184). Furthermore, αIL-10R treatment after the establishment of infection enhanced viral control and led to an increase in IFNγ production from antigen-specific CD8 T cells (183, 184). Since then, elevated IL-10 levels have been observed to correlate with viral load in HIV infected patients and *in vitro* blockade of the IL-10 pathway led to increased proliferation of HIV-specific CD8 T cells (185). More recently, the cytokine IL-21 was shown to play a critical role in regulating the functionality and survival of CD8 T cells (186-188). The absence of IL-21 signaling in either IL-21-/- or IL-21R-/- mice infected with LCMV clone 13 or high dose of LCMV Docile led to severe exhaustion and a gradual loss of the antigen-specific CD8 T cell

population, and that in turn led to impaired viral control (186-188). It is unclear, however, whether IL-21 production (primarily from CD4 T cells) is higher or lower during chronic viral infection, as the two studies using LCMV clone 13 showed higher mRNA but lower protein as compared to LCMV Arm (186, 188). Along with these two cytokines, the 'inflammatory' environment of LCMV clone 13 is markedly different than LCMV Arm infection (S. Blackburn, unpublished data), and it is likely that there are other factors present (or absent) during LCMV clone 13 that could regulate T cell dysfunction. Chapter 4 investigates the role of persistent 'inflammation' in the differentiation of exhausted CD8 T cells. We find that 'inflammation' alone has a profound impact on memory differentiation, does not alter other key aspects of functional exhaustion such as the expression of inhibitory receptors. Our results could have important implications in the design of vaccination strategies, particularly of people who are chronically infected, and have the potential to further our understanding of how functional exhaustion is regulated during chronic viral infection.

Chapter 2

A role for the transcriptional repressor Blimp-1 in CD8 T cell exhaustion during chronic viral infection

Abstract

After an acute infection, virus-specific CD8 T cells undergo memory differentiation once antigen has been cleared, resulting in a pool of highly functional memory CD8 T cells. During chronic infection, however, virus-specific CD8 T cells follow an altered program of differentiation, resulting in a population of functionally exhausted CD8 T cells. While multiple inhibitory pathways have been shown to play an important role in regulating different aspects of functional exhaustion, the transcriptional mechanism that leads to exhaustion has not been elucidated. In this study, we describe a major role for Blimp-1 in CD8 T cell exhaustion during chronic viral infection. We find that Blimp-1 represses the acquistion of important memory properties and promotes functional exhaustion by regulating the expression of inhibitory receptors. Furthermore, our studies indicate that while high expression of Blimp-1 promotes functional exhaustion, moderate levels of Blimp-1 are required for effector function such as cytotoxcity. Thus, we define Blimp-1 as a transcriptional rheostat that balances expression of inhibitory receptors, memory differentiation and effector function.

Introduction

Functionally exhausted CD8 T cells have been identified and described in many different models of chronic viral infection. The hallmarks of functional exhaustion include the hierarchical loss of effector function (14) and the high expression of multiple inhibitory receptors (158-160, 164, 173). However, the molecular mechanisms that underlie these properties have not been investigated. Gene expression studies show there are a significant number of genes that are differentially expressed by exhausted CD8 T cells, including those encoding transcription factors such as Blimp-1 (158). This suggests that there may be a unique transcriptional program guiding the differentiation of these cells. In this chapter, we examine the role of transcription factor Blimp-1 in the regulation of functional exhaustion during chronic viral infection.

Blimp-1 is a zinc-finger containing transcriptional repressor that is perhaps best known for regulating fate decisions during the differentiation of activated B cells (189-191). High expression of Blimp-1 leads to the repression of the memory B cell program and promotes the terminal differentiation of plasma cells (190). Blimp-1 also plays a role in regulating the fate decisions of non-hematopoeitic cells as well, including germ cells (192, 193) and hair follicle stem cells (194). More recently, Blimp-1 was shown to be expressed in T cells, in which it regulates homeostasis and activation (150, 151). Inactivating Blimp-1 through mutation or deletion in naïve T cells led to an activated phenotype and the accumulation of effector and memory T cells (150, 151). Blimp-1 also regulates the expression of effector molecules such as IFNγ (195), IL-2 (196) and

granzyme B (197) in T cells, and is induced by TCR signals as well as cytokines such as IL-2 and IL-4 (197, 198).

Based on the role that Blimp-1 plays in regulating the fate decisions and differentiation in numerous cell types, we examined whether Blimp-1 could be playing a similar role in regulating the differentiation of exhausted CD8 T cells. We find that Blimp-1 is highly upregulated in antigen-specific CD8 T cells during chronic viral infection, and that expression correlated with severity of dysfunction and expression of inhibitory receptors. The high inhibitory receptor levels and memory repression associated with CD8 T cell exhaustion were both reversed upon conditional deletion of Blimp-1. However, haploinsufficient mice controlled virus more rapidly than either wt or the full conditional knockouts and indicated that some Blimp-1 expression is necessary to maintain some level of effector function. Thus, our study suggests that Blimp-1 plays an important role in the regulation of exhaustion during chronic viral infection, and suggests that Blimp-1 acts as a transcriptional rheostat, promoting CD8 T cell effector function when moderately expressed and CD8 T cell exhaustion when highly expressed.

Materials and Methods

Animals and viruses. Four to six week old C57BL/6 mice were purchased from NCI. B6.129X1-Gt(ROSA)26Sor$^{tm1(EYFP)Cos/J}$ (Rosa26-YFP) mice were purchased from Jackson Laboratories. BAC transgenic Blimp-1 YFP reporters were obtained from Eric Meffre (Yale University, New Haven, CT). *Prdm1*$^{flox/flox}$ (f/f)

mice were obtained from Kathryn Calame (Columbia University, New York, NY), granzyme B-Cre mice were obtained from Joshy Jacob (Emory University, Atlanta, GA) and CD4-Cre mice were obtained from Steven Reiner (University of Pennsylvania, Philadelphia, PA). *Prdm1*$^{f/f}$ mice were crossed with either granzyme B-Cre or CD4-Cre mice to generate *prdm1*$^{f/f}$ x Cre+, *prdm1*$^{f/f}$ x Cre-, *prdm1*$^{f/+}$ x Cre+, *prdm1*$^{f/+}$ x Cre- and *prdm1*$^{+/+}$ x Cre+ mice. Rosa26-YFP mice were crossed with granzyme B-Cre mice to generate Rosa26-YFP+ x Cre+ and Rosa26-YFP- x Cre+ mice. Mice were infected with $2x10^5$ plaque forming units (PFU) of LCMV Armstrong (Arm) i.p. or $2x10^6$ PFU LCMV clone-13 (Cl-13) i.v. as described (89). Virus was grown and viral titers were determined by plaque assay as described (89). All animals were used in accordance with IACUC procedures.

Lymphocyte isolation and flow cytometry. Lymphocyte isolation from lymphoid and non-lymphoid tissues, surface stains, intracellular cytokine stains and CD107 assay were performed as previously described (89, 199). All antibodies were purchased from Biolegend except for CD127, CD160, TNFa, IL-2 (eBioscience), 2B4 (eBioscience, BD Biosciences) LAG-3 (AbD Serotec), granzyme B (Caltag) and MIP-1α (R&D Systems). LIVE/DEAD dead cell stain, CFSE, CD62L, streptavidin-APC and streptavidin-Quantum dot 655 were purchased from Invitrogen. MHC class I peptide tetramers were made and used as described previously (89). All flow cytometry data was acquired on an LSRII (BD Biosciences) and analyzed by FlowJo (Treestar). Pie charts were created

using the Pestle and SPICE programs, written by Mario Roederer (Vaccine Research Center, NIAID, NIH).

Quantitative PCR. Cells were sorted by flow cytometry on a FACSAria (BD Biosciences). RNA extraction was performed using the Trizol protocol (Invitrogen). cDNA was generated using the High Capacity cDNA Archive Kit (Applied Biosystems). Relative quantification real-time PCR was performed on an ABI Prism 7000 using inventoried primers purchased from Applied Biosystems. All samples were normalized to an endogenous control of HPRT, and gene expression was measured as a fold-change over expression in naïve $CD44^{lo}$ CD8 T cells.

***In vitro* killing assays.** CD8 T cells were purified with magnetic beads (Miltenyi Biotec) from splenocytes of CKO, het and wt mice. Briefly, splenocytes were labeled with magnetic beads specific for CD8α and then run through a MACS LS separation column according to the manufacturer's protocol. Target cells were either labeled with GP33 peptide or SIINFEKL peptide and CFSE labeled at two different concentrations. Equal numbers of DbGP33+ CD8 T cells were plated at a 2:1 ratio with the labeled target cells and total cell numbers were normalized with naïve splenocytes. The cells were incubated at 37C for 16-20 hrs and specific lysis was calculated as described (200).

Results

Blimp-1 is highly expressed in functionally exhausted CD8 T cells during chronic viral infection.

In order to investigate the role of Blimp-1 in CD8 T cell exhaustion during chronic viral infection, we began by examining the kinetics of Blimp-1 expression in antigen-specific CD8 T cells by quantitative PCR (qPCR). DbGP33-specific CD8 T cells were sorted from the spleens of LCMV Arm or LCMV clone 13 infected mice at d8, 15 and 30 post-infection (p.i.). We found that at an early effector time point (d8 p.i.), there was little difference in Blimp-1 expression in DbGP33-specific CD8 T cells from either an acute or chronic viral infection (Fig 1a). During an acute infection, as the antigen-specific effector CD8 T cell population differentiated into a memory population, the levels of Blimp-1 slowly decreased. However, during chronic viral infection, Blimp-1 expression was greatly upregulated between d8 and d15 p.i., and remained high out to d30 p.i. (Fig 1a). Blimp-1 expression was also examined using a Blimp-1 YFP reporter mouse infected with either LCMV Arm or LCMV clone 13. After infection, only activated, $CD44^{hi}$ CD8 T cells expressed YFP (data not shown). At 8 days p.i., Blimp-1 YFP levels were similar in antigen-specific CD8 T cells during both Arm and clone 13 infections. While Blimp-1 YFP expression slowly decreased after d8 p.i. during acute infection, YFP MFI increased after d8 p.i. during chronic infection (Fig 1b). The difference in Blimp-1 YFP MFI between antigen-specific CD8 T cells from acute or chronic infection was not isolated to a single tissue, as we found Blimp-1 YFP expression to be higher in antigen-specific CD8 T cells in

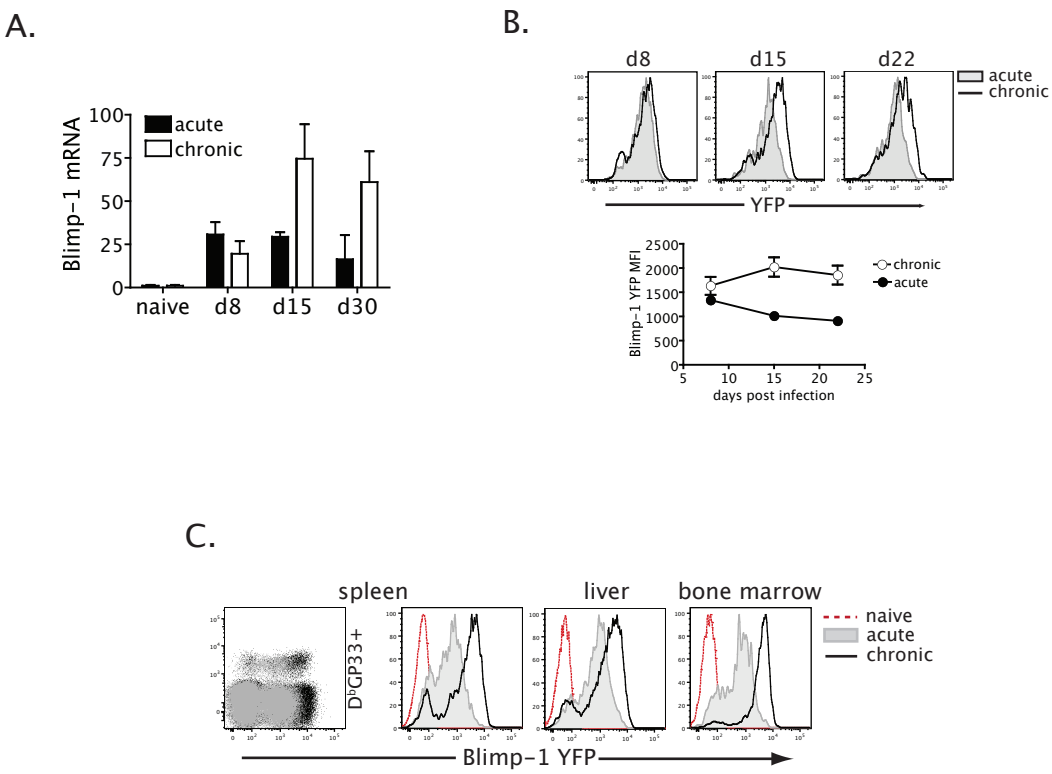

Figure 1. Blimp-1 is highly expressed in exhausted antigen-specific CD8 T cells during chronic viral infection. A) Blimp-1 message by qPCR. DbGP33+ T cells were sorted by FACS from LCMV Arm (acute) or clone 13 (chronic) infected mice at the indicated d.p.i.. Naive controls were $CD44^{lo}$ CD8 T sorted from Arm immune. Error bars show range of expression in triplicate wells. B) Kinetics of Blimp-1 reporter expression after LCMV Arm or clone 13 infection. Histograms are gated on DbGP33+ CD8 T cells from the blood of LCMV Arm (shaded) or LCMV clone 13 (open) infection. Data points in graph show Blimp-1 YFP MFI of DbGP33+ CD8 T cells from LCMV Arm (black circles) or LCMV clone 13 (white circles) at the indicated d.p.i.. Error bars are standard error of the mean (SEM). N=2-3 mice per time point. D) Blimp-1 YFP reporter MFI in tissues 30 d.p.i. with LCMV Arm or clone 13. Dot plot is gated on total CD8 T cells. Histograms are gated on DbGP33+ CD8 T cells from LCMV Arm (shaded) or LCMV clone 13 (open) infection. Open red histograms show $CD44^{lo}$ CD8 T cells from LCMV Arm infection.

multiple lymphoid and non-lymphoid tissues during chronic infection as compared to acute infection (Fig 1c). Thus, Blimp-1 was globally overexpressed in exhausted antigen-specific CD8 T cells during chronic viral infection.

Blimp-1 correlates with severity of dysfunction and/or terminal differentiation during in exhausted CD8 T cells.

The upregulation of Blimp-1 expression in virus-specific CD8 T cells during chronic viral infection corresponded with the onset of certain features that are the hallmarks of functional exhaustion, such as the high expression of inhibitory receptors. To determine whether Blimp-1 expression correlated with inhibitory receptor expression, we first examined Blimp-1 expression in subsets of PD-1 expressing antigen-specific CD8 T cells during chronic viral infection. DbGP33-specific PD-1^{hi} and PD-1$^{int/lo}$ cells were sorted and Blimp-1 mRNA was measured by qPCR. We found that Blimp-1 expression was approximately 2-fold higher in the more terminally differentiated PD-1^{hi} subset compared to the PD-1$^{int/lo}$ subset (Fig 2a). Furthermore, subsets of antigen-specific CD8 T cells that were high for other inhibitory receptors such as LAG-3, CD160 and 2B4 expressed higher Blimp-1 YFP than subsets that were low (Fig 2b). When we examined the number of different inhibitory receptors co-expressed on each cell, we found that those cells which expressed the highest number of inhibitory molecules had the highest Blimp-1 YFP MFI (Fig 2c). We also observed a correlation between Blimp-1 YFP expression and the severity of functional exhaustion during chronic viral infection. During LCMV clone 13, CD8 T cells

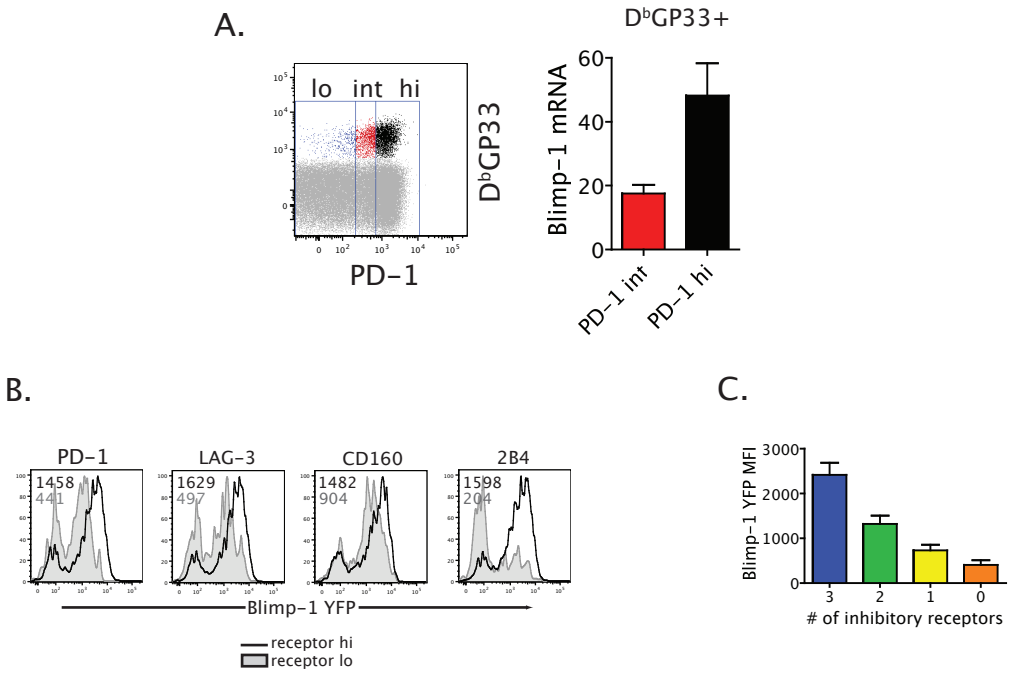

Figure 2. Blimp-1 expression correlates with expression of inhibitory receptors on antigen-specific CD8 T cells during chronic viral infection. A) Blimp-1 mRNA in PD-1^{hi} and PD-1^{int} subsets of exhausted CD8 T cells. Plot is gated on wt CD8 T cells from spleen at d30 post LCMV clone 13 infection. For qPCR, PD-1^{hi} and PD-1^{int} DbGP33+ CD8 T cells were sorted from the spleens of LCMV clone 13 infected wt mice. Graph shows fold increase in Blimp-1 expression over naive CD44^{lo} CD8 T cells sorted from LCMV Arm immune mice. Error bars show range of expression in triplicate wells. B) Blimp-1YFP reporter expression in inhibitory receptor hi vs. lo subsets of exhausted CD8 T cells at d30 post LCMV clone 13 infection (spleen). Histograms are gated on inhibitory receptor lo (shaded) or hi (open) DbGP33+ CD8 T cells. Numbers in gray are YFP MFI of shaded histograms, numbers in black are YFP MFI of open histograms. C) Correlation of Blimp-1 expression and number of inhibitory receptors expressed. Blimp-1 YFP reporter mice were infected with LCMV clone 13 and inhibitory receptor expression was determined on DbGP33+ CD8 T cells from the spleen at d30 p.i.. Boolean gating established the populations that expressed a combination of 3, 2, 1 or no inhibitory receptors and Blimp-1 YFP MFI was determined for each subgroups. Graph represents two independent experiments.

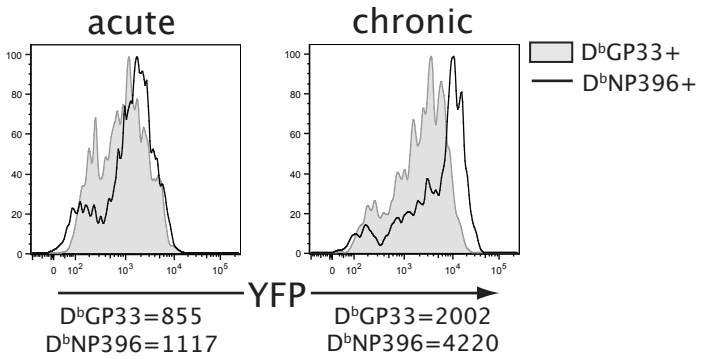

Figure 3. Blimp-1 expression correlates with severity of functional exhaustion. Blimp-1 YFP reporter expression in CD8 T cells specific for different epitopes during LCMV Arm or clone 13 infection. Histograms are gated on DbGP33+ CD8 T cells (shaded) or DbNP396+ CD8 T cells (open). Numbers beneath histograms show YFP MFI of each population.

specific for immunodominant epitopes such as DbNP396 become severely exhausted and are often physically deleted from the CD8 T cell response. We found that DbNP396-specific CD8 T cells had a higher Blimp-1 YFP MFI than DbGP33-specific CD8 T cells during LCMV clone 13, while Blimp-1 driven YFP expression was similar between these two populations after LCMV Arm infection (Fig 3). Thus, Blimp-1 expression was higher in exhausted virus-specific CD8 T cells during chronic infections than in fully functional CD8 T cells after acute infection, and Blimp-1 expression correlated with increased severity of T cell dysfunction and/or terminal differentiation.

Blimp-1 alters the differentiation of virus-specific CD8 T cells during chronic viral infection.

Blimp-1 has been shown to play a role in T cell activation and homeostasis (150, 151), and based on the dramatic increase of Blimp-1 expression we wanted to determine whether Blimp-1 played a role in regulating functional exhaustion during chronic viral infection. To examine this, *prdm1*, the gene encoding Blimp-1, was conditionally deleted. The *prdm1*$^{flox/flox}$ mice (191) were crossed to mice expressing Cre under the CD4 promoter, which deletes Blimp-1 in T cells at the double positive stage of T cell development. The *prdm1*$^{flox/flox}$ x CD4-Cre mice will be referred to as CD4-Cre CKO mice. CD4-Cre CKO mice and wt littermates were infected with LCMV Cl-13 and both viral control and T cell responses were examined ~30 days p.i.. Deletion of Blimp-1 in antigen-specific CD8 T cells restored a memory phenotype, as the cells were

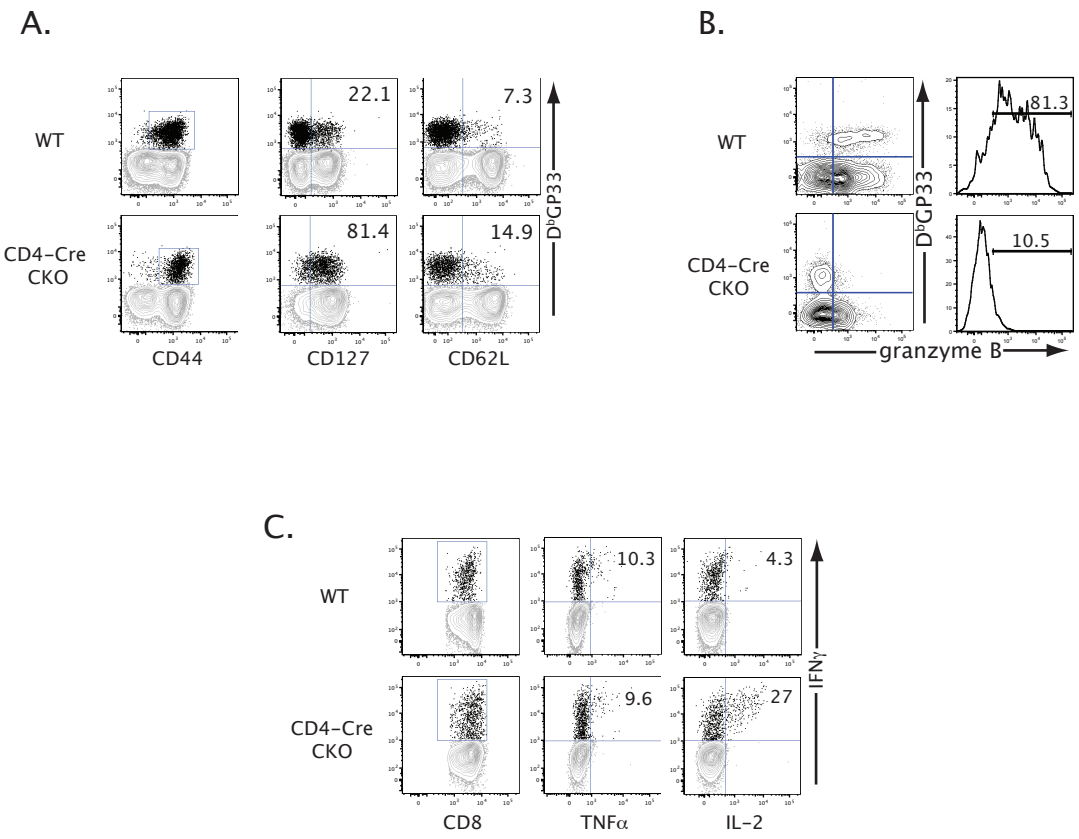

Figure 4. CD8 T cell responses in CD4-Cre driven Blimp-1 conditional knockout mice. CD4-Cre CKO and wt littermates were infected with LCMV clone 13 and CD8 T cells were analyzed in the spleen at d30 p.i.. A) Phenotype of wt and CD4-Cre CKO antigen-specific CD8 T cells. Plots are gated on total CD8 T cells. Numbers in plots show percent of DbGP33+ CD8 T cells that are high for each marker. B) Granzyme B expression in wt and CD4-Cre CKO antigen-specific CD8 T cells. Plots are gated on total CD8 T cells, histograms are gated on DbGP33+ CD8 T cells. Numbers above gates show percent of DbGP33+ CD8 T cells that are granzyme B^{hi}. C) Effector function of wt and CD4-Cre CKO antigen-specific CD8 T cells. Splenocytes were stimulated with GP33 peptide for 5 hrs at 37C. Plots are gated on total CD8 T cells. Numbers in plots show percent of IFNγ+ CD8 T cells that also produce a second cytokine.

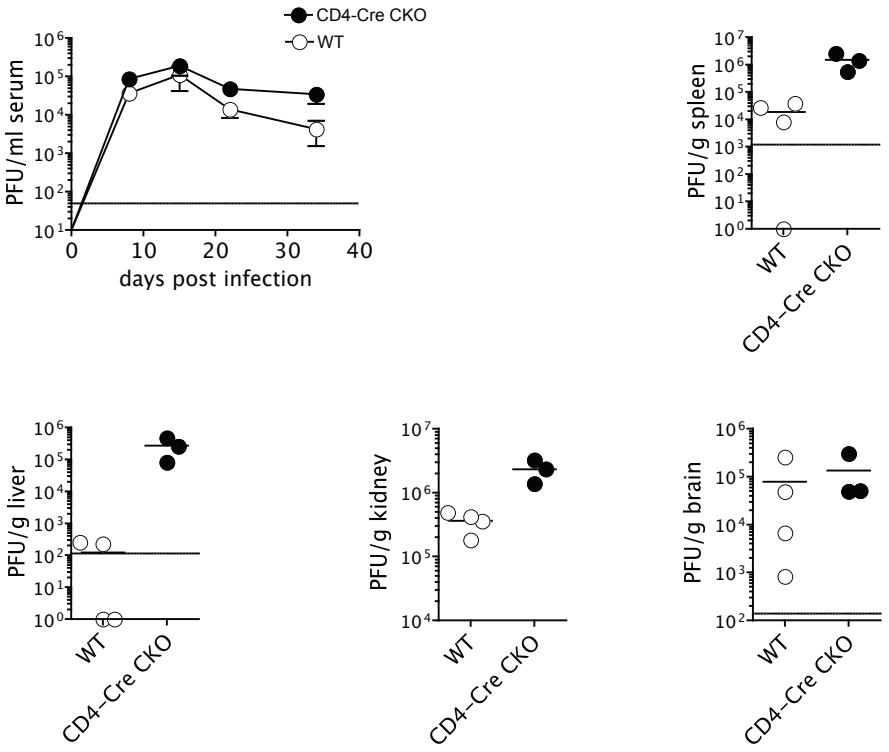

Figure 5. CD4-Cre driven Blimp-1 conditional knockouts have poor viral control. CD4-Cre CKO mice and wt littermates were infected with LCMV clone 13 and viral titers were measured in multiple tissues. LCMV viral titers were measured by plaque assay at the indicated days for serum and at 30 d.p.i. for all other tissues. N=3-5 for data points in serum graph. Error bars are SEM. Dashed line shows limit of detection for each tissue. Data is representative of three independent experiments.

CD127^{hi} and CD62L^{hi} (Fig 4a). Virus-specific CD8 T cells lacking Blimp-1 expressed less granzyme B than wt virus-specific CD8 T cells, but produced higher levels IL-2 (Fig 4b,c). However, despite acquiring a memory phenotype and having slightly improved effector function, the CD4-Cre CKO mice were unable to control the infection (Fig 5). Viral titers were higher in multiple tissues in the CD4-Cre CKO mice as compared to wt mice (Fig 5).

Since CD4-Cre conditionally deletes Blimp-1 prior to T cell activation and Blimp-1 has been shown to play a role during activation (150, 151), it is difficult to ascertain whether the differences observed between the CD4-Cre CKO and wt mice are due to an altered T cell compartment and improper activation, or due to the action of Blimp-1 on the differentiation of exhausted CD8 T cells. Thus, to avoid any potential complications arising from the lack of Blimp-1 during T cell activation, *prdm1*$^{flox/flox}$ mice were crossed with mice expressing the Cre recombinase under the human GranzymeB promoter (gzmB-Cre) (201). These conditional knockout mice will heretofore be referred to as CKO mice.

To first determine the activity of the gzmB-Cre, we crossed the gzmB-Cre mice to Rosa26-stop$^{fl/fl}$-YFP mice (Rosa26-YFP), which have a floxed stop codon upstream of the gene encoding YFP within the Rosa26 locus. Upon Cre activation, the stop codon is excised and YFP is expressed. Granzyme B is expressed in CD8 T cells 1-2 days after activation (72) and accordingly, we observed YFP expression in CD25+ CD8 T cells 3 days post-infection with LCMV clone 13 (Fig 6). By d6 p.i., the majority of antigen-specific CD8 T cells were YFP+, indicating Cre recombination had occurred in most of the responding CD8

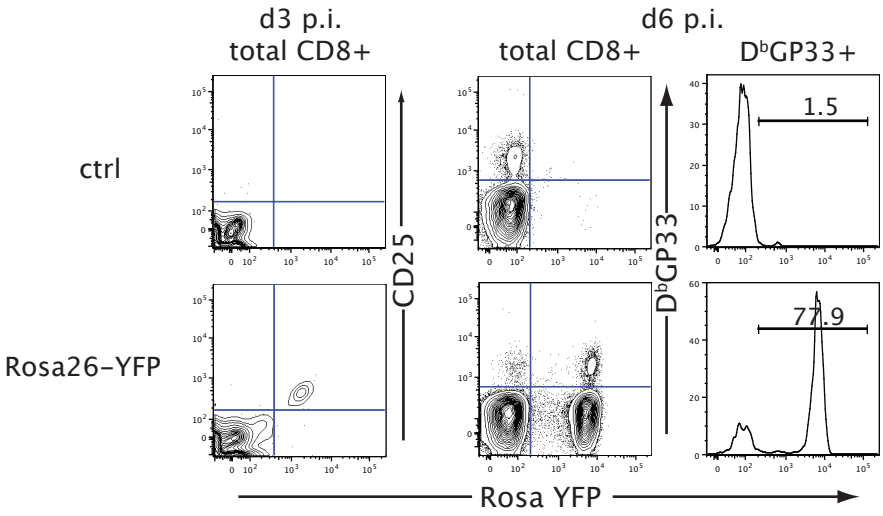

Figure 6. Efficiency of hGranzymeB-Cre. Ctrl or Rosa26-f/stop/f-YFP x gzmB-Cre mice were infected with LCMV clone 13 and YFP expression was measured in the blood (d3 p.i.) or in the spleen (d6 p.i.). Plots are gated on total CD8 T cells. Histograms are gated on DbGP33+ CD8 T cells. Numbers above gates show percent of DbGP33+ CD8 T cells that express YFP.

T cells. It is still possible, however, that previously expressed Blimp-1 protein could temporarily persist after the deletion of *prdm1*.

CKO and wildtype (wt) littermates were infected with LCMV clone 13, and T cell responses and viral control were examined 30 days p.i.. Unlike the CD4-Cre CKO mice, the CKO mice had viral titers in the serum and tissues that were similar to wt through the first 20-30 days p.i. (Fig 7a). The frequency of antigen-specific CD8 T cells in the CKO mice was slightly elevated in the spleen and lymph node, and similar to wt in other tissues such as the bone marrow and liver (Fig 7b). In particular, the DbNP396-specific population, which is often deleted during chronic viral infection, was rescued to a greater degree in the CKO mice than populations specific for GP33 or another LCMV epitope, GP276 (Fig 7b). Contraction of the virus-specific CD8 T cell population in the CKO mice also appeared to be slightly delayed compared to wt (Fig 7b). Like the CD4-Cre CKO mice, virus-specific CD8 T cells from the CKO mice also expressed CD127 and CD62L at a level similar to memory CD8 T cells from Arm immune mice (Fig 7c). Virus-specific CD8 T cells from the CKO cells also showed only a modest improvement in the production of the cytokines. While IL-2 production was restored to levels near to that produced by memory CD8 T cells from Arm immune mice for both DbGP33- and DbNP396-specific CD8 T cells, TNFα production was still much lower than after an acute infection (Fig 7d, data not shown). CKO virus-specific CD8 T cells also expressed lower levels of the chemokine MIP-1α than wt (Fig 7d). Together, our data show that while Blimp-1 alone may not regulate effector function in exhausted CD8 T cells, high levels of

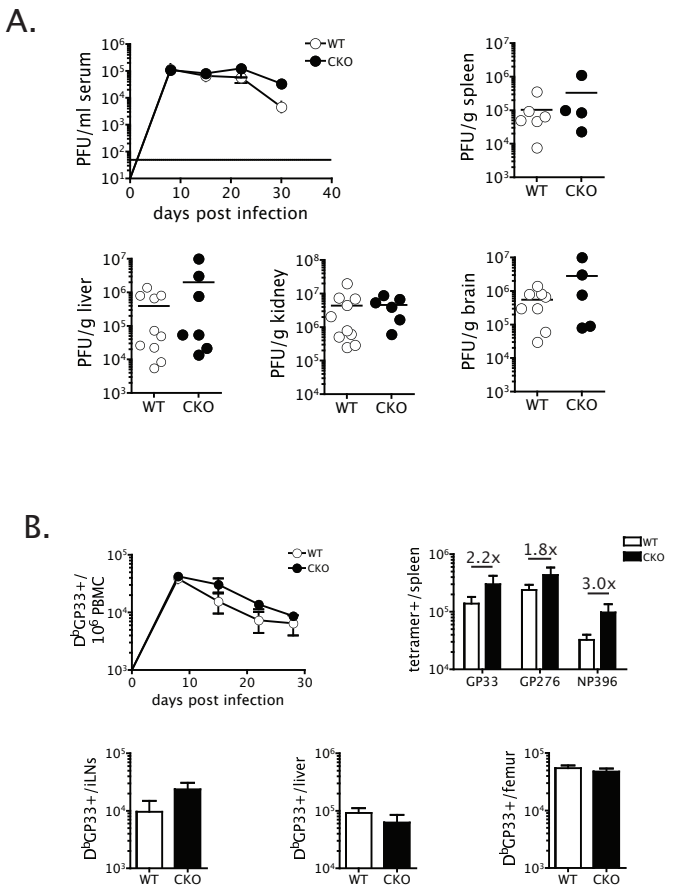

Figure 7. CD8 T cell responses in gzmB-Cre driven Blimp-1 conditional knockout mice. CKO mice and wt littermates were infected with LCMV clone 13 and viral control and CD8 T cell responses were measured at 30 d.p.i. unless otherwise noted. A) Viral control. Viral load was measured by plaque assay at the indicated days in the serum and d30 p.i. in all other tissues. Error bars in serum are SEM, dashed line shows limit of detection. N=3-10 mice per time point. Data is representative of four independent experiments. B) Absolute number of antigen-specific CD8 T cells in wt and CKO mice. Frequency of DbGP33+ CD8 T cells was measured in the blood at indicated time points. Absolute number of tetramer+ CD8 T cells was measured in each tissue at d30 p.i.. Numbers above spleen graph show fold increase of CKO over wt for each tetramer+ population. Error bars are SEM, and n=4-8 for each tissue. Data represents three independent experiments. (Figure continued on next page.)

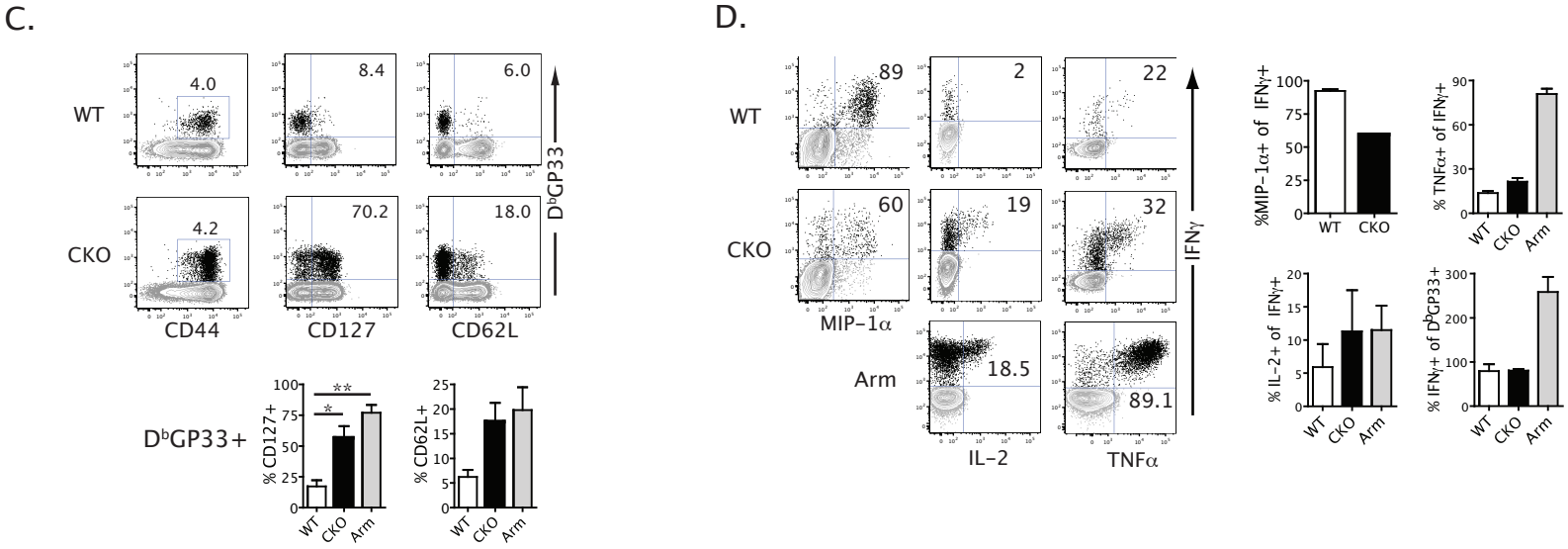

Figure 7 (cont). CD8 T cell responses in gzmB-Cre driven Blimp-1 conditional knockout mice. C) Phenotype of wt and CKO CD8 T cells in the spleen. Plots are gated on total CD8 T cells. Numbers in leftmost plots show percent of total CD8 T cells that are DbGP33+. Numbers in middle and righthand plots indicate percent of DbGP33+ CD8 T cells that are positive for each marker. Graphs represent two independent experiments, Arm indicates marker expression for memory CD8 T cells from Arm immune mice (~d30 p.i.). Error bars are SEM. For CD127, *p=0.04, **p=0.002 by Student's t-test. D) Effector function of wt and CKO antigen-specific CD8 T cells compared to memory CD8 T cells (d30+ Arm immune). Splenocytes were stimulated with GP33 peptide for 5 hrs. at 37C. Plots are gated on CD8 T cells. Numbers in each plot show the percent of IFNγ+ CD8 T cells that also produce the second indicated cytokine. Numbers are graphed to the right.

Blimp-1 may repress memory CD8 T cell differentiation during chronic viral infection.

Blimp-1 regulates the expression of inhibitory receptors on exhausted CD8 T cells.

Since high Blimp-1 levels are associated with the high expression of inhibitory receptors on exhausted CD8 T cells (Fig 2), we next examined the expression of inhibitory molecules in the CKO mice. At d30 post LCMV clone 13 infection, DbGP33-specific CD8 T cells from wt mice expressed high levels of PD-1 and LAG-3, and subsets of the DbGP33-specific CD8 T cell population also expressed high levels of CD160 and 2B4 (Fig 8a). In the CKO mice, however, the DbGP33-specific CD8 T cells expressed nearly 2-fold lower levels of both PD-1 and LAG-3 (Fig 8a). Furthermore, the subsets of $CD160^{hi}$ and $2B4^{hi}$ DbGP33-specific CD8 T cells present in the wt population was absent in the CKO mice (Fig 8a). We also assessed the number of different inhibitory molecules expressed by each cell in the CKO and wt mice by multi-parameter flow cytometry. We found that within the DbGP33-specific CD8 T cell population, the majority of the cells expressed a combination of three or all four inhibitory receptors at once (Fig 8a). However, within the CKO mice, very few cells expressed all four inhibitory receptors. Rather, most of the population expressed either no inhibitory receptors or just one (Fig 8a). This pattern of expression was not isolated to just the DbGP33-specific population. Total activated $CD44^{hi}$ CD8 T cells from the CKO mice also had lower levels of PD-1 and LAG-3 than the wt

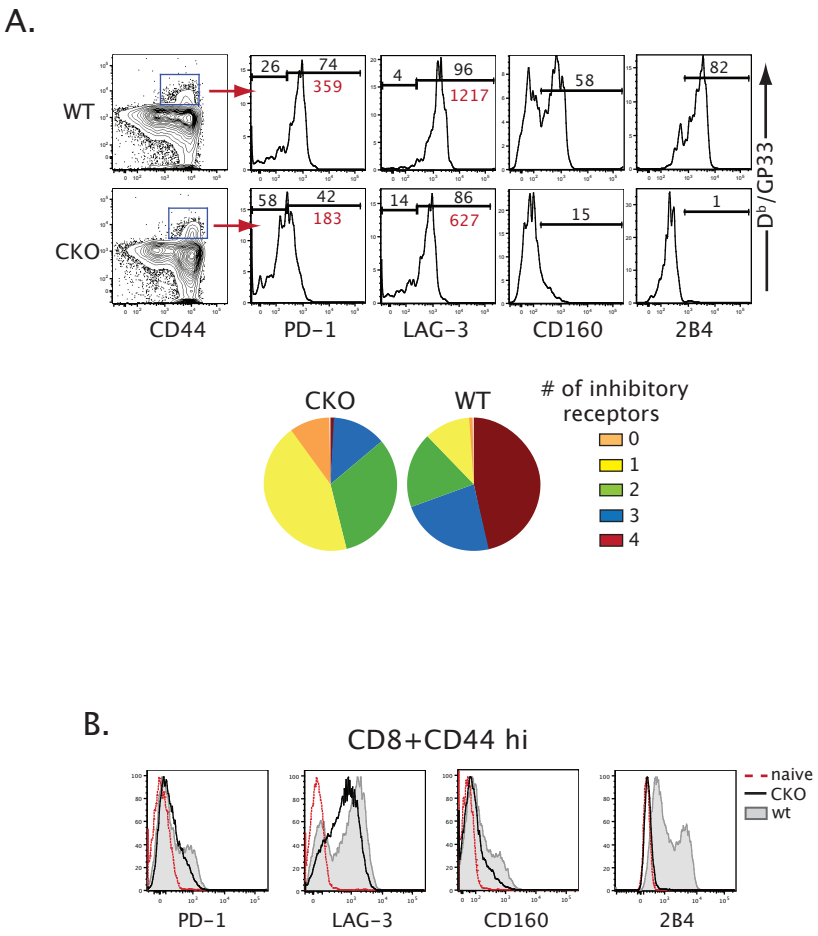

Figure 8. Blimp-1 regulates inhibitory receptor expression on antigen-specific CD8 T cells during chronic viral infection. CKO and wt littermates were infected with LCMV clone 13 and CD8 T cells from the spleen were analyzed 30 d.p.i.. A) Inhibitory receptor expression on antigen-specific CD8 T cells from wt and CKO mice. Plots are gated on total CD8 T cells. Histograms are gated on DbGP33+ CD8 T cells. Numbers above the gates indicate the percentage of DbGP33+ CD8 T cells in each gate. Numbers in red are the MFI of each marker. Pie charts show DbGP33+ CD8 T cell populations from wt and CKO mice. Each colored slice indicates the fraction of DbGP33+ CD8 T cells that express a combination of 4, 3, 2, 1 or no inhibitory receptors. B) Inhibitory receptor expression in total $CD44^{hi}$ CD8 T cells in wt (shaded) or CKO (open) mice. Open red histograms represent $CD44^{lo}$ CD8 T cells from wt mice.

CD44^{hi} CD8 T cells, and very few activated CD8 T cells from the CKO mice expressed any CD160 or 2B4 (Fig 8b). Together, our data show that Blimp-1 plays a key role in regulating the elevated levels and co-expression of inhibitory receptors on exhausted CD8 T cells during chronic viral infection.

Blimp-1 haploinsufficiency leads to rapid viral control during chronic viral infection.

Lower expression of inhibitory receptors on the CKO CD8 T cells suggested that long-term viral control may be enhanced. However, since Blimp-1 has been shown to regulate effector functions such as granzyme B expression (197), we also infected mice that were conditionally haploinsufficient for Blimp-1 (*prdm1*$^{fl/+}$ x gzmB-Cre; conditional hets) in order to examine the impact of decreased Blimp-1 expression on long-term viral control without the complication of altered T cell function. When infected with LCMV clone 13, wt mice controlled viremia about 2 months p.i., and CKO mice maintained high serum titers (Fig 9a). Conditional haploinsufficiency for Blimp-1, however, resulted in viremia control that was more rapid than either the CKO or wt mice (Fig 9a). The conditional het mice also had lower viral titers in multiple tissues compared to wt or CKO mice (Fig 9a). To determine whether haploinsufficiency of Blimp-1 would also impact inhibitory receptor expression, we examined PD-1 at day 15 p.i., a time point when viral titers between the three groups of mice were similar (Fig 9b). Virus-specific CD8 T cells from the conditional het mice expressed significantly lower levels of PD-1 compared to wt mice (Fig 9b). Furthermore, conditional het mice

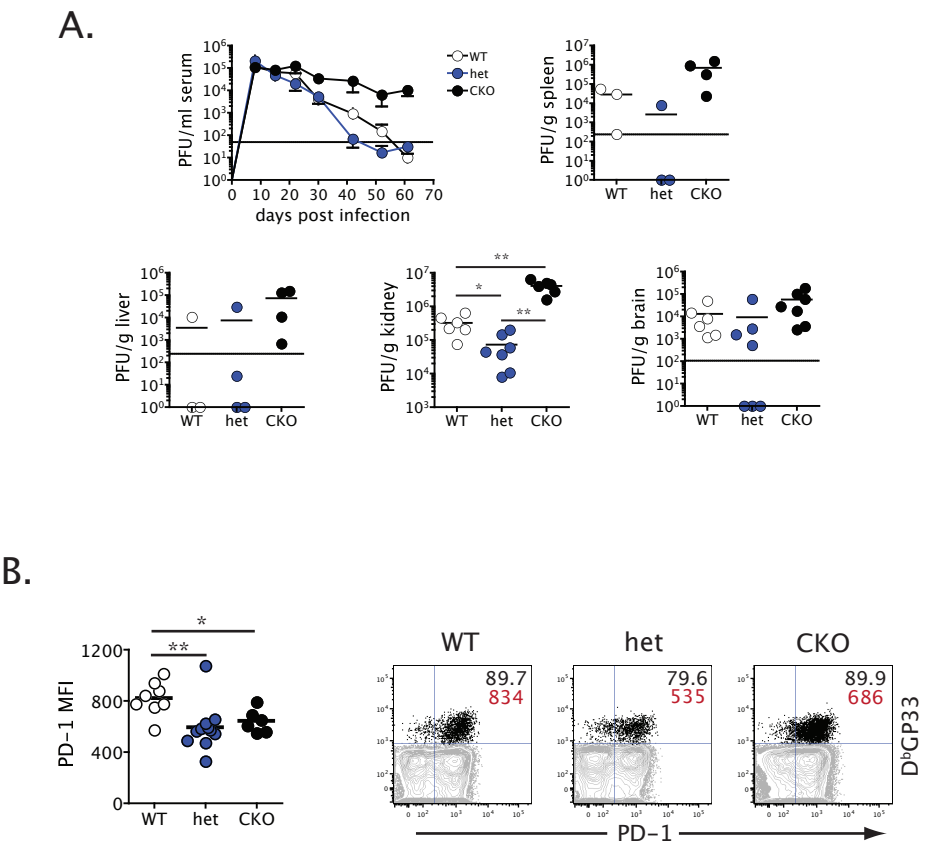

Figure 9. Blimp-1 haploinsufficiency in antigen-specific CD8 T cells leads to rapid viral control. A) Viral titers in CKO, het and wt littermates d60 post LCMV clone 13 infection. Viral load was determined in the serum on the indicated days and in the tissues by plaque assay. Dashed lines represent limit of detection for each tissue. Data is representative of three independent experiments, n=3-17. For kidney titers, wt vs. het *p=0.03, wt vs. CKO **p=0.003, het vs. CKO **p=0.002 by Student's t-test. B) PD-1 expression on antigen-specific CD8 T cells from CKO, het and wt mice. PD-1 MFI was measured on DbGP33+ CD8 T cells from the blood at d15 p.i. Data is representative of three independent experiments. Wt vs. het **p=0.007, wt vs. CKO *p=0.01 by Student's t-test. Plots are gated on total CD8 T cells from the blood. Numbers in red indicate the PD-1 MFI of the DbGP33+ CD8 T cells.

also appeared to express slightly less PD-1 than CKO mice (Fig 9b). Thus, while complete Blimp-1 insufficiency (CKO) resulted in poor long-term viral control, limiting Blimp-1 expression to a single intact copy of *prdm1* was sufficient to both enhance viral control and decrease PD-1 expression on virus-specific CD8 T cells.

Blimp-1 conditional heterozygosity leads to better maintenance of cytotoxicity than conditional knockouts.

To determine the cellular basis behind the difference in the kinetics of virus control, we first examined the number of antigen-specific CD8 T cells in the wt, conditional het and CKO mice. The frequency of DbGP33-specific CD8 T cells in all three groups remained similar up to the first month p.i. (Fig 10a). However, the frequency of DbGP33-specific CD8 T cells in the blood continued to decline in the CKO mice whereas the frequency stabilized in both the wt and conditional hets and led to significant differences between the three groups (Fig 10a). When we examined the absolute number of antigen-specific CD8 T cells in the spleen, however, we found that the general trend did not change between the first and second months p.i. (Fig 10b,c). We did observe, however, a small decrease in the number of DbNP396-specific CD8 T cells in the CKO mice between months 1 and 2 p.i., while this population was maintained in the conditional het mice (Fig 10c). We next examined the functional properties of virus-specific CD8 T cells from the CKO, conditional het and wt mice. The ability of virus-specific CD8 T cells from CKO and conditional het mice to co-produce

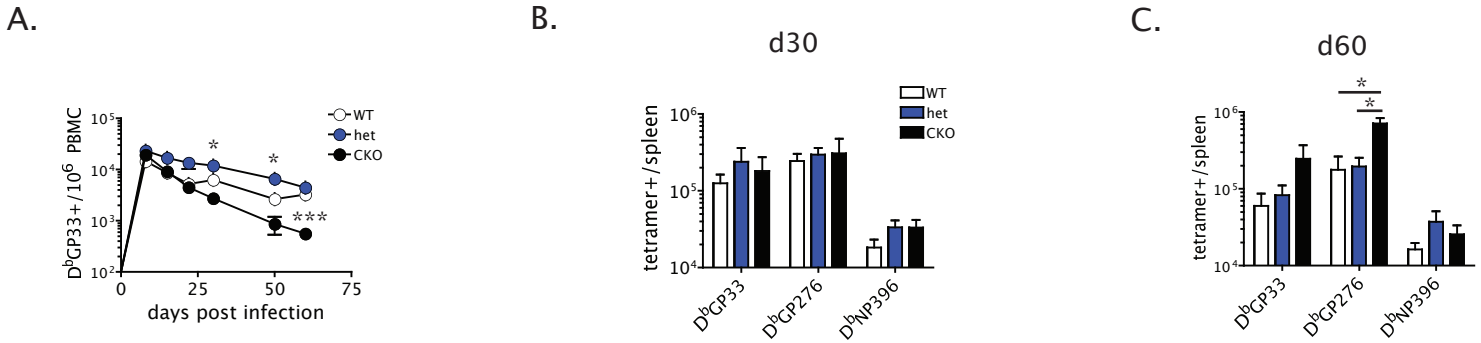

Figure 10. Absolute number of antigen-specific CD8 T cells in CKO, haploinsufficient and wt mice. CKO, conditional het and wt littermates were infected with LCMV clone 13. Absolute numbers of LCMV-specific CD8 T cells were measured in the blood and spleen at d30 or d60 p.i.. A) Frequency of DbGP33+ CD8 T cells was measured in blood at the indicated time points. Error bars are SEM, n=4-15 per time point. Graph is representative of three independent experiments. At d30, wt vs CKO *p=0.04, het vs. CKO *p=0.008. At d50, wt vs. het *p=0.02, het vs. CKO *p=0.006. At d60, het vs. CKO ***p=0.0008. All statistics measured by Student's t-test. B) Absolute number of tetramer+ CD8 T cells in the spleen at 30 d.p.i.. Data is representative of two independent experiments. N=3-4 mice per group. Error bars are SEM. C) Absolute number of tetramer+ CD8 T cells in the spleen at d60 p.i. Data is representative of two independent experiments. N=3-4 mice per group. Error bars are SEM. For het vs. CKO, *p=0.02, for wt vs. CKO *p=0.02 by Student's t-test.

effector cytokines TNFα or IFNγ was not significantly different from wt cells, although the amount of IFNγ produced per cell was reduced in the CKO mice (Fig 11a,b). One copy of *prdm1*, however, was sufficient to restore co-production of MIP-1α and IFNγ to wt levels, and was also sufficient to repress IL-2 production (Fig 11a). Virus-specific CD8 T cells from CKO, conditional het and wt mice were all equally capable of degranulation as measured by CD107 (Fig 11b). Blimp-1 has been implicated in the expression of granzyme B (197), and when examined, we found that antigen-specific CD8 T cells from the CKO were deficient in the expression of this cytotoxic granule as compared to the conditional het or wt mice (Fig 11c). The loss of granzyme B expression is most likely due to loss of Blimp-1 expression rather than any competition between the hGzmB-Cre and endogenous granzyme B promoters, as CD8 T cells from the CD4-Cre CKO mice also show loss of granzyme B (Fig 4b). While the killing ability of exhausted CD8 T cells is not as potent as memory CD8 T cells, exhausted CD8 T cells do retain residual cytotoxicity (89). To test whether decreased granzyme B would impact the ability of CKO antigen-specific CD8 T cells to kill infected target cells, an *in vitro* killing assay was performed. CD8 T cells from the CKO mice were deficient in their ability to lyse peptide-coated target cells at both early and late time points during chronic LCMV infection (Fig 11d). Wt and conditional het CD8 T cells, while not as efficient as memory CD8 T cells, acquired and maintained their ability to kill (Fig 11d,e). Thus, conditional deficiency indicated a role for Blimp-1 in controlling some aspects of T cell exhaustion, but complete loss of Blimp-1 also compromised a key CD8 T cell

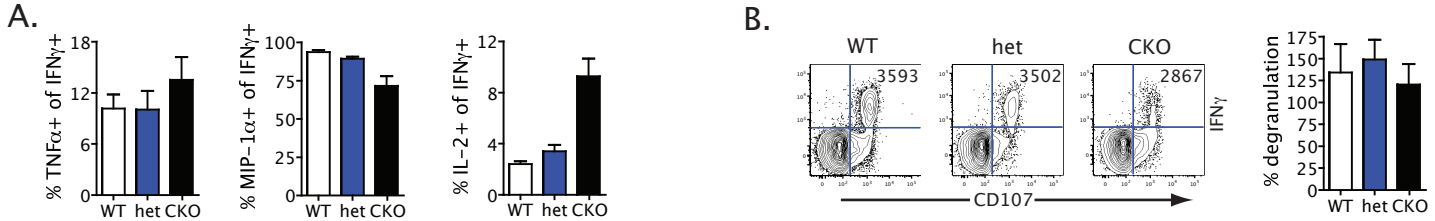

Figure 11. Blimp-1 regulates cytotoxicity in antigen-specific CD8 T cells during chronic viral infection. CKO, het and wt littermates were infectd with LCMV clone 13 and analyzed at d30 p.i unless otherwise noted. A) Function of CD8 T cells from CKO, het and wt mice. Splenocytes were stimulated with GP33 peptide for 5 hrs at 37C, and percent of IFNγ+ CD8 T cells that also produced the second indicated cytokine was determined by ICS. Data are representative of two independent experiments, n=4-6. Error bars are SEM. B) Splenocytes were stimulated with GP33 peptide and degranulation based on CD107 staining was determined. Plots are gated on total CD8 T cells. Numbers in plots indicate MFI of IFNγ. Graph shows number of DbGP33+ CD8 T cells that are also CD107+. Data represents two independent experiments. Error bars are SEM. (Figure continued on next page.)

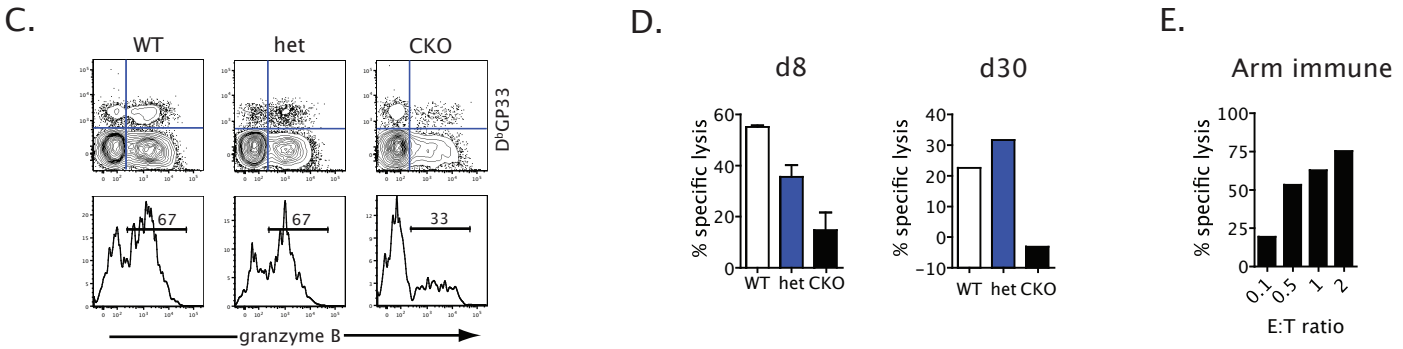

Figure 11 (cont.) Blimp-1 regulates cytotoxicity in antigen-specific CD8 T cells during chronic viral infection. C) Granzyme B expression in CD8 T cells from CKO, het or wt mice. Dot plots are gated on total CD8 T cells from the spleen and histograms are gated on DbGP33+ CD8 T cells. Numbers above the gates indicate the percent of DbGP33+ CD8 T cells that express granzyme B. D) Specific killing by CD8 T cells from CKO, het and wt mice as determined by an in vitro killing assay on d8 and d30 p.i. with LCMV clone 13. Equal number of DbGP33+ CD8 T cells from the spleen were plated with GP33 peptide labeled target cells at a 2:1 effector to target ratio. After 16-20 hrs, specific lysis was calculated. Error bars are SEM for triplicate wells. E) Specific killing by memory CD8 T cells from Arm immune mice.B6 mice containing ~$3x10^4$ P14 T cels were infected with LCMV Arm. After more than 30 days, P14 CD8 T cells from the spleen were plated at the indicated effector to target ratios with GP33 peptide labeled target cells. After 20 hrs, specific lysis was calculated.

effector function that is partially maintained by exhausted CD8 T cells with either one or both copies of *prdm1*.

Blimp-1 acts in a cell-intrinsic manner in exhausted CD8 T cells.

While the data described above suggest that Blimp-1 regulates key aspects of CD8 T cell exhaustion such as the expression of inhibitory receptors, they do not rule out the possibility that Blimp-1 may be acting in a manner that is not cell-intrinsic. Use of the gzmB-Cre may lead to loss of Blimp-1 from other cell types, and differences in the infectious environment, such as pathogenesis and viral load, between the three groups of mice could impact CD8 T cell differentiation. In order to address these issues, mixed bone marrow (BM) chimeras were generated by injecting equal numbers of B and T cell depleted bone marrow from Ly5.1+ wt and Ly5.2+ CKO mice into irradiated Ly5.1+ wt recipients (Fig 12a). Once fully reconstituted, the BM chimeras were infected with LCMV clone 13 and the CD8 T cells were analyzed four weeks p.i..

In the BM chimeras, virus-specific CD8 T cells lacking Blimp-1 displayed a phenotype that was distinct from the wt virus-specific CD8 T cells. As in the separate mice, CKO CD8 T cells from the BM chimeras acquired a memory-like phenotype with high levels of CD127 and CD62L, while wt cells remained $CD127^{lo}$ and $CD62L^{lo}$ (Fig 12b). Also, as in the separate mice, CKO virus-specific CD8 T cells from the BM chimeras also co-produced more IFNγ and IL-2, slightly more TNFα, and less MIP-1α than wt virus-specific CD8 T cells (Fig 12c). Furthermore, granzyme B expression was also decreased in the CKO antigen-

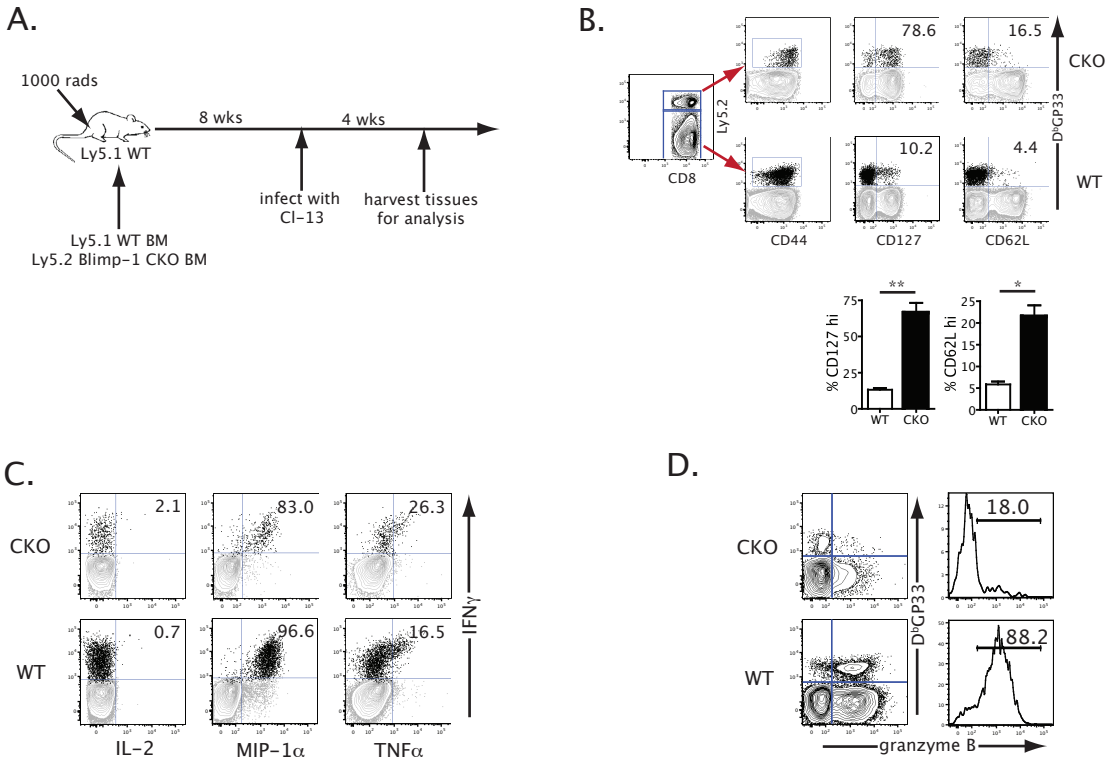

Figure 12. The role of Blimp-1 in CD8 T cells during chronic viral infection is cell-intrinsic. A) Experimental design for the generation of mixed BM chimeras reconstituted with *prdm*$^{f/f}$ x gzmB-Cre (part B-E) or *prdm1*$^{f/f}$ x CD4-Cre (part F) BM. B) Phenotype of CD8 T cells from mixed BM chimeras. Leftmost plot is gated on total CD8 T cells from the spleen. Top row of plots is gated on Ly5.2+ CD8 T cells (CKO) and bottom row is gated on Ly5.2- CD8 T cells (wt). Numbers in plots indicate the percentage of DbGP33+ CD8 T cells positive for each marker. Numbers are graphed to the right. Data are representative of two experiments, n=4. Error bars are SEM. For CD127, **p=0.005 and for CD62L, *p=0.01 by Student's t-test. C) Effector function of CD8 T cells from mixed BM chimeras. Splenocytes were stimulated with GP33 peptide for 5 hrs at 37C. Top row of plots indicate the percentage of IFNγ+ CD8 T cells that also produce a second cytokine. D) Granzyme B expression in CD8 T cells from mixed BM chimeras. Plots are gated on CD8 T cells from the spleen. Histograms are gated on DbGP33+ CD8 T cells. Top row is CKO and bottom is wt. Numbers above gates show percent of DbGP33+ CD8 T cells that express granzyme B.

specific CD8 T cells as compared to wt (Fig 12d). When inhibitory receptor expression was assessed in the BM chimeras, we found that virus-specific CD8 T cells lacking Blimp-1 expressed less PD-1 and slightly less LAG-3 than the wt cells, while the subset of $CD160^{hi}$ and $2B4^{hi}$ cells present in the wt virus-specific CD8 T cell population was absent in the CKO population (Fig 13a). When the co-expression of inhibitory receptors was examined in the BM chimeras, we again found that a much larger fraction of the wt virus-specific CD8 T cell population expressed three or four inhibitory receptors at once as compared to the CKO virus-specific CD8 T cells (Fig 13a). In parallel experiments, mixed BM chimeras using wt and CD4-Cre CKO bone marrow were also generated. We observed similar patterns of memory differentiation, effector function and inhibitory receptor expression as we observed with the CKO BM chimeras (data not shown, Fig 13b), suggesting that the changes observed in the CKO CD8 T cells were not due to the timing of cre-mediated recombination or deletion of Blimp-1 in cytotoxic cell lineages other than CD8 T cells. Together, our data suggest that Blimp-1 has a cell-intrinsic role in regulating central features of T cell exhaustion such as inhibitory receptor expression, and identify Blimp-1 as a transcriptional regulator of CD8 T cell exhaustion during chronic viral infections.

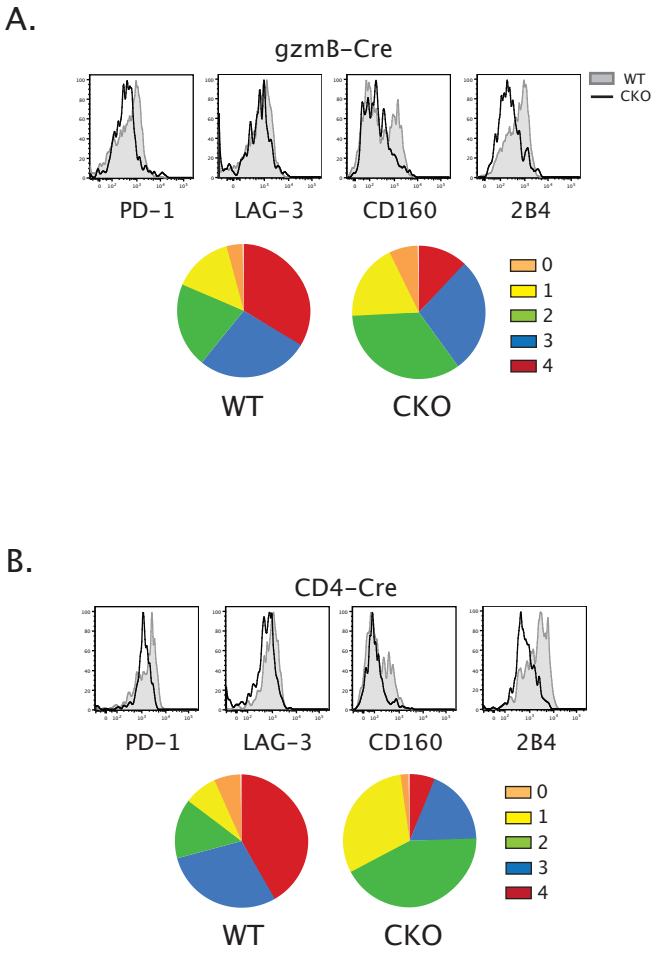

Figure 13. Blimp-1 cell-intrinsically regulates the expression of inhibitory receptors on exhausted CD8 T cells during chronic viral infection. BM chimeras generated with CKO and wt or CD4-Cre CKO and wt were infected with LCMV clone 13. A) Inhibitory receptor expression on CD8 T cells from mixed BM chimeras. Histograms are gated on wt DbGP33+ (shaded) or CKO DbGP33+ (open) CD8 T cells. Pie charts show either wt or CKO DbGP33+ CD8 T cells. Colored slices indicate the fraction of the total population that expressed a combination of 4, 3, 2, 1 or no inhibitory markers. B) Inhibitory receptor expression on CD8 T cells from mixed CD4-Cre BM chimeras. Histograms are gated on wt DbGP33+ (shaded) or CKO DbGP33+ (open) CD8 T cells. Pie charts show either wt or CKO DbGP33+ CD8 T cells.

Discussion

In this study, we identify an important role for the transcriptional repressor Blimp-1 in regulating several defining features of CD8 T cell exhaustion during chronic viral infection. Exhausted CD8 T cells had substantially higher expression of Blimp-1 compared to functional effector or memory CD8 T cells generated following acute infection. This higher expression of Blimp-1 correlated with high inhibitory receptor levels and repression of memory T cell properties. Conditional deletion of Blimp-1 led to the restoration of some key aspects of memory (i.e. CD127 and CD62L expression, IL-2 production) and decreased expression of inhibitory receptors. Despite this, however, the full CKO mice did not resolve chronic infection, while haploinsuffcient mice controlled infection more rapidly than both wt and CKO mice. This suggested that while overexpression of Blimp-1 can promote functional exhaustion and the repression of memory differentiation, some Blimp-1 is necessary in order to acquire and/or maintain important effector functions, such as cytotoxicity. Thus, Blimp-1 appears to be acting as a molecular rheostat, acting to regulate effector function at low levels, while both promoting functional exhaustion and repressing memory at high levels.

CD8 T cell exhaustion is a common feature of many chronic viral infections in both animal models as well as in humans and is a likely reason for poor control of infection in these settings (14). High and sustained expression of PD-1 has emerged as a hallmark of T cell exhaustion, and blocking the PD-1:PD-L pathway can re-invigorate immune responses during persisting infections (160,

162, 171). In this study we found that Blimp-1 expression was 2-3 times higher in the more terminally differentiated PD-1^{hi} subset of exhausted CD8 T cells compared to the PD-1$^{int/lo}$ subset, which can be 'revived' by antibody blockade (163). Other inhibitory receptors, including LAG-3, 2B4 and CD160, are also upregulated by exhausted CD8 T cells and these pathways cooperate to negatively regulate CD8 T cell responses during chronic viral infection (158, 160). Blimp-1 appeared to cell-intrinsically regulate expression of these additional inhibitory receptors since the absence of Blimp-1 resulted in reduced expression of PD-1, LAG-3, 2B4 and CD160 by virus-specific CD8 T cells at later time points during chronic infection. However, PD-1 levels were higher on CKO virus-specific CD8 T cells at early time points (d8 p.i.) but had decreased below wt levels by d15 p.i. (data not shown). This suggests at least two different mechanisms by which Blimp-1 regulates high inhibitory receptor expression: 1) direct (or indirect) transcriptional regulation, or 2) survival of highly exhausted CD8 T cell subsets. The expression of inhibitory molecules occurs in a hierarchical manner, with PD-1 and LAG-3 most widely expressed, followed by 2B4 (158, 160). Only the most severely exhausted CD8 T cells appear to upregulate CD160 (158, 160). The loss of CD160 and 2B4 expression as well as the decrease in the PD-1^{hi} and LAG-3^{hi} subpopulations could indicate that expression of these inhibitory receptors does not occur, or the most highly dysfunctional cells have undergone apoptosis. Knockdown of Blimp-1 has been shown to decrease the survival of transformed plasma cells (202), thus it is possible that Blimp-1 may be acting in a similar manner in virus-specific CD8 T

cells early during chronic viral infection. Further work is necessary to determine the exact mechanism by which Blimp-1 regulates this aspect of functional exhaustion.

Despite lower expression of inhibitory receptors in the absence of Blimp-1, these CD8 T cells remained poor cytokine producers. One possibility is that other negative regulatory pathways compensate in this setting. Alternatively, Blimp-1 could positively regulate expression of antiviral cytokines, either directly or indirectly. A third possibility is that Blimp-1 controls one "module" of the transcriptional program of T cell exhaustion which includes inhibitory receptor expression and memory repression, while another layer of transcriptional control also influences expression of antiviral cytokines. Such an idea of overlapping transcriptional modules is emerging for Foxp3+ Tregs (203, 204). Future studies are necessary to dissect additional transcriptional pathways associated with T cell exhaustion and Blimp-1-independent regulation of cytokine production. It will also be important to compare functional changes in exhausted CD8 T cells that occur following antibody-mediated inhibitory receptor blockade versus temporal deletion of Blimp-1.

The amount of Blimp-1 expressed also appeared to have a crucial impact on CD8 T cell differentiation and exhaustion. Mice with one intact copy of the *prdm1* gene were not intermediate between wt and CKO mice, but rather achieved more efficient control of infection than wt or CKO mice. Many transcription factors function as transcriptional on/off switches (205). In contrast, our data suggest that Blimp-1 could act as a molecular rheostat in CD8 T cells

during chronic infection, mediating different cell fates or transcriptional events at different expression levels. Other transcription factors can function in a graded fashion, and some molecules that possess this property include master regulators of cell fate and differentiation such as Nanog, Sox2 and Oct-3/4 (206, 207). The transcription factor PU.1 also acts in a graded manner during hematopoietic differentiation (208). Accumulating evidence also points to the importance of quantitative changes in the expression of transcription factors such as Tbet and Eomes in memory CD8 T cell differentiation following acute infection (60, 148, 149). The timing of conditional deletion also appears to have an impact on the functionality of exhausted CD8 T cells. Conditional deletion prior to activation (CD4-Cre) results in poor viral control that is apparent by 1 month p.i., in contrast to deletion post-activation (gzmB-Cre), suggesting that Blimp-1 may be required upon activation to acquire even residual effector function. Thus, our data on both temporal expression patterns and conditional deficiency suggest that low or intermediate expression of Blimp-1 is required for some effector functions and could be important in fate decisions between memory and terminal effector cell differentiation following acute infection, while overexpression of Blimp-1 promotes CD8 T cell exhaustion and represses memory differentiation.

Our studies also point to a critical role for sustained cytolytic potential during chronic viral infections. Recent work has demonstrated the importance of cytotoxicity in long-term control of chronic infections in humans (209-212). While exhausted CD8 T cells are known to have partial defects in killing compared to highly functional effector or memory CD8 T cells (89), some residual cytotoxicity

by antigen-specific CD8 T cells can be maintained *in vivo* (213). Blimp-1 conditional het mice controlled virus substantially faster than either the wt or CKO mice, and this difference corresponded to sustained cytolysis in the conditional het mice compared to the CKO mice. However, conditional het mice controlled virus *in vivo* more rapidly than wt mice as well, despite similar killing and cytokine production *in vitro*. It is possible that lower PD-1 expression by conditional het mice may lead to improvements in effector function *in vivo* that are not obvious *in vitro*, as has been observed with blockade of other inhibitory pathways such as LAG-3 (160). Conditional het mice also had slightly higher total numbers of antigen-specific CD8 T cells than wt mice including more DbNP396-specific CD8 T cells. Higher numbers of virus-specific CD8 T cells and improved effector functions *in vivo* by conditional het mice could account for this more efficient control of infection compared to wt mice, but future studies are necessary to investigate these issue further.

In summary, we have identified Blimp-1 as a transcriptional regulator of functional exhaustion and repressor of memory differentiation in CD8 T cells during chronic viral infection. These studies provide a framework to begin dissecting Blimp-1 targets, regulation of Blimp-1 activity and additional transcriptional pathways involved in T cell dysfunction during chronic infection. While there are clearly additional transcription factors and transcriptional pathways that contribute to T cell exhaustion, our results identify Blimp-1 as a transcriptional regulator of CD8 T cell exhaustion during chronic viral infection.

Chapter 3

Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic viral infection

Abstract

Efficient maintenance of memory CD8 T cells is central to long-term protective immunity. IL-7 and IL-15 driven homeostatic proliferation is essential for long-term memory CD8 T cell persistence following acute infections. During chronic infections, however, virus-specific CD8 T cells respond poorly to these cytokines. Yet, virus-specific CD8 T cells often persist for long periods of time during chronic infections. We have addressed this apparent paradox by examining the mechanism for maintaining virus-specific CD8 T cells during chronic infection. We find that homeostatic cytokines (e.g. IL-7/15), inflammatory signals and priming of recent thymic emigrants are not sufficient to maintain virus-specific CD8 T cells over time during chronic infection. Rather, our results demonstrate that viral peptide is required for virus-specific CD8 T cell persistence during chronic infection. Moreover, this viral antigen-dependent maintenance results in a dramatically different type of T cell division than is normally observed during memory T cell homeostasis. Rather than undergoing slow, steady homeostatic turnover during chronic viral infection, CD8 T cells undergo extensive peptide-dependent division, yet cell numbers remain relatively stable. These results indicate that antigen-specific CD8 T cell responses during

persisting infection are maintained by a mechanism distinct from that following acute infection.

Introduction

A cardinal property of memory CD8 T cells that differentiate after acute infection is their long-term, antigen-independent survival and self-renewal, which is driven by the homeostatic cytokines IL-7 and IL-15 (22). The ability to respond to these homeostatic cytokines is a property that is gradually acquired as memory CD8 T cells differentiate (64), and is mediated by the upregulation IL-7Rα (CD127) and IL-15Rβ (CD122) (22). During chronic viral infection, expression of these receptors is impaired (14, 174, 176), and *in vitro* responses of IL-7 and IL-15 are poor (174, 180). CD127 levels are also decreased on antigen-specific CD8 T cells during human chronic infections such as HIV and HCV (14). When transferred to naïve recipients, exhausted CD8 T cells that are 'rested' in an infection-free environment do not adopt a memory phenotype (i.e. upregulate CD127 or CD62L) and slowly decline in number over time (174). Despite this, exhausted antigen-specific CD8 T cells persist long-term within the original chronically infected host (90, 174). This suggests that the mechanism of maintenance utilized by exhausted antigen-specific CD8 T cells during chronic viral infection is very different from the cytokine-driven homeostasis of memory CD8 T cells after an acute infection.

Despite numerous observations that the IL-7 and IL-15 pathways are impaired during chronic viral infection, the exact mechanism by which exhausted CD8 T cells are maintained is unknown. Several possibilities exist: 1) IL-7 and IL-15 may be expressed at a different level or different manner during chronic infection, 2) an unknown cytokine or growth factor present only during chronic

infection, 3) some unidentified cell-cell contact mediating survival, 4) the input of primed recent thymic emigrants (182) or 5) persistent antigen. This chapter will explore these different possibilities to determine the signal(s) that are necessary to maintain exhausted CD8 T cells.

Our work from Chapter 2 shows that Blimp-1 may be a key factor in repressing memory differentiation during chronic viral infection. In this chapter, we examine the consequences of altered memory differentiation and how homeostasis of the dysfunctional antigen-specific CD8 T cell population is impacted. Our data indicate that IL-7 and IL-15 are not necessary *in vivo* for the persistence of these antigen-specific CD8 T cells during chronic viral infection. By using an adoptive transfer system with a variant LCMV clone 13 strain, we show that cognate antigen is required for the maintenance of virus-specific CD8 T cells, and that primed RTEs do not have to join the existing pool of exhausted CD8 T cells to maintain stable numbers. Furthermore, rather than undergoing the slow, steady homeostatic proliferation that is characteristic of memory CD8 T cells, exhausted CD8 T cells undergo rapid, antigen-driven division. Thus, our study identifies a unique homeostatic mechanism of exhausted CD8 T cells and has implications for the maintenance of protective immunity during the treatment of chronic infections.

Materials and Methods

Animals and viruses. Four to six week old female C57BL/6 mice were purchased from the Jackson Laboratories, and four to six week old female congenic B6-Ly5.2/Cr mice were purchased from NCI. Thymectomized and sham thymectomized C57BL/6 mice were purchased form Charles River Laboratories. IL-15-/- mice were originally obtained from Michael Caligiuri (Ohio State University, Columbus, OH). Mice were infected with $2x10^6$ plaque forming units (PFU) of LCMV clone 13 or the V35A clone 13 variant virus i.v. or $2x10^5$ PFU of LCMV Armstrong i.p. as described (89). The V35A variant of LCMV clone 13 was isolated following infection of C57BL/6 mice containing LCMV-specific TCR transgenic T cells (P14 cells). Briefly, a small number of naïve P14 splenocytes was adoptively transferred to C57BL/6 mice followed by LCMV clone 13 infection. Virus was isolated from viremic mice approximately 1 month post infection by plaque purification and sequenced through the GP33-41 encoding region. Several clones were identified with the same Val to Ala mutation at residue 35, a mutation that has been previously observed (214). Virus was grown and viral titers were determined by plaque assay as described (89). The αIL-7Rα antibody was purified by the hybridoma core at the Wistar Institute from the supernatant of the A7R34 hybridoma and used as described (63). All mice were used in accordance with institutional IACUC procedures.

Lymphocyte isolation and flow cytometry. Lymphocyte isolation from lymphoid and non-lymphoid tissues and surface and intracellular stains were

performed as previously described . All antibodies were purchased from BD Biosciences except for CD127, Ly5.2 (eBioscience) and granzyme B (Caltag). APC-streptavidin was purchased from Invitrogen. MHC class I peptide tetramers were made and used as described previously (89).

CFSE labeling and adoptive transfers. After wt clone 13 or V35A clone 13 infected mice were no longer viremic (2-3 months post-infection), wt clone 13 donor mice were sacrificed and their spleens were harvested. CD8 T cells were purified from splenocytes using magnetic beads (MACS beads, Miltenyi Biotec). Briefly, splenocytes were labeled with magnetic beads specific for CD8α and then run through a MACS LS separation column (Miltenyi Biotec) according to the manufacturer's protocol. The purified CD8 T cells were then labeled with CFSE as described previously (89, 174). Purified CD8 T cells were adoptively transferred intravenously to each recipient mouse. Between 2-2.5x10^5 DbGP33-specific CD8 T cells were transferred in the experiments shown. In each individual experiment identical numbers of DbGP33 tetramer+ CD8 T cells were adoptively transferred to each separate recipient mouse. Donor populations were monitored in the peripheral blood by retro-orbital blood collection as described previously (174).

Results

Exhausted CD8 T cells persist long-term despite low expression of homeostatic cytokine receptors.

After an acute infection, antigen-specific CD8 T cells expand into a pool of effector cells, undergo contraction and are subsequently stably maintained as a population of memory CD8 T cells in the absence of antigen (66) (Fig 14a). To examine the persistence of exhausted antigen-specific CD8 T cells after a chronic viral infection, C57BL/6 mice were infected with either LCMV Armstrong (Arm) or LCMV clone 13, and the frequency of DbGP33-specific CD8 T cells was tracked in the blood. The kinetics of the DbGP33-specific CD8 T cell response was similar between LCMV Arm and clone 13 infection, and like memory CD8 T cells in the Arm immune mice, exhausted CD8 T cells were stably maintained for approximately one year post infection (Fig 14a).

A key feature of memory CD8 T cells is their high expression of the homeostatic cytokine receptors CD127 and CD122 (22). DbGP33-specific CD8 T cells from Arm immune mice all expressed high levels of CD127 and CD122 in both lymphoid and non-lymphoid tissues (Fig 14b). However, DbGP33-specific CD8 T cells from chronically infected mice expressed lower levels of both receptors as compared to memory CD8 T cells from the Arm immune mice (Fig 14b). The distribution of CD127 expression was much broader, with a significant fraction of the total population being $CD127^{lo}$, and the MFI of CD122 expression was consistently lower on exhausted CD8 T cells than memory CD8 T cells in both lymphoid and non-lymphoid tissues (Fig 14b). Thus, while antigen-specific

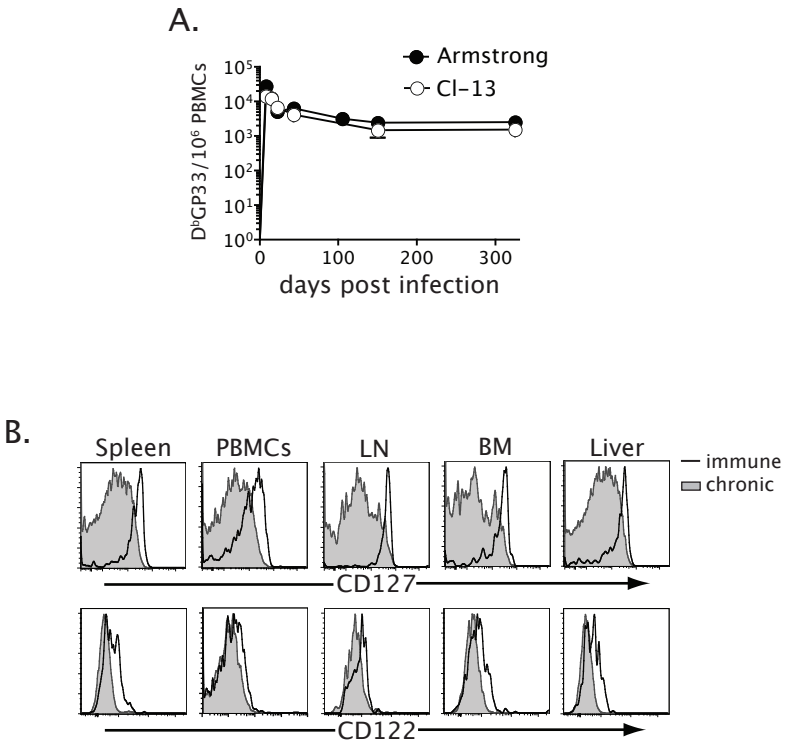

Figure 14. Exhausted antigen-specific CD8 T cells persist but do not express high levels of homeostatic cytokine receptors. A) Longitudinal analysis of DbGP33+ CD8 T cells in the blood after LCMV Arm or clone 13 infection. N=3-9 per time point. Error bars are SEM. B) Analysis of CD127 and CD122 expression on DbGP276+ CD8 T cells from tissues of Arm immune (>d30 p.i.) and clone 13 infected mice (2-4 months p.i.). Histograms are gated on DbGP276+ CD8 T cells from Arm immune (open) or clone 13 (shaded) infected mice. Similar results were obtained for DbGP33+ CD8 T cells (not shown).

exhausted CD8 T cells persist long-term in chronically infected mice, they do not upregulate the expression of key homeostatic cytokine receptors.

Exhausted CD8 T cells do not require IL-7 and IL-15 for long-term maintenance.

As memory CD8 T cells generated after an acute infection use IL-7 and IL-15 for their long-term survival and self-renewal, we wanted to examine whether exhausted CD8 T cells, despite their low expression of CD127 and CD122, could also use these two cytokines *in vivo* for their maintenance. To test this, IL-15-/- or wt mice were infected with LCMV Arm or clone 13. Two to three months post-infection, wt mice were left untreated, and IL-15-/- mice were treated with an antibody that blocks the interaction with IL-7 and IL-7Rα (A7R34). This approach has been previously used to block IL-7 signals, including those necessary for memory CD8 T cell homeostasis (63, 127, 215, 216). As expected, DbGP33-specific CD8 T cells in the untreated Arm immune wt controls were stably maintained in the blood, while the frequency of DbGP33-specific CD8 T cells in the Arm immune IL-15-/- mice declined significantly after A7R34 treatment (Fig 15a). However, in mice infected with LCMV clone 13, there was no difference in the frequency of DbGP33-specific CD8 T cells between untreated wt and IL-15-/- mice treated with A7R34 (Fig 15a). When compared directly, it was apparent that lack of signals from the IL-7 and IL-15 pathways had a much greater impact on the maintenance of antigen-specific memory CD8 T cells in Arm immune mice than exhausted CD8 T cells from chronically infected mice (Fig 15a). We also observed a decrease in the number of memory DbGP33-specific CD8 T cells in

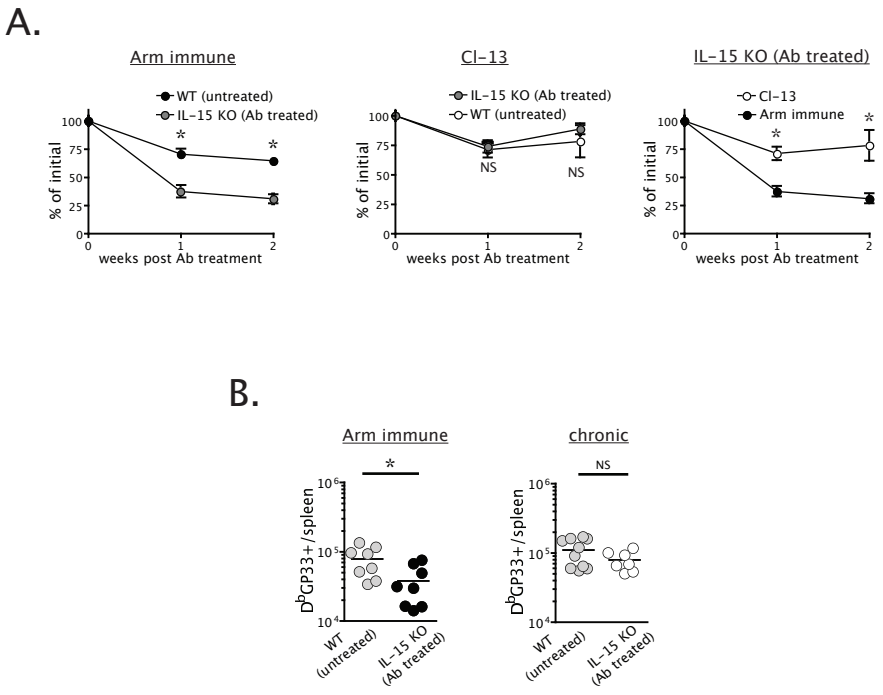

Figure 15. Virus-specific CD8 T cells do not require IL-7 and IL-15 to persist in chronically infected hosts. A) IL-15 KO and wt mice were infected with LCMV Arm or clone 13. After 2-3 months p.i., the IL-15 KO mice were treated with 200 υg αIL-7Rα antibody (Ab) i.p. every 2-3 days for 2 weeks, and the maintenance of virus-specific CD8 T cells was compared to untreated wt mice. Left graph shows Arm-infected Ab treated IL-15 KO and untreated wt mice. Middle graph shows clone 13-infected Ab treated IL-15 KO and untreated wt mice. Right graph displays a direct comparison of the same LCMV Arm and clone 13 infected Ab treated IL-15 KO groups from the first two graphs. All graphs show DbGP33+ CD8 T cell frequency in the blood as a percentage of the DbGP33+ CD8 T cell frequency on the first day of treatment (initial). Graphs represent three independent experiments, n=8-11 per time point. *p<0.05 by Student's t-test. Error bars are SEM. B) Absolute number of DbGP33+ CD8 T cells in the spleens of LCMV Arm immune and clone 13 infected wt untreated and IL-15 KO Ab treated mice at the end of treatment. Graphs represent two independent experiments, n=8-10 per group. *p=0.03 by Student's t-test. Error bars are SEM.

the spleens of Arm immune IL-15-/- mice treated with A7R34, but not in those infected with LCMV clone 13 (Fig 15b). Thus, unlike memory CD8 T cells generated after an acute infection, exhausted CD8 T cells present after a chronic infection do not depend on the homeostatic cytokines IL-7 and IL-15 for their long-term maintenance *in vivo*.

Recent thymic emigrants are not required for the maintenance of an antigen-specific CD8 T cell population.

As IL-7 and IL-15 do not appear necessary for the long-term persistence of the exhausted antigen-specific CD8 T cell population, it appears that another mechanism is being engaged. As exhausted CD8 T cells that are adoptively transferred to naïve recipients decline in number over time (174), it is likely that a factor unique to the environment of chronic viral infections is providing the maintenance signal. One possibility is that recent thymic emigrants (RTEs) are being primed by persisting antigen and joining the pre-existing pool of antigen-specific CD8 T cells. Previous findings using thymectomized mice suggest that recent RTEs are not required for the maintenance of an antigen-specific CD8 T cell population (181). However, Vezys et al showed that thymic emigrants can be primed by persisting antigen during both chronic LCMV and polyoma virus (PyV) infection, and that RTEs were necessary for the stable maintenance of PyV-specific CD8 T cells (182). Also, the primed thymic emigrants contributed to the dynamic phenotype of the total antigen-specific CD8 T cell population (182). Thus, in order to determine whether thymic emigrants contribute to the

maintenance of virus-specific CD8 T cells during chronic LCMV infection, thymectomized, sham thymectomized or intact B6 (control) mice were infected with LCMV clone 13 and the virus-specific CD8 T cell response was tracked over time. The frequency of DbGP33-specific CD8 T cells in the thymectomized mice was very similar to frequencies in either the intact B6 (exp 1) or the sham thymectomized mice (exp 2) (Fig 16a). The absolute number of virus-specific CD8 T cells in the spleens of thymectomized and control mice was nearly indistinguishable for the three epitopes tested, and the number of DbGP33-specific CD8 T cells in the liver and BM of the two groups was also very similar (Fig 16b). To test whether the lack of RTEs in the thymectomized mice had an impact on the overall phenotype of the antigen-specific CD8 T cell population, we analyzed the expression of several different markers associated with mature memory CD8 T cells (137). We found no significant difference between the thymectomized and control mice in CD127, CD62L, CD43 or CXCR3 expression (Fig 16c). There was also no difference in the ability of virus-specific CD8 T cells to produce antiviral cytokines upon *in vitro* stimulation (data not shown). Thus, in the LCMV model system, while RTEs can be primed by persistent antigen as shown by Vezys et al, they are not necessary for the long-term maintenance of an exhausted antigen-specific CD8 T cell population.

Variant LCMV clone 13 virus does not elicit a DbGP33-specific CD8 T cell response.

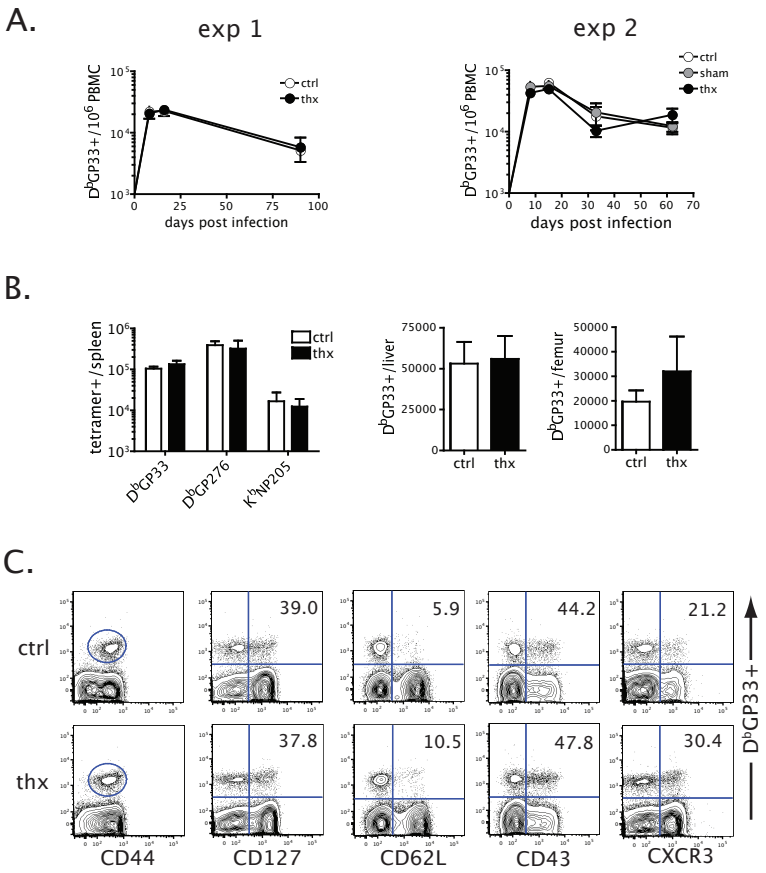

Figure 16. Thymic output is not necessary for the maintenance of virus-specific CD8 T cells during chronic LCMV infection. Thymectomized (thx), sham thymectomized or unmanipulated B6 (ctrl) mice were infected with LCMV clone 13 and CD8 T cells were analyzed ~3 months p.i. unless otherwise noted. A) Frequency of DbGP33+ CD8 T cells in the blood of thx, sham or ctrl mice at the indicated time points. N=2-5 per time point. Error bars are SEM. B) Absolute number of antigen-specific CD8 T cells in thx or ctrl mice. Number of antigen-specific CD8 T cells was measured in the indicated tissues. N=2 per group. Error bars are SEM. C) Phenotype of antigen-specific CD8 T cells in thx or ctrl mice. Plots are gated on total CD8 T cells from the spleen. Numbers in plots indicate percent of DbGP33+ CD8 T cells that are positive for each marker.

To examine the mechanism by which antigen-specific CD8 T cells persist during chronic viral infection, we used a variant strain of LCMV clone 13 with a mutation in the GP33 epitope (V->A mutation at residue 35, V35A). This mutation in the epitope abolishes binding of the GP33 epitope to Db MHC class I molecules (214). Infection with the V35A virus establishes a chronic infection with viremia that is nearly identical to wt clone 13 (Fig 17a). Unlike wt clone 13, infection with the V35A virus does not generate a DbGP33-specific response, although responses to four other epitopes were similar (Fig 17b).

To test whether the V35A virus could stimulate memory DbGP33-specific CD8 T cells, CD8 T cells were purified from the spleens of Arm immune mice, CFSE labeled and adoptively transferred to mice infected with V35A after the control of viremia (~2 months p.i.). The donor DbGP33-specific CD8 T cells failed to expand, and rather appeared to undergo division that was reminiscent of the slow, steady homeostatic proliferation that is characteristic of resting memory CD8 T cells (22) (Fig 17c,d). Donor memory CD8 T cells specific for the DbGP276 epitope, however, proliferated extensively upon adoptive transfer to V35A infected recipients, and the majority of the population became CFSE negative (Fig 17c,d). Thus, infection with V35A causes a chronic viral infection with viral titers that are similar to wt LCMV clone 13. Furthermore, while the mutated GP33 epitope in the V35A virus is does not activate a naïve or memory GP33-specific response, other epitopes can generate CD8 T cell responses that are similar to wt LCMV clone 13.

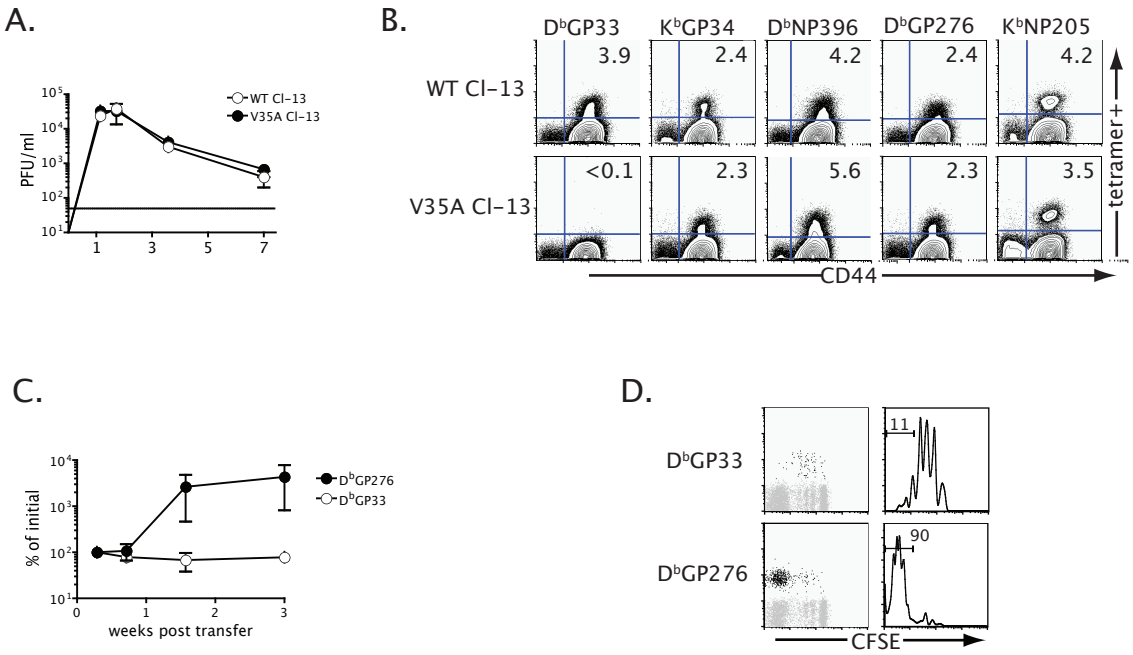

Figure 17. The V35A clone 13 variant virus causes chronic infection similar to wt clone 13. B6 mice were infected with wt or V35A variant clone 13. A) Viral load was determined in the blood of mice infected with wt or V35A variant clone 13 by plaque assay. Dashed line shows limit of detection. Error bars are SEM. B) Tetramer staining of splenocytes on day 8 p.i. Plots are gated on total CD8 T cells. Numbers in plots show percent of CD8 T cells positive for each tetramer. C) CD8 T cells from Arm immune (>d30 p.i.) were adoptively transferred into V35A-infected Ly5.1+ B6 mice (~2-7 months p.i.). Donor Ly5.2+ DbGP276+ and DbGP33+ CD8 T cells were monitored in the blood. Kinetics of expansion were normalized to number at d5 post-transfer. N=3-4 per time point. Data are representative of two independent experiments. Error bars are SEM. D) CFSE profiles are shown for the donor (Ly5.2+) DbGP33+ and DbGP276+ CD8 T cells in V35A-infected recipients 3 weeks post-transfer for the experiment in part C. Plots are gated on donor Ly5.2+ CD8 T cells. Histograms are gated on donor tetramer+ CD8 T cells. Numbers over gates in the histograms show percent of cells that are CFSE-. Data are representative of two independent experiments.

Cognate virus-derived antigen is required for the maintenance of antigen-specific CD8 T cell during chronic viral infection.

In order to determine the factor(s) that could be providing the maintenance signal to exhausted antigen-specific CD8 T cells, the V35A virus was used to narrow the possibilities to either antigen or some other factor present in the chronically infected environment. An adoptive transfer system was used in which Ly5.2+ donor mice were infected with wt LCMV clone 13, and Ly5.1+ recipient mice were infected with either wt LCMV clone 13 or V35A virus, all at the same time (Fig 18a). After all three groups controlled viremia (~2-3 months p.i.), CD8 T cells were purified by magnetic beads from the spleens of the donor wt LCMV clone 13 infected mice, and equal numbers of DbGP33-specific CD8 T cells were transferred to all recipient mice (Fig 18a). This strategy allowed us to compare the maintenance of the donor DbGP33-specific population in recipient mice where cognate antigen is either present or absent. A donor population of DbGP33-specific CD8 T cells was present and measurable at both early and late time points post transfer, as was a control population of donor DbGP276-specific CD8 T cells (Fig 18b). As a second strategy, by tracking both donor populations in the same V35A infected recipient mouse, we could internally control any variability between different recipients.

When donor DbGP33-specific CD8 T cells were transferred to wt LCMV clone 13 infected recipients, the population was stably maintained over time (Fig 18c). However, when transferred to a recipient infected with V35A, the frequency of the donor DbGP33-specific population decreased over time (Fig 18c).

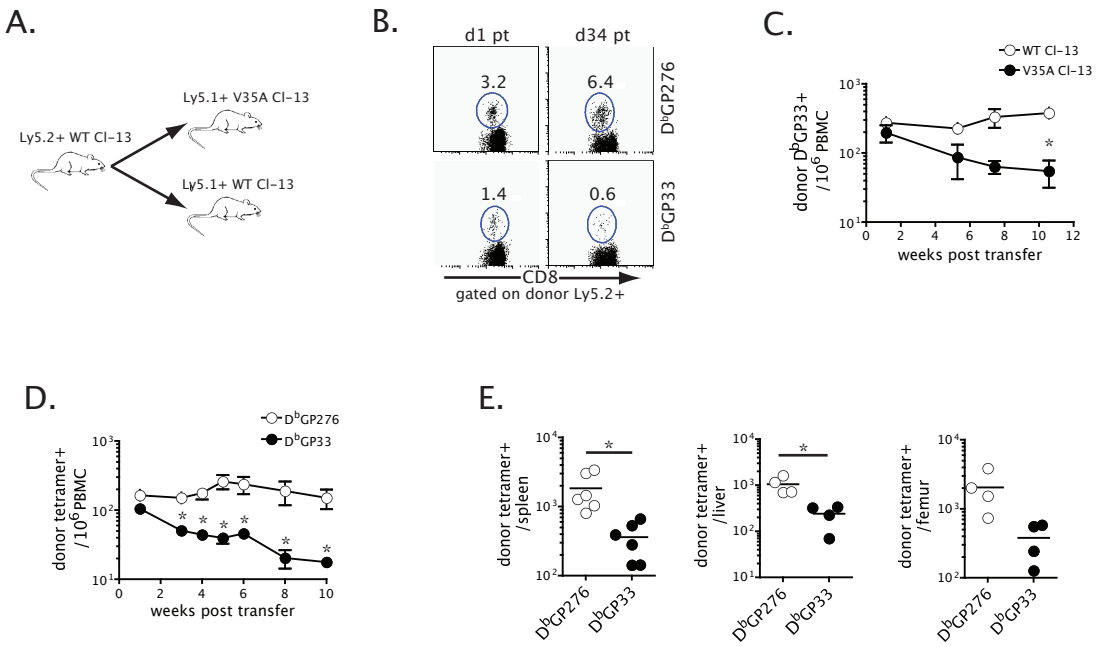

Figure 18. Virus-specific CD8 T cells from chronically infected mice do not persist without cognate antigen. A) Experimental schematic. Donor Ly5.2+ B6 mice were infected with wt clone 13, and recipient Ly5.1+ B6 mice were infected with either wt or V35A clone 13 on the same day. 2-3 months later, CD8 T cells were purified from the spleens of donor Ly5.2+ mice using magnetic beads and equal numbers of DbGP33+ CD8 T cells were transferred to the recipients. B) Representative analysis of DbGP33+ and DbGP276+ donor Ly5.2+ CD8 T cells in the blood of recipient mice. Numbers are the percent of donor CD8 T cells that are tetramer+. C) Frequency of DbGP33+ donor cells in the blood of wt and V35A clone 13 infected recipients. At the last time point, wt vs V35A *p=0.008 by Student's t-test. Graph is representative of two independent experiments. D) Frequency of DbGP33 and DbGP276+ donor CD8 T cells in the blood of V35A clone 13 infected recipients. Difference between DbGP33+ and DbGP276+ frequency is significant at indicated time points ($p<0.05$ by Student's t-test). Graph is representative of five independent experiments, n=2-3 per group. E) Number of donor tetramer+ CD8 T cells in indicated tissues of V35A-infected recipients. BM represents two femurs. Graphs represent 2-3 independent experiments, n=3-6 per group. *$p<0.04$ by Student's t-test. Error bars are SEM.

Furthermore, within the same V35A infected recipient, while the donor DbGP33-specific population slowly declined in the absence of specific peptide, the donor DbGP276-specific CD8 T cells persisted (Fig 18d). The disappearance of donor DbGP33-specific CD8 T cells was not simply due to a migration of these cells out of the blood, as the number of donor DbGP33-specific CD8 T cells was lower in both lymphoid and non-lymphoid tissues such as the spleen, liver and bone marrow (Fig 18e). Together, our data suggest that virus-derived cognate antigen is the signal that is necessary to maintain a long-term population of antigen-specific CD8 T cells during chronic viral infection.

Virus-specific CD8 T cells are maintained by rapid, antigen-driven division during chronic viral infection.

Following an acute infection, memory CD8 T cells undergo a slow, steady homeostatic proliferation to mediate their survival and self-renewal (22). As the signal that maintains antigen-specific CD8 T cells during chronic viral infection is very different from that of memory CD8 T cells, we next wanted to determine whether the virus-specific CD8 T cells underwent a similar type of division. As a control, CD8 T cells from the spleens of Arm immune mice were isolated, labeled with CFSE and transferred to naïve, congenically marked recipients. As expected, the antigen-specific memory CD8 T cells underwent typical homeostatic proliferation, where only a portion (~50%) of the population divided no more than 5 times over the course of four weeks (Fig 19a,c,d). In order to examine division during chronic viral infection, an adoptive transfer system

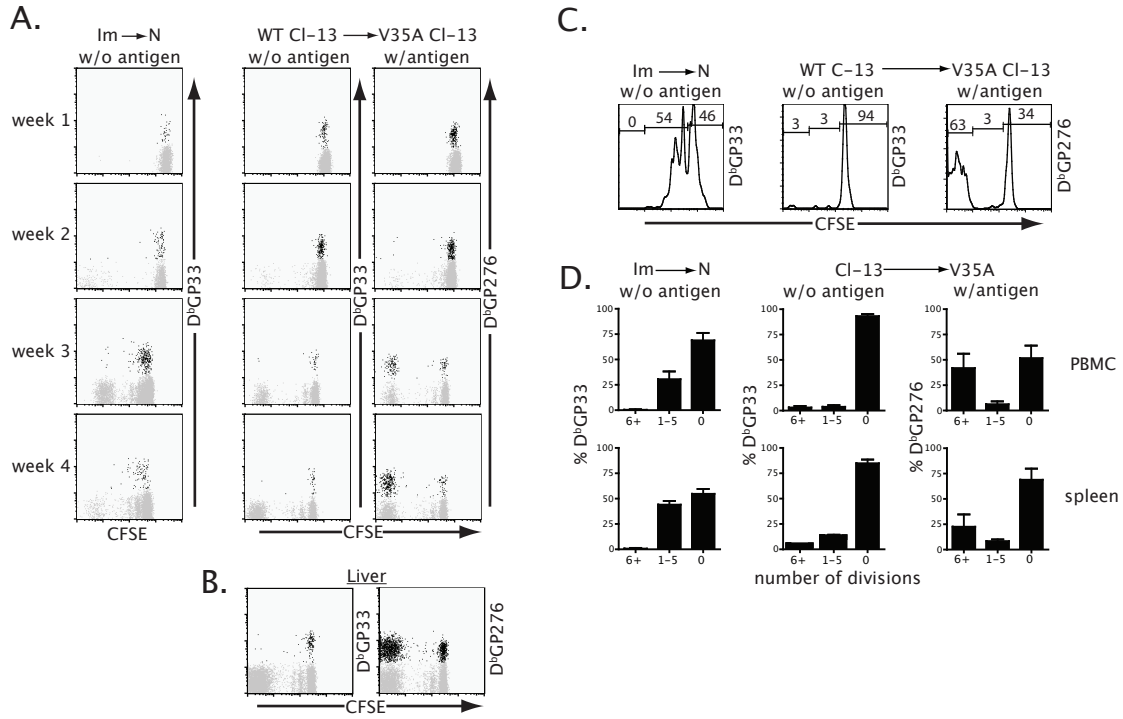

Figure 19. Virus-specific CD8 T cells are maintained by extensive proliferation during chronic infection. The experimental approach from Fig 18a was used to monitor cell division history of CFSE labeled donor cells during chronic LCMV infection. A) Prolferation patterns of donor virus-specific CD8 T cells in the blood. Left column shows homeostatic proliferation of memory CD8 T cells from Arm immune mice (>d30 p.i.) after adoptive transfer to naive mice. Middle column shows division of DbGP33+ CD8 T cells in V35A-infected recipients (without antigen). Right column shows division of DbGP276+ CD8 T cell population from same V35A recipient (with antigen). B) Proliferation of wt clone 13-derived donor virus-specific CD8 T cells in the liver of V35A-infected recipients at 4 weeks post-transfer. C) Histograms of donor tetramer+ CD8 T cells in the blood at 4 weeks post-transfer. Numbers indicate the percent of virus-specific CD8 T cells that have undergone no division (right gate), 1-5 divisions (middle) or 6+ divisions (left). All histograms are gated on Ly5.2+ CD8 tetramer+ populations. D) Graphs indicate percent of donor tetramer+ CD8 T cells that have undergone 0, 1-5 or 6+ divisions. Top row is from the blood, bottom is from the spleen at 4 weeks post-transfer. Error bars are SEM. Data is representative of 2-4 independent experiments.

identical the one previously described (Fig 18a) was used with CFSE labeled donor CD8 T cells. Briefly, Ly5.2+ donor mice were infected with wt LCMV clone 13, and Ly5.1+ recipient mice were infected with V35A. After control of viremia, CD8 T cells were purified from the spleens of the donor mice, CFSE labeled and adoptively transferred to the recipient mice. The donor populations were tracked in the blood both in the presence (DbGP276) and absence (DbGP33) of cognate antigen. Over the course of four weeks, the donor DbGP33-specific CD8 T cell population did not appear to undergo any sort of division in the absence of antigen, and indeed, declined in frequency over time (Fig 19a,c,d). However, when the donor DbGP276-specific CD8 T cell population was examined, the pattern of division presented was strikingly different from that of memory CD8 T cells. Donor DbGP276-specific CD8 T cells transferred to recipients chronically infected with V35A virus did not divide during the first two weeks post transfer (Fig 19a). However, in the third week post transfer, a small population of CFSE negative donor DbGP276-specific CD8 T cells appeared, and this CFSE negative population increased in the fourth week (Fig 19a,c,d). This was in stark contrast to the homeostatic proliferation of memory CD8 T cells, were no cells fully diluted CFSE over the course of four weeks (Fig 19a,c,d). This rapid, antigen-driven division was not isolated to the blood, as it was also observed in the spleen and liver (Fig 19b,d). In sum, our data suggest that during chronic viral infection, antigen-specific CD8 T cells are maintained by a mechanism of rapid proliferation that is driven by specific, virus-derived peptide rather than cytokine-driven homeostatic proliferation.

Discussion

In this chapter, we have defined the mechanism of virus-specific CD8 T cell maintenance during chronic viral infection and have also uncovered a division pattern that is distinct from the homeostatic turnover of memory T cells. Our studies indicated that viral antigen was necessary for long-term persistence of virus-specific CD8 T cells during chronic infection, and also that the environment of chronic infection, including any inflammatory signals and altered IL-7 and IL-15 expression, was not sufficient to maintain virus-specific CD8 T cells in chronically infected mice. It remains possible that environmental factors may play an accessory role, but they alone cannot sustain virus-specific CD8 T cells during chronic LCMV infection.

During chronic LCMV infection, many virus-specific CD8 T cells had divided more than 5-6 times in ~1 month in the presence of viral antigen, yet the number of these virus-specific CD8 T cells numbers remained relatively stable. This observation suggests that either a very small subset of $CFSE^{hi}$ cells are recruited to divide or that the antigen-driven division of this CD8 T cell population is accompanied by extensive cell death. This pattern of division may help explain the immunodominance changes and the immune “inflation” that has been observed during several persisting infections (89, 217, 218). A difference between death and division rates during homeostatic proliferation following acute infection may lead to only a subtle change in cell numbers since the rate of division is slow. In contrast, during chronic infection, where the persisting virus-specific CD8 T cell population undergoes extensive division over the course of

several weeks, a minor discrepancy between the rates of division and death could be amplified to a large change in cell numbers. Therapeutic manipulation of the rate of either recruitment into division or death may provide a novel means to modulate antigen-specific CD8 T cell responses to persisting viral antigen.

The CFSE profiles in the present study reveal populations of both extensively divided and completely undivided virus-specific CD8 T cells during persisting infection. It is unclear why some virus-specific CD8 T cells proliferate extensively during chronic infection while others did not. The transfer of memory CD8 T cells from Arm immune mice to chronically infected recipients results in complete division of all DbGP276-specific donor cells (see Figure 2). This observation suggests that lack of antigen encounter is an unlikely explanation for the undivided population of virus-specific CD8 T cells from chronically infected mice. A second possibility is that only a subpopulation of the virus-specific CD8 T cells generated during chronic infection are capable of the peptide-dependent division. It will be important to determine whether heterogeneity exists in these T cell populations based on antigen-dependent maintenance potential or division history.

The present study suggests that during chronic infections, virus-specific CD8 T cells acquire a mechanism of maintenance distinct from naïve CD8 T cells or effector and memory CD8 T cells generated following acute infection. During chronic infection, virus-specific CD8 T cells appear to rely on TCR signals for persistence. However, unlike naïve CD8 T cells, MHC and self-peptide alone cannot supply the TCR signal required by virus-specific CD8 T cells during

chronic infection. Instead, viral antigen is necessary for long-term persistence. Unlike memory CD8 T cells that undergo proliferative expansion in response to antigen, virus-specific CD8 T cells present during chronic infection undergo extensive division, but the population does not expand dramatically in number in response to low levels of persisting viral antigen (compare the DbGP276 response in Figure 17c and 18d). Whether all virus-specific CD8 T cells undergo this antigen-driven division, or only a small subset of cells are recruited to divide, the observations are consistent with previous studies that found reduced proliferative potential of virus-specific T cells during chronic infection (89, 174, 210). This altered responsiveness to homeostatic signals and to persisting viral antigen suggests that during chronic infections, virus-specific CD8 T cells undergo a fundamentally different pattern of differentiation compared to memory CD8 T cells that develop in the absence of persisting antigen following acute infection. Thus, during chronic viral infection, antigen-specific CD8 T cells appear to be "tuned" to respond to low levels of antigen in a dramatically different manner than memory CD8 T cells generated following acute infections.

A recent study suggested that ongoing thymic output can result in priming of new virus-specific CD8 T cells during persisting infections (182). In the current study, we found that chronic infection of thymectomized mice did not have a significant impact on the maintenance, function or phenotype of the antigen-specific CD8 T cell population. We also used congenically marked donor populations and found that the number of tetramer+ CD8 T cells remains reasonably stable for ~10 weeks when antigen is present (see Figure 18). These

results suggest that RTEs are not necessary to maintain a virus-specific CD8 T cell population during chronic LCMV infection. However, our results do not exclude a qualitative contribution from RTEs primed on persisting antigen in the periphery. It will be interesting to determine if a subpopulation of recently primed CD8 T cells possesses distinct functional characteristics compared to the majority of the antigen-specific population that has been maintained since early during the initial infection.

In summary, the present work defines specific viral antigen, rather than IL-7 and IL-15, inflammatory cytokines or new recruitment from RTEs, as an essential signal that governs the persistence of virus-specific CD8 T cells during chronic LCMV infection in mice. In addition, we have identified a new mechanism for this type of antigen-dependent, long-term persistence by extensive cellular division without dramatic changes in cell numbers. These observations may provide a framework to re-evaluate a long-standing debate about the role of persisting antigen in the maintenance of immunological memory. Finally, the antigen-dependent maintenance mechanism described in this study may have implications for therapeutic interventions if either the rate of proliferation or cell death can be modulated *in vivo* to alter the size or quality of antigen-specific CD8 T cell populations during persisting infections.

Chapter 4

A role for chronic inflammation in the regulation of CD8 T cell dysfunction during chronic infection

Abstract

Chronic infections represent a major social and economic problem around the world. The control of disease burden can be further complicated by the co-infections and secondary infections that accompany many chronic infections. Epidemiologically, chronic infections have been shown to have a negative impact on the control of secondary infections or co-infections. However, there is little understanding as to how established chronic infections impact cellular responses to heterologous infections. CD8 T cell exhaustion and failed memory differentiation are features that are shared by many chronic infections and may contribute to poor pathogen control and diminished protective immunity. The factors that regulate altered memory CD8 T cell differentiation during chronic infection are not well understood. In this study, we examine the impact of prolonged inflammation on CD8 T cell differentiation and find that effector CD8 T cells differentiating to memory are more sensitive to alteration by chronic inflammation than established memory CD8 T cells. Furthermore, memory CD8 T cells that differentiate in the presence of persistent inflammation have poor secondary responses and provide less protection. Together, these data suggest that established, persistent inflammation can impact memory differentiation in a

bystander fashion, and may lead to decreased efficacy of preventative vaccination strategies and diminished immunity to heterologous pathogens during chronic infection.

Introduction

After an acute infection, memory differentiation occurs in the absence of antigen, but also in the absence of any factors generated by host-pathogen interactions (14). However, during chronic infection, altered memory differentiation occurs not only in the presence of persistent antigen, but also in the continuous presence of all factors that arise from host-pathogen interactions (i.e. cytokines, chemokines, TLR ligands, etc.), which will heretofore be referred to as 'inflammation'. While the role of persistent antigen has been well described in regulating functional exhaustion (89, 219), the role that inflammation plays has not been as well studied. Recently, some of these factors, including the immunoregulatory cytokine IL-10 (183, 184) and IL-21 (186-188), have been shown to play an important role in determining the chronicity of LCMV infection and the functionality of the responding CD8 T cells. However, it is not entirely clear from these studies whether the impact on the cellular response is due to altered pathogenesis of the infection, or due to the direct effect of these cytokines on the T cells themselves. In this chapter, we investigate the impact that chronic 'inflammation' has on the differentiation of dysfunctional T cells during chronic infection at two stages – the effector to memory transition, and the T_{EM} to T_{CM} transition.

In addition to providing a greater understanding of how functional exhaustion is regulated during chronic infection, these studies could have important implications for understanding the biology of co-infections. There is a considerable amount of epidemiological evidence that suggests that chronic

infections, even those that are not overtly immunosuppressive such as HIV, can have a negative impact on responses to secondary, heterologous infections. Malaria infections are associated with increased HIV viral loads, particularly during febrile episodes (220), leading to increased transmission and thousands of additional cases of HIV infection (221). Infection with helminths such as schistosomes may also lead to increased viral replication of patients co-infected with HIV (222). Even in situations where a chronic infection is not involved, co-infection can have a negative impact on secondary infections. For example, acute respiratory infections such as influenza and RSV and other viral infections such as measles can lead to increased incidences of bacterial co-infections (223, 224). Furthermore, these bacterial infections often become more severe and lead to the onset of bacterial sepsis (224). While co-infections and their impact on disease control have been well documented, there is little understanding of how established chronic infection affects the development of cellular responses to heterologous infections.

The two previous chapters examined cell-intrinsic regulation and cell-intrinsic properties of functionally exhausted CD8 T cells. In this chapter, we address the effect that cell-extrinsic factors generated by interaction of the host and pathogen has on the regulation of T cell dysfunction. We find that chronic inflammation has a profound impact on the differentiation of memory CD8 T cells. Non-LCMV-specific CD8 T cells adoptively transferred to chronically infected recipients did not acquire several key memory properties. However, some features that are normally associated with functional exhaustion, such as the

upregulation of inhibitory receptors, were not impacted by inflammation alone. Our results suggest that distinct aspects of T cell dysfunction may be regulated by different signals. We believe that our study furthers our understanding of how functional exhaustion is regulated during chronic infections. Furthermore, our results could have important implications when designing treatment strategies to control disease burden, particularly for those involving prophylactic or therapeutic vaccination of chronically infected patients.

Materials and Methods

Animals and viruses. Four to six week old female C57BL/6 mice and four to six week old female congenic B6-Ly5.2/Cr were purchased from NCI. OTI TCR Tg mice were obtained from Wolfgang Weninger (Sydney Medical School, Australia). Vesicular stomatitis virus recombinantly expressing OVA (VSV-OVA) was propogated and titered as previously described (125). *Listeria monocytogenes* recombinantly expressing OVA (LM-OVA) was also propogated and titered as previously described (225). ME-49 strain of *Toxoplasma gondii* (toxo) was obtained from David Roos, and was grown and titered as previously described (226). LCMV virus was grown and titers were determined by plaque assay as previously described (89). Mice were infected with $2x10^5$ plaque forming units (PFU) i.p. of LCMV Armstrong, $2x10^6$ PFU i.v. of LCMV clone 13, $2x10^6$ PFU i.v. of VSV-OVA, $1.5x10^4$ colony forming units (CFU) i.v. of LM-OVA for primary challenges or 100 parasites i.p of toxo. Secondary challenges were

performed with 2.5-5x10^5 CFU i.v. of LM-OVA. All animals were used in accordance with IACUC procedures.

Lymphocyte isolation and flow cytometry. Lymphocyte isolation from lymphoid and non-lymphoid tissues, surface stains and intracellular cytokine stains were performed as described previously (89). All antibodies were purchased from Biolegend except for CD127, TNFa, IL-2 (eBioscience), CD62L (Invitrogen). LIVE/DEAD dead cell stain was purchased from Invitrogen. MHC class I tetramers were made and used as described previously (89). All flow cytometry data was acquired on an LSRII (BD Biosciences) and analyzed by FlowJo (Treestar).

Adoptive transfers. Naïve OTI TCR Tg mice were sacrificed and spleens were harvested. 5x10^5 splenocytes were adoptively transferred i.v. into congenically marked B6-Ly5.2/Cr naïve recipient mice. Recipients were infected with an OVA expressing pathogen and were sacrificed 8-30 days post infection (p.i.). Spleens were harvested, and CD8 T cells were purified by magnetic beads (MACS beads, Miltenyi Biotec). Briefly, splenocytes were incubated with beads specific for CD8a and then run through a MACS LS separation column (Miltenyi Biotec) according to the manufacturer's protocol. Frequency of OTI CD8 T cells was determined by flow cytometry. Equal numbers of OTI CD8 T cells were adoptively transferred to either infected or naïve congenically marked B6-Ly5.2/Cr recipients. Donor populations were monitored by retro-orbital bleeding

as described previously (174). The same adoptive transfer method was used for secondary challenges.

Results

Established chronic infections alter CD8 T cell differentiation after heterologous challenge.

In order to determine the role of chronic inflammation in regulating memory CD8 T cell differentiation, we first employed a direct co-infection model. Mice were infected with LCMV clone 13 and allowed to rest for 30 days. At d30 p.i., the LCMV clone 13 infected mice and naïve controls were infected with *Listeria monocytogenes* expressing recombinant OVA protein (LM-OVA). Response to the OVA peptide SIINFEKL was tracked over time and analyzed at 30 days post LM-OVA challenge (Fig 20a). As the LM-OVA is cleared within a week and the SIINFEKL epitope is not expressed by LCMV, we could be confident that the antigen-specific CD8 T cells responding to SIINFEKL would be differentiating in the absence of persistent antigen but in the presence of the chronic inflammation generated by LCMV clone 13 infection. 30 days after LM-OVA challenge, the KbSIINFEKL-specific CD8 T cells in the control mice had differentiated into a population of memory CD8 T cells uniformly expressing high levels of CD127, and with a small subset beginning to upregulate CD62L (Fig 20b). In contrast, the KbSIINFEKL-specific population in the LCMV clone 13 co-infected mice expressed low levels of CD127 and very few cells were $CD62L^{hi}$ (Fig 20b). When the effector function of these cells was assessed, we found that

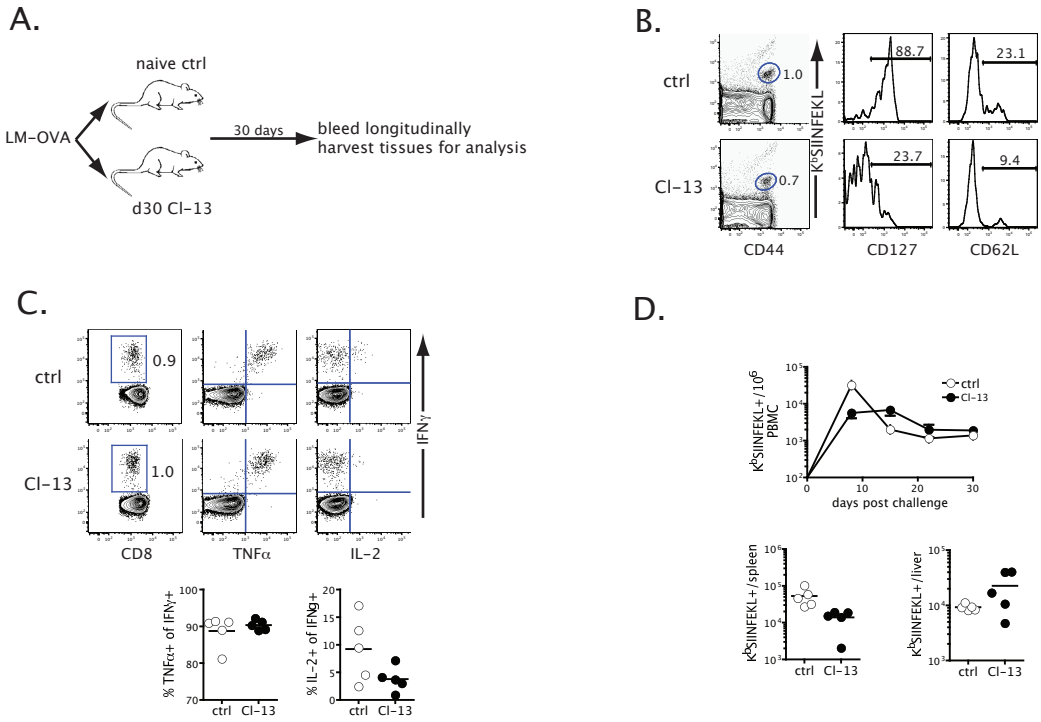

Figure 20. Chronic viral infection alters immune response to heterologous challenge. A) Schematic of experiment. B6 mice were infected with LCMV clone 13. 30 d.p.i., LCMV clone 13 infected mice and naive B6 were challenged with LM-OVA. The OVA-specific response was analyzed 30 days after LM-OVA challenge. B) Phenotype of OVA-specific CD8 T cells in ctrl and clone 13 infected mice. Plots are gated on total CD8 T cells. Histograms are gated on OVA-specific CD8 T cells. Numbers in plots show percent of total CD8 T cells that are OVA-specific. Numbers in histograms show percent of OVA-specific CD8 T cells that are positive for each marker. C) Effector function of OVA-secific CD8 T cells. Splenocytes were stimulated with OVA peptide for 5 hrs at 37C. All plots are gated on total CD8 T cells. Number in plot shows percent of total CD8 T cells that produce IFNγ. Graphs indicate percent of of IFNγ+ CD8 T cells that also produce the second indicated cytokine. D) Number of OVA-specific CD8 T cells. Top graph shows frequency of OVA-specific CD8 T cells in the blood at the indicated time points. Bottom graphs show absolute number of OVA-specific CD8 T cells in each indicated tissue. Error bars are SEM. N=5 at each time point.

90-95% of the memory KbSIINFEKL-specific CD8 T cells in the control mice were capable of co-producing IFNγ and TNFα, and a substantial fraction expressed both IFNγ and IL-2 (Fig 20c). In the LCMV clone 13 co-infected mice, however, while IFNγ and TNFα co-production appeared to be intact, IL-2 production, a key memory property, was impaired (Fig 20c). The frequency of KbSIINFEKL-specific CD8 T cells in the blood also differed between the control and LCMV clone 13 co-infected mice. The peak of the antigen-specific CD8 T cell response in the LCMV clone 13 co-infected mice was slightly delayed and not as great as in the control mice (~d8 p.i.), although the frequency of KbSIINFEKL-specific CD8 T cells in the blood after the contraction phase was similar (~d30 p.i.) (Fig 20d). The absolute number of KbSIINFEKL-specific CD8 T cells was not significantly different in the spleen or liver between the two groups, though there was a trend towards fewer antigen-specific CD8 T cells in the spleens of the LCMV clone 13 co-infected mice and more in the liver (Fig 20d). Together, these results indicate that an established chronic viral infection can effect the differentiation of CD8 T cells responding to a heterologous infection by repressing the memory development and decreasing the magnitude of the effector response.

Inflammation alone can repress memory CD8 T cell differentiation during chronic viral infection.

While the results from the direct co-infection model indicate that an established chronic viral infection can alter the CD8 T cell response to a

heterologous infection, a number of caveats complicate the interpretation of the data. Co-infections can change the pathogenesis of both infections (220, 221, 227) and LCMV clone 13 can infect APCs such as macrophages and DCs (87, 88), which raises the possibility that antigen-presentation and activation of the KbSIINFEKL-specific CD8 T cells may not be normal. To isolate the impact of chronic inflammation and focus directly on the memory differentiation stage, we switched to an adoptive transfer model. $5x10^5$ OTI splenocytes were transferred to naïve recipients and challenged with vesicular stomatitis virus expressing the OVA protein (VSV-OVA). At d8 p.i., CD8 T cells from the spleens of the infected OTI peripheral chimeras were purified and equal numbers of effector OTI CD8 T cells were transferred to control Ly5.1+ recipients that were either naïve or infected with VSV-OVA, and to Ly5.1+ recipients that had been challenged with LCMV clone 13 8 days prior (Fig 21a). Little difference was observed regardless of whether naïve or VSV-OVA infected recipients were used as controls. At d8 p.i., the effector OTI donor cells were mostly $CD127^{lo}$ and $CD62L^{lo}$ (Fig 21b). By adoptively transferring an effector OTI CD8 population generated by an acute infection, we could ensure that the OTI CD8 T cells had been properly primed and were capable of differentiating into normal memory CD8 T cells.

After about one month post-transfer, the donor OTI CD8 T cells that had been adoptively transferred to the control recipient mice expressed high levels of CD127, and were beginning to upregulate CD62L (Fig 21c). As in the direct co-infection model, however, the donor OTI CD8 T cells transferred to the LCMV clone 13 infected recipients remained $CD127^{lo}$ and $CD62L^{lo}$ (Fig 21c). Effector

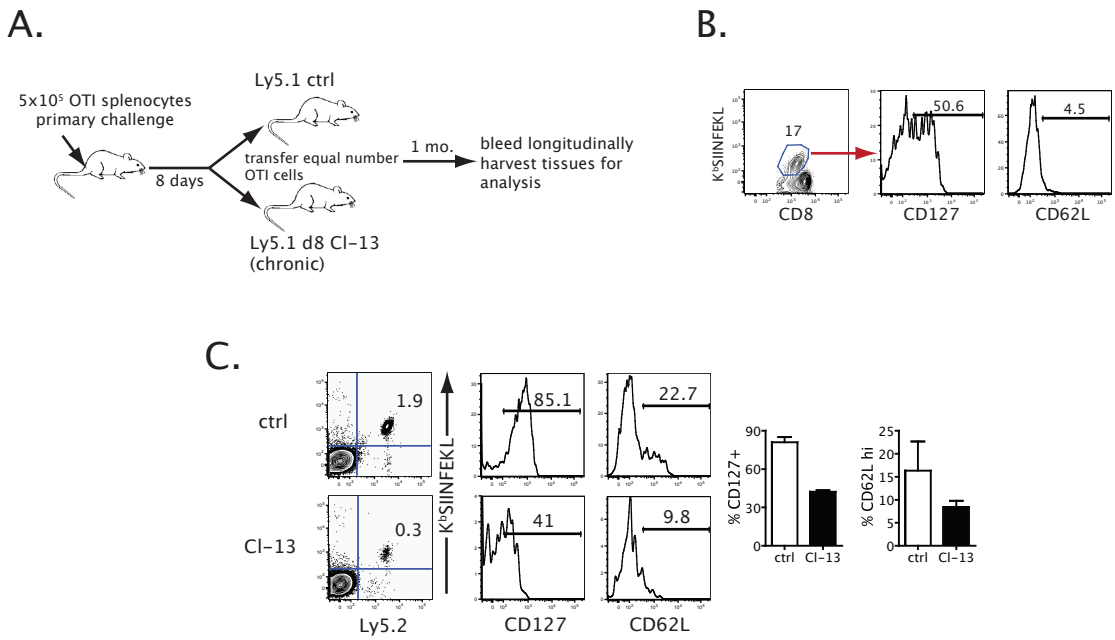

Figure 21. Memory CD8 T cell differentiation is repressed by chronic inflammation. A) Schematic of experiment. 5x10^5 OTI splenocytes were adoptively transferred to naive B6 recipients. Mice were challenged with VSV-OVA and one group of Ly5.1+ B6 recipients were infected with LCMV clone 13 at the same time. Control Ly5.1+ B6 mice were either challenged with VSV-OVA, LCMV Arm or naive. No difference was observed between different controls. At d8 p.i.,CD8 T cells were purified by magnetic beads from the spleens of the donor mice and equal numbers of OTI CD8 T cells were transferred to LCMV clone 13 infected and ctrl recipients. Donor OTI CD8 T cells were analyzed ~1 month post-transfer. B) Phenotype of donor OTI CD8 T cells pre-transfer. Plot is gated on total CD8 T cells. Histograms are gated on OTI CD8 T cells. Number in plot is percent of total CD8 T cells that are KbSIINFEKL+. Numbers in histograms show the percent of the OTI CD8 T cell population that is positive for each marker. C) Phenotype of donor OTI CD8 T cells in the spleen 1 month post-transfer. Plots are gated on total CD8 T cells, histograms are gated on donor OTI CD8 T cells. Numbers in plots indicate the percent of total CD8 T cells that are donor OTI. Numbers in histograms show percent of donor OTI population that is positive for each marker. Numbers are graphed to the right. N=2 per group, data is representative of seven independent experiments. Error bars are SEM. (Figure continued on next page.)

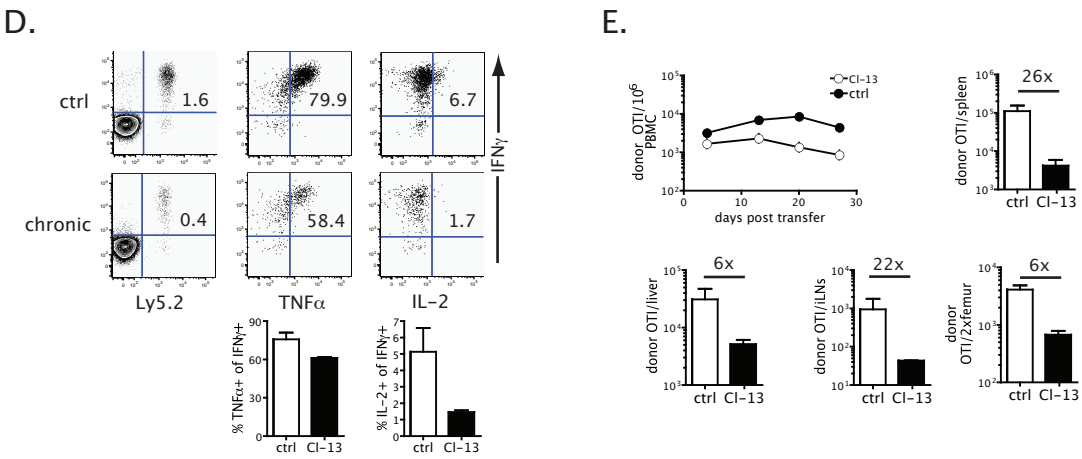

Figure 21 (cont.). Memory differentiation is repressed by chronic inflammation. D) Effector function of donor OTI CD8 T cells. Splenocytes were stimulated with OVA peptide for 5 hrs at 37C. Left plots are gated on total CD8 T cells, middle and right plots are gated on donor CD8 T cells. Number in left plots show percent of total CD8 T cells that produce IFNγ after OVA stim. Numbers in middle and left plots show percent of IFNγ+ CD8 T cells that also produce the second indicated cytokine. Numbers are graphed below. N=2 per group, data is representative of seven independent experiments. Error bars are SEM. E) Number of OTI CD8 T cells in clone 13 infected or ctrl recipients. Frequency of donor OTI CD8 T cells was measured in the blood at the indicated time points. Absolute number of donor OTI CD8 T cells was measured in the indicated tissues. Numbers above bar graphs indicate the fold increase in donor OTI numbers in ctrl recipients over LCMV clone 13 infected recipients. N=2 per groups, data is representative of seven independent experiments. Error bars are SEM.

functions of donor OTI CD8 T cells transferred to LCMV clone 13 infected recipients were also somewhat compromised. The frequency of donor OTI CD8 T cells that co-produced IFNγ and TNFα was slightly lower in the chronically infected recipients, and IL-2 production was substantially, if not significantly, diminished (Fig 21d). The survival of donor OTI CD8 T cells was also decreased in the chronically infected recipients compared to the controls. The frequency of the donor OTI CD8 T cells in the blood of the control mice remained relatively stable over 30 days, whereas the frequency declined in the blood of the LCMV clone 13 recipients (Fig 21e). More strikingly, the absolute number of donor OTI CD8 T cells was greatly decreased in both lymphoid and non-lymphoid tissues. The difference in recovery of donor OTI CD8 T cells ranged from 6-fold (liver and bone marrow) to 26-fold (spleen), indicating a systemic decrease rather than redistribution of donor OTI CD8 T cells due to differences in migration within the two recipient groups (Fig 21e). Together, these data suggest that, like the direct co-infection model, chronic inflammation alone can impact the differentiation of CD8 T cell responses. More specifically, chronic inflammation appears to be able to repress memory differentiation, preventing the acquisition of memory properties such as CD127 and CD62L expression and IL-2 production.

Chronic inflammation alone does not regulate expression of inhibitory receptors.

Along with the repression of memory differentiation, the expression of inhibitory receptors is a cardinal feature of functional exhaustion. In particular, the high expression of PD-1 has been associated with many human and murine

models of chronic viral infection (14), and of the numerous inhibitory molecules upregulated during chronic LCMV infection, PD-1 is one of the most commonly expressed by exhausted CD8 T cells (160). Recent reports have also suggested that PD-1 expression can be upregulated by γ chain cytokines (228), some of which are elevated during chronic viral infection (data not shown). To determine whether chronic 'inflammation' alone could regulate this property of functional exhaustion, we examined the donor OTI CD8 T cells for PD-1 expression after adoptive transfer to control or LCMV clone 13 infected recipients. At 2 days post adoptive transfer, donor OTI CD8 T cells transferred to both control and chronically infected recipients expressed similar levels of PD-1 (Fig 22a). It should be noted that effector and memory CD8 T cells also express PD-1 after acute infection (162, 163) though the majority of these cells are PD-$1^{int/lo}$ rather than PD-1^{hi} as most exhausted CD8 T cells are (data not shown) (163). Inhibitory receptor expression was also not upregulated by chronic inflammation over time, as donor OTI CD8 T cells in both control and LCMV clone 13 infected recipients retained similar PD-1 expression at d30 post adoptive transfer (Fig 22b). Furthermore, the level of PD-1 expression on the donor cells was lower than on exhausted CD8 T cells specific for LCMV (Fig 22b). Thus, while chronic inflammation alone seems capable of altering some key memory properties of differentiating CD8 T cells, another signal such as antigen may be required to regulate other aspects of T cell dysfunction during chronic viral infection, such as the high expression of inhibitory receptors.

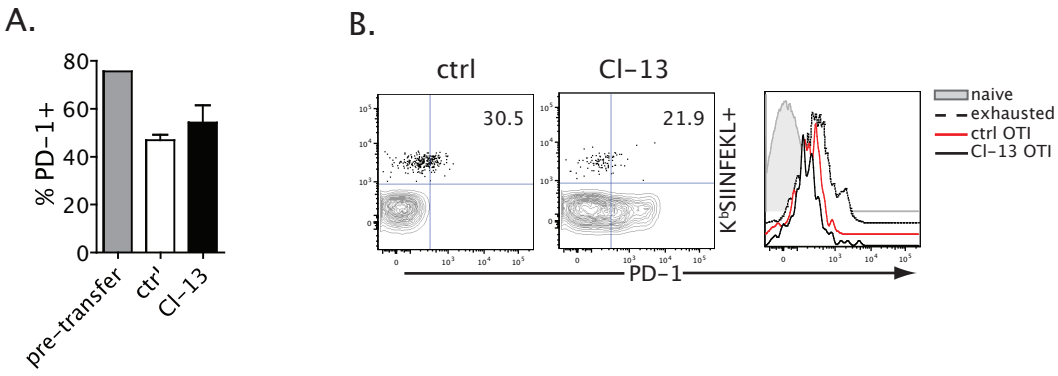

Figure 22. Chronic inflammation alone does not lead to high PD-1 expression. A) PD-1 expression pre- and post-transfer. PD-1 expression on donor OTI CD8 T cells just prior to adoptive transfer to ctrl or LCMV clone 13 infected recipients (pre-transfer), and two days post-transfer in the blood (ctrl, Cl-13). Error bars are SEM. Data is representative of seven independent experiments. B) PD-1 expression 1 month post-transfer in the spleen. Plots are gated on total CD8 T cells. Numbers in plots indicate the percent of donor OTI CD8 T cells that are PD-1$^{int/hi}$. Histograms are gated on tetramer+ CD8 T cells, with the exception of naive. Naive (shaded histogram) is gated on CD44^{lo} CD62L^{hi} cells in ctrl mice. Exhausted (dashed) is gated on DbGP33+ CD8 T cells in LCMV clone 13 infected recipients. Ctrl OTI (red) and Cl-13 OTI (black) are gated on donor OTI CD8 T cells from ctrl and LCMV clone 13 infected recipients, respectively. Data is representative of seven independent experiments.

Different types of chronic inflammation can repress memory CD8 T cell differentiation.

While LCMV clone 13 is a very useful model for different types of chronic viral infection, it is possible that the inflammation induced by this pathogen is unique in its ability to repress memory differentiation. To test whether our results were specific to LCMV, we also used *Toxoplasma gondii* (toxo) as another model of chronic infection. While both LCMV and toxo induce inflammation that is skewed towards Th1, LCMV is primarily driven by type I interferons (92, 229), while toxo induces IL-12 mediated inflammation (230, 231). The adoptive transfer system described previously was used (Fig 21a), but with toxo infected Ly5.1+ recipients as our experimental group. As observed with LCMV clone 13, donor OTI CD8 T cells transferred to toxo infected recipients did not appear to differentiate into memory CD8 T cells, as the cells remained $CD127^{lo}$ (Fig 23a). There was also a drastic difference in the survival of donor OTI CD8 T cells between the control and toxo infected groups. The frequency of donor OTI CD8 T cells was much lower in toxo infected recipients compared to the controls immediately after adoptive transfer, and remained low over 30 days post transfer (Fig 23b). The recovery of donor OTI CD8 T cells from the tissues of toxo infected recipients was also significantly lower in the spleen and liver, and a similar trend was observed in the lymph nodes, indicating that the decrease in donor cell numbers was not simply to due to migration (Fig 23b). The parallels between the LCMV clone 13 model and the toxo model suggest that different types of chronic inflammation (i.e. type I interferon driven vs. IL-12 driven) may

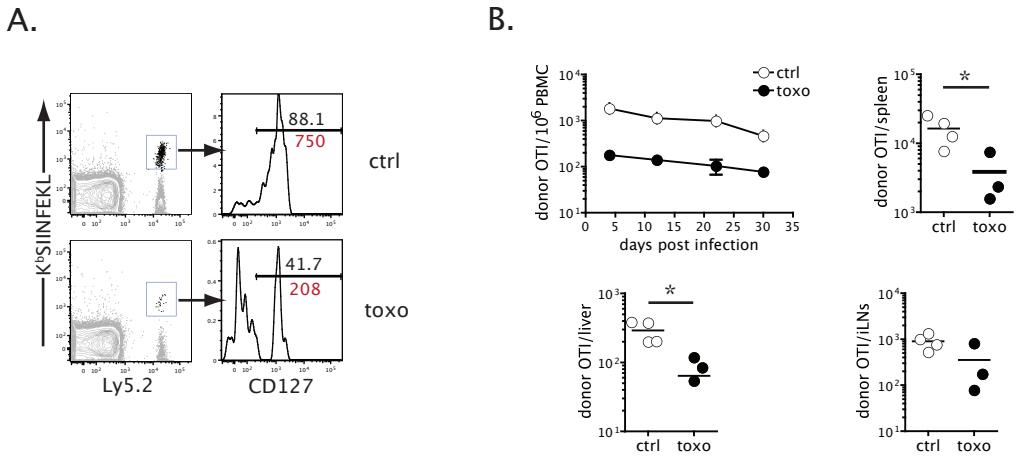

Figure 23. Chronic inflammation during toxoplasma infection represses memory CD8 T cell differentiation. Experimental design outlined in Fig. 21 was used, but with toxoplasma infected recipients instead of LCMV clone 13 infected recipients. A) Phenotype of donor OTI CD8 T cells in ctrl and toxo infected recipients in the blood 18 d.p.i.. Plots are gated on total CD8 T cells, histograms are gated on donor OTI CD8 T cells. Numbers in plots indicate the percent of total CD8 T cells that are KbSIINFEKL+. Black numbers in histograms show percent of donor OTI population that is $CD127^{hi}$. Red numbers show MFI of CD127 expression. B) Number of donor OTI CD8 T cells in ctrl and toxo infected recipients 1 month post-transfer. Frequency of donor OTI CD8 T cells was determined in the blood at the indicated time points. Absolute number of donor OTI CD8 T cells was measured in each of the indicated tissues. N=3-4 per group. For spleen, *p=0.04, for liver, *p=0.02 by Student's t-test. Error bars are SEM. All data are representative of two independent experiments.

repress memory differentiation, and that there may be factors common to different types of chronic infections that can alter memory CD8 T cell differentiation.

Established memory CD8 T cells are plastic but less susceptible to chronic inflammation than differentiating effector CD8 T cells.

Our data thus far indicate that the memory CD8 T cell differentiation pathway can be altered by the inflammation present during chronic infections. It is not clear, however, whether the responsiveness of CD8 T cells to their environment is maintained throughout the memory phase. There is evidence that tissue microenvironments may be able to dictate the properties of memory CD8 T cells (232, 233), and the memory CD8 T cell population continues to differentiate long after the initial infection is cleared (64, 135, 137). To test the plasticity of memory CD8 T cells, we examined whether established memory CD8 T cells could be influenced by chronic inflammation using our adoptive transfer system. $5x10^5$ OTI splenocytes were transferred to naïve recipients, which were challenged with VSV-OVA. At approximately one month p.i., memory OTI CD8 T cells that were generated were transferred to either control Ly5.1+ recipients or Ly5.1+ LCMV clone 13 recipients that had been infected 30 days prior (Fig 24a). The transferred memory OTI CD8 T cells were mostly $CD127^{hi}$, starting to upregulate CD62L and were capable of producing IL-2 (Fig 24b).

At 30 days post transfer, donor OTI CD8 T cells in the control recipients retained high CD127 expression and further increased expression of CD62L (Fig

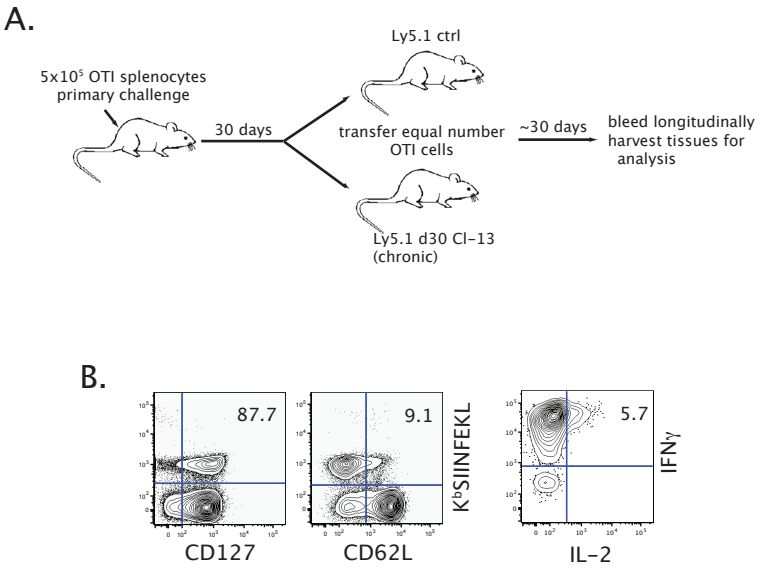

Figure 24. Established memory CD8 T cells are more resistant to alteration by chronic inflammation than effector CD8 T cells. A) Schematic of experiment. 5x10^5 OTI splenocytes were adoptively transferred to naive donor B6 mice and challenged with VSV-OVA. One group of recipient Ly5.1+ B6 recipients was infected with LCMV clone 13 at the same time. Another ctrl recipient group was left naive or infected with VSV-OVA or LCMV Arm. No difference was observed between the ctrl groups. 30 d.p.i., CD8 T cells were purified from the spleens of donor mice and equal numbers of donor OTI CD8 T cells were adoptively transferred to Ly5.1+ LCMV clone 13 infected recipients and Ly5.1+ ctrl recipients. Donor OTI CD8 T cells were analyzed 1 month post-transfer. B) Donor OTI CD8 T cells pre-transfer (30 d.p.i.). Left and middle plots are gated on total CD8 T cells. Numbers in plots indicate the percent of donor OTI CD8 T cells that are positive for each marker. Right plot is gated on donor CD8 T cells. Splenocytes were stimulated with OVA peptide for 5 hrs at 37C. Number indicates the percent of IFNγ+ cells that co-produce IL-2. Data is representative of three independent experiments. (Figure continued on next page.)

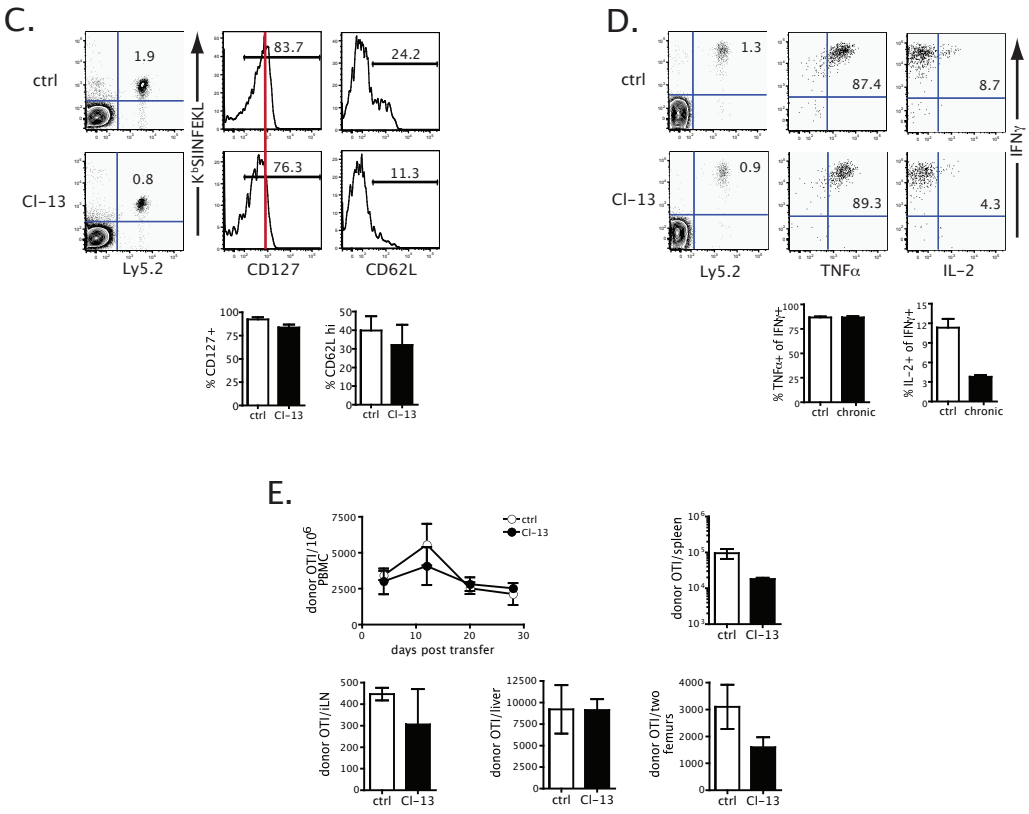

Figure 24 (cont.) Established memory CD8 T cells are more resistant to alteration by chronic inflammation than effector CD8 T cells. C) Phenotype of donor OTI CD8 T cells in the spleen 1 month post-transfer. Plots are gated on total CD8 T cells, histograms are gated on donor OTI cells. Numbers in plots show percent of total CD8 T cells that are donor OTI. Numbers in histograms indicate the percent of donor OTI that are positive for each marker. Numbers are graphed below. N=3 per group. Error bars are SEM. D) Effector function of donor OTI cells. Splenocytes from recipient mice were stimulated with OVA peptide for 5 hrs at 37C. Left plots are gated on total CD8 T cells. Numbers indicate percent of total CD8 T cells that produce IFNγ. Middle and right plots are gated on donor CD8 T cells. Numbers show the percent of IFNγ+ cells that also produce the second indicated cytokine. Numbers are graphed below. N=3 per group, error bars are SEM. E) Number of donor OTI CD8 T cells. Frequency of donor OTI CD8 T cells were measured in the blood at the indicated time points. Absolute number of donor OTI CD8 T cells were determined in each tissue. N=3 per group, error bars are SEM. All data is representative three independent experiments.

24c). The majority of donor OTI CD8 T cells transferred to LCMV clone 13 infected recipients also remained $CD127^{hi}$, though the level of expression was lower than in the control recipients (Fig 24c). The upregulation of CD62L by a memory CD8 T cell population is an indicator of the transition from effector to central memory (68, 135, 143, 144), and though a subset of donor OTI CD8 T cells maintained high CD62L expression in the chronically infected recipients, no further upregulation was observed (Fig 24b,c). Other molecules associated with memory 'maturation', including CD27 and CXCR3, were also not as highly expressed on donor OTI CD8 T cells in chronically infected recipients as compared to controls (data not shown). While TNFα and IFNγ co-production by the donor OTI CD8 T cells did not appear to be compromised, IL-2 and IFNγ co-production did not increase in the LCMV clone 13 infected recipients as it did in the controls (Fig 24d). Unlike donor effector OTI CD8 T cells, the survival of donor memory OTI CD8 T cells did not appear to be greatly affected by chronic inflammation. The frequency of donor OTI CD8 T cells in the blood remained similar and stable over the course of a month post transfer, and the absolute number of donor OTI CD8 T cells did not differ greatly between control and chronically infected groups in both lymphoid and non-lymphoid tissues (Fig 24e). Thus, our data indicate that while established memory CD8 T cells do not appear to be actively altered by low levels of persistent inflammation, the transition from effector memory to central memory seems to be prevented or delayed by prolonged inflammation.

Extent of memory CD8 T cell differentiation alteration is determined by severity of chronic inflammation and differentiation state of the cell.

Thus far, our data indicate that the transition from effector to memory CD8 T cell is highly susceptible to alteration by chronic inflammation, while established memory CD8 T cells retain their memory properties but do not appear to transition from effector to central memory. However, the transferred effector and memory OTI CD8 T cells were likely exposed to different amounts of inflammation. During LCMV clone 13 infection, more virus is present at the early d8 time point than at d30 p.i., at which point the virus is beginning to be cleared from tissues such as the spleen and liver (89). Furthermore, the CD8 T cell response, which peaks at ~d8 p.i., is more functional at this early time point than at the later d30 time point (89). Thus, due to the differences in pathogen burden and effector functions of the immune response, it is likely that both the severity and composition of the inflammation present is different at early (d8) and late (d30) stages of the infection. In order to determine whether our observations were due to the differentiation stage of the donor OTI CD8 T cells or due to the level of chronic inflammation, we adoptively transferred effector OTI CD8 T cells (d8) into recipient mice 30 days post infection with LCMV clone 13 (low inflammation) (Fig 25a). We also transferred memory OTI CD8 T cells (d30) into recipient mice 8 days post infection with LCMV clone 13 (high inflammation) (Fig 26a).

Donor effector OTI CD8 T cells that were transferred into d30 LCMV clone 13 infected recipients unexpectedly did not display a sharp decrease in CD127

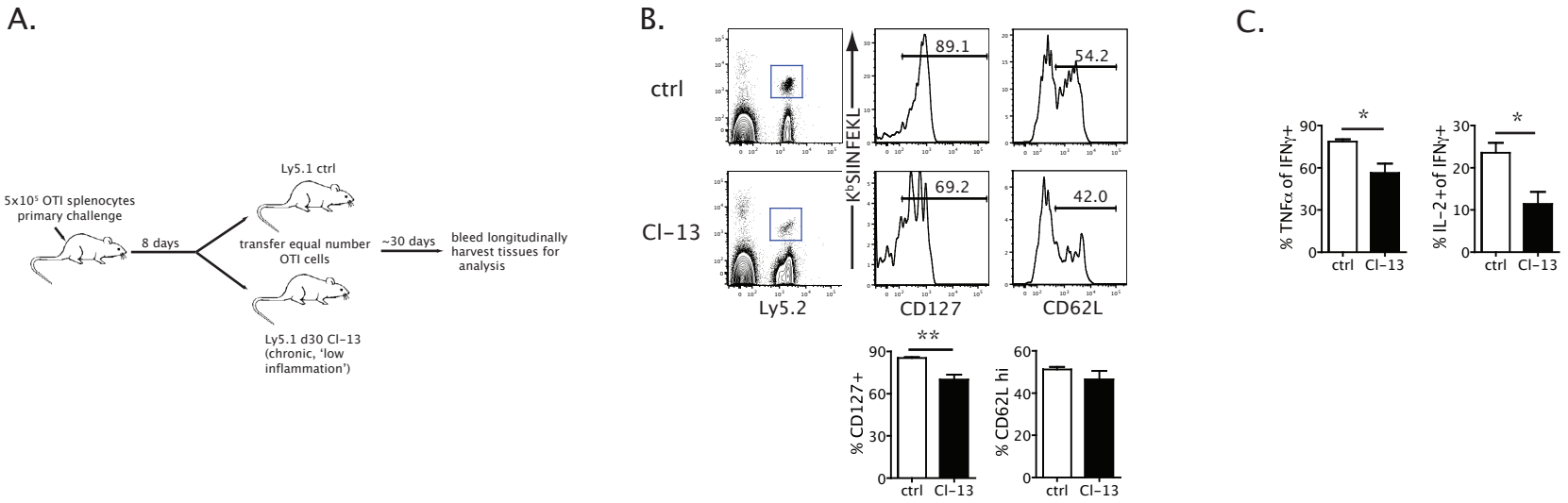

Figure 25. Low levels of chronic inflammation have a less profound impact on effector CD8 T cells differentiating into memory CD8 T cells. A) Schematic of experiment. Experimental design from Fig 21a and 24a was used, except d8 effector OTI CD8 T cells were transferred into recipient mice infected with LCMV clone 13 30 days prior (d8 to 30). Donor OTI CD8 T cells were analyzed one month post-transfer. B) Phenotype of donor OTI CD8 T cells in the spleen. Plots are gated on total CD8 T cells, histograms are gated on donor OTI CD8 T cells. Numbers in histograms show percent of donor OTI CD8 T cells that are positive for each marker. Numbers are graphed below. For CD127, **p=0.002 by Student's t-test. N=4 per group, error bars are SEM. C) Effector function of donor OTI CD8 T cells. Splenocytes from recipient mice were stimulated with OVA peptide for 5 hrs at 37C. Graphs show percent of IFNγ+ that also produce the second indicated cytokine. For TNFα *p=0.05, for IL-2 *p=0.02 by Student's t-test. N=4 per group, error bars are SEM.

expression 30 days post transfer. (Fig 25b). While significant, the fold difference in CD127 expression between the control and chronically infected recipients was not as great as observed during the adoptive transfer to d8 LCMV clone 13 infected recipients (high inflammation) (Fig 21c, 27). There was also little difference in CD62L expression between the two groups (Fig 25b). Cytokine production, however, was still compromised by low levels of inflammation, and donor OTI CD8 T cells from the d30 LCMV clone 13 infected recipients had significantly less TNFα and IL-2 production as compared to the controls (Fig 25c). It should be noted that this data is preliminary, as the experiment has only been performed once. When memory OTI CD8 T cells were transferred to d8 LCMV clone 13 infected recipients (high inflammation), CD127 expression decreased significantly, and CD62L expression did not increase 30 days post-transfer (Fig 26b). Effector functions, however did appear to be greatly compromised. Co-production of IFNγ with either TNFα or IL-2 by the donor OTI CD8 T cells was only slightly decreased in the chronically infected recipients (Fig 26c). These data suggest certain memory CD8 T cell properties, such as high CD127 expression and upregulation of CD62L, are more susceptible to the effects of chronic inflammation in differentiating effector CD8 T cells than in established memory CD8 T cells (Fig 27). Together, our data indicate that CD8 T cell differentiation can be impacted by both high and low levels of chronic inflammation at all stages of the immune response, including the effector to memory transition as well as the effector memory to central memory transition.

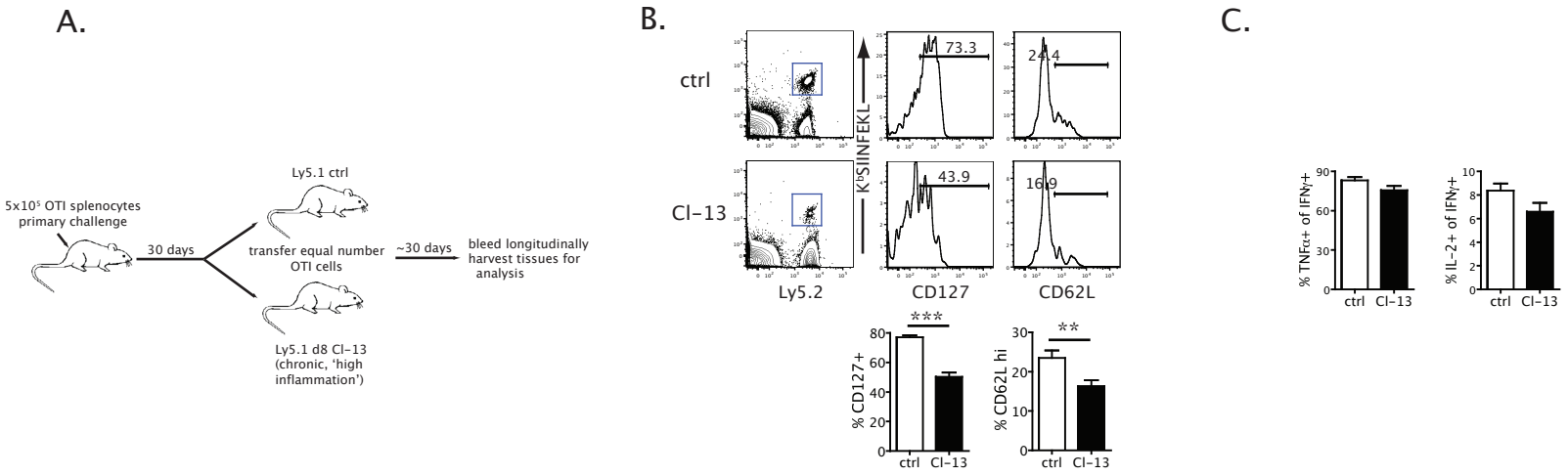

Figure 26. Memory CD8 T cells are more resistant than effector CD8 T cells to high levels of chronic inflammation. A) Schematic of experiment. Experimental design from Fig 21a and 24a were used, except memory OTI CD8 T cells were transferred to recipients at d8 post-LCMV clone 13 infection (d30 to 8). Donor OTI CD8 T cells were analyzed 1 month post-transfer. B) Phenotype of donor OTI CD8 T cells in the spleen. Plots are gated on total CD8 T cells, histograms are gated on donor OTI. Numbers in histograms show percent of donor OTI that are positive for each marker. Numbers are graphed below. For CD127, ***p<0.0001, for CD62L *p=0.001. N=8, error bars are SEM. C) Effector function of donor OTI CD8 T cells. Splenocytes from recipient mice were stimulated with OVA peptide for 5 hrs at 37C. Graphs represent percent of IFNγ+ cells that also produce the second indicated cyokine. N=4, error bars are SEM. All data is representative of two independent experiments.

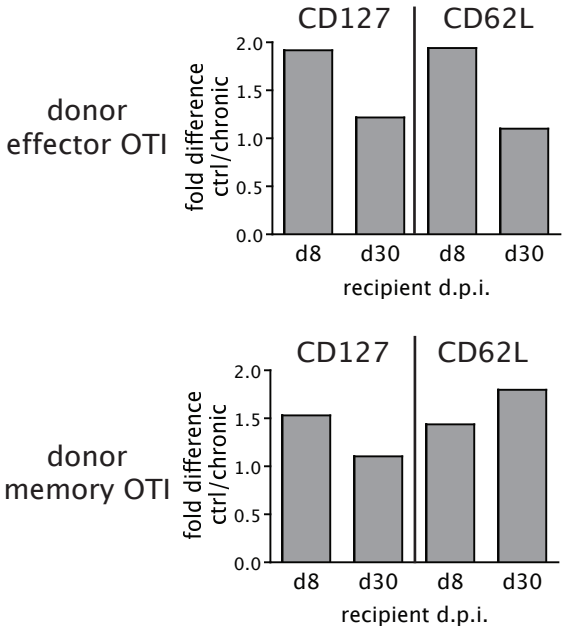

Figure 27. Summary of CD127 and CD62L expression. Graphs show fold difference in marker expression of donor OTI CD8 T cells from ctrl recipients over LCMV clone 13 infected recipients. Top graph shows all experiments performed with donor effector OTI CD8 T cells (d8) transferred to early (d8) or late (d30) time points post-LCMV clone 13 infection of recipients. Bottom graph shows all experiments performed with donor memory OTI CD8 T cells (d30) transferred to early (d8) or late (d30) time points post-LCMV clone 13 infection of recipients. Data is representative of 1-7 independent experiments.

Secondary responses of memory CD8 T cells may be compromised by exposure to chronic inflammation.

Our results thus far indicate that chronic inflammation appears capable of repressing memory CD8 T cell differentiation. As the ability to mount a rapid, potent secondary response is a critical property of functional memory CD8 T cells (73), we examined whether exposure to chronic inflammation could affect this feature. In order to assess the intrinsic potential of the donor CD8 T cells to respond to a secondary challenge, a second round of adoptive transfers were performed (Fig 28a). In brief, experiments were carried out as described in Fig 21a, 24a, 25a and 26a. For each experiment, after a month post transfer, equal numbers of OTI CD8 T cells from the spleens of either LCMV clone 13 infected recipients or control recipients were transferred to new, naïve, Ly5.1+ recipients after CD8 purification by magnetic beads (Fig 28a). These new Ly5.1+ recipients were then challenged with LM-OVA (Fig 28a). Five to six days later, the secondary OTI CD8 T cell responses were analyzed and bacterial burden was measured.

Regardless of the differentiation state of OTI CD8 T cells at the time of the first adoptive transfer or the level of chronic inflammation, OTI CD8 T cells that had been transferred from chronically infected groups did not accumulate as much after secondary challenge as those that had been transferred from the control groups (Fig 28b). Compared to the controls, the difference in magnitude of secondary responses was greatest for effector donor OTI CD8 T cells that had differentiated in the presence of high inflammation (d8 to 8) (Fig 28b). The

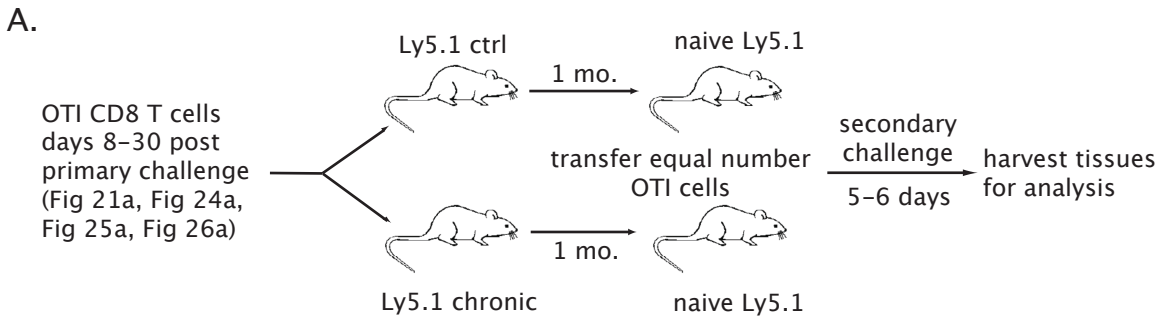

Figure 28. Exposure to chronic inflammation compromises secondary responses. A) Schematic of experiment. 1 month after the transfers described in Fig 21a (d8 to 8), 24a (d30 to 30), 25a (d8 to 30), and 26a (d30 to 8), equal numbers of donor OTI cells from either ctrl or LCMV clone 13 infected recipients were transferred to new naive Ly5.1+ B6 recipients. The new recipients were challenged with LM-OVA, and secondary responses were measured 5-6 days post-challenge. (Figure continued on next page.)

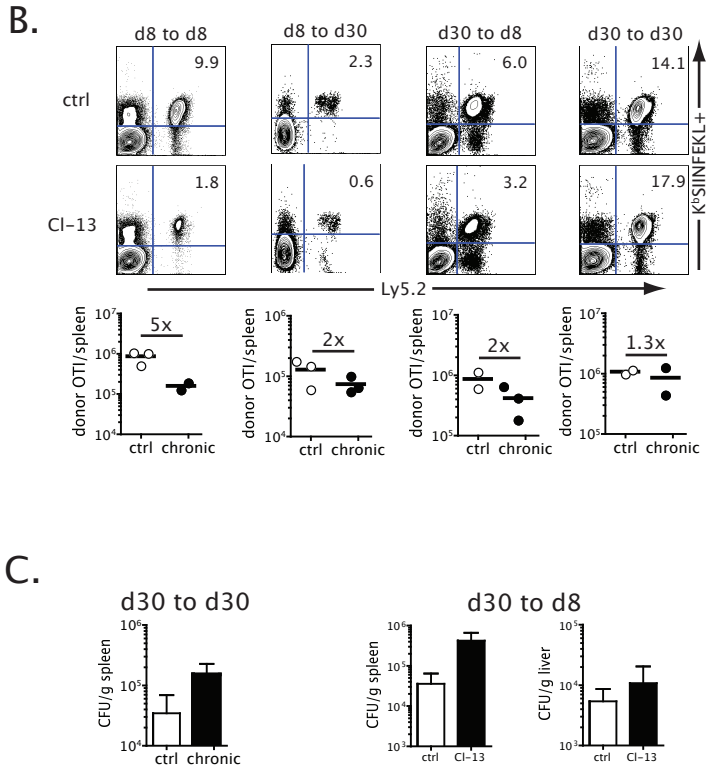

Figure 28 (cont.) Exposure to chronic inflammation compromises secondary responses. B) Accumulation of donor OTI CD8 T cells after in the spleen. Top row shows donor OTI CD8 T cells that rested in the ctrl group for 1 month, bottom row shows donor OTI CD8 T cells that rested in the LCMV clone 13 infected group for 1 month prior to secondary challenge. All plots are gated on total CD8 T cells. Numbers in plots show percent of total CD8 T cells that are donor OTI. Absolute number of donor OTI CD8 T cells are graphed below. Numbers above each graph show fold difference in accumulation between donor OTI CD8 T cells from ctrl vs. LCMV clone 13 infected groups. Data is represenative of 1-2 independent experiments. C) Bacterial burden after secondary challenge. Left graph shows secondary challenge after transfer of memory OTI to d30 LCMV clone 13 infected recipients, middle and right graphs show secondary challenge after transfer of memory OTI to d8 LCMV clone 13 infected recipient. Bacterial burden in each indicated tissue was measured by colony growth on BHI agar at 37C for 24 hrs.

difference was minimal for memory OTI CD8 T cells that had differentiated in a situation of low inflammation (d30 to 30) (Fig 28b). However, preliminary data indicate that these memory OTI CD8 T cells (d30 to 30) were not as efficient in controlling bacterial burden upon secondary challenge (Fig 28c). Memory CD8 T cells that differentiated in a situation of high inflammation (d30 to 8) also appeared to be less protective, although it is difficult to compare the impact that exposure to low vs high inflammation may have had on protective immunity (Fig 28c). Thus, our data suggest that memory differentiation in the presence of chronic inflammation alone can lead to the development of memory CD8 T cells that may provide suboptimal protection.

Discussion

In this study, we describe a role for inflammation in the dysregulation of memory CD8 T cell differentiation during chronic infection. By using an adoptive transfer system with OTI TCR transgenic CD8 T cells that do not recognize any LCMV epitopes, we find that chronic 'inflammation' alone prevented the acquisition of several key memory properties during the effector to memory transition, including the upregulation of CD127 and CD62L, and high IL-2 production, but did not lead to upregulation of inhibitory receptor expression. The conversion of established T_{EM} to T_{CM} could also be prevented by persistent inflammation, although the impact was not as profound as on the effector to memory transition. Both the activation state of the CD8 T cell (effector or T_{EM})

and the level of inflammation (early or late during chronic infection) were important in determining the severity of dysfunction. Regardless of the situation, the cellular changes that occurred in the donor CD8 T cells resulted in impaired secondary responses.

During chronic viral infections, CD8 T cells can become functionally exhausted and undergo altered memory differentiation. Although these two major defects are often observed together (14), there are certain models of chronic viral infection, such as γHV, where altered memory differentiation is uncoupled from loss of effector function and observed alone in dysfunctional CD8 T cells (179, 234). This raises the question as to whether different factors regulate distinct aspects of T cell dysfunction during chronic infection. Our data suggest that chronic inflammation alone may regulate altered memory CD8 T cell differentiation, and that additional signals are required to mediate the loss of effector function (89, 90) and upregulation of inhibitory receptors (23) that are characteristic of functional exhaustion. While expression of memory markers such as CD127 is downregulated by chronic inflammation, it is not yet clear whether this has any functional consequences for the cell. The expression of CD127 can be regulated by both IL-7 (235, 236) and TCR stimulation (63). However, in human T cells, TCR signaling causes a more 'permanent' downregulation of CD127 than IL-7 signaling (237). Furthermore, forced expression of CD127 does not impart any selective advantage in the differentiation of memory CD8 T cells after acute infection (95), suggesting that expression of this marker does not ensure memory differentiation. Conversely,

this suggests that loss of CD127 may not necessarily deter a precursor cell from acquiring memory properties, such as the ability to use IL-7 for homeostasis. In an LCMV model of chronic viral infection, as this work and other studies have shown, exhausted virus-specific CD8 T cells do not utilize IL-7 and IL-15 efficiently, both *in vitro* and *in vivo* (Chapter 3) (174, 180). Exhausted virus-specific CD8 T cells appear to become 'addicted' to antigen (Chapter 3), and it is not yet clear whether chronic stimulation through the TCR as well as prolonged inflammation are necessary to limit reliance on homeostatic cytokines and induce antigenic 'addiction'. In the future, it will be important to determine whether defective memory differentiation regulated by chronic inflammation alone is comparable to defective memory differentiation of exhausted CD8 T cells that have been stimulated by both persistent inflammation and antigen.

Effector cells and T_{EM} are both populations that evolve over time, as memory precursors differentiate to long-lived memory and T_{EM} convert to T_{CM} (66). Adoptive transfers performed with effector CD8 T cells and effector memory CD8 T cells indicated that effector memory CD8 T cells, while plastic, were less affected by chronic inflammation than effector CD8 T cells, regardless of the severity of inflammation. This implies a cell-intrinsic resistance to alteration. One possibility is that memory CD8 T cells do not express or downregulate expression of the receptors that are required to recognize input from the chronic inflammatory environment. Another possibility is that gene expression patterns in memory CD8 T cells are more firmly imprinted than in effector CD8 T cells. Differentiation and cell fate decisions are accompanied by

epigenetic control and chromatin remodeling in numerous different cell types. Epigenetic marks are used extensively in multiple different cells during development (238, 239). Chromatin remodeling can also occur in more mature cell types, including CD4 T cells within the Th1/Th2 paradigm (37). Epigenetic regulation leads to heritable changes in gene expression and can reduce (or increase) the accessibility to certain genes by cell-extrinsic signals (37, 238, 239). Memory CD8 T cells acquire histone modifications that aid their function (115), and rapid production of IL-2 is mediated by DNA demethylation (114). These epigenetic markers of differentiation could potentially render memory CD8 T cells partially refractory to the effects of chronic inflammation. Still, memory CD8 T cells are not completely unresponsive to persistent inflammation, as conversion from T_{EM} to T_{CM} is halted or delayed. Thus, the question remains as to whether memory CD8 T cells generated after an acute infection ever reach a terminally differentiated, non-plastic state. It will be important to determine whether memory properties, such as the ability to mount robust secondary responses, always remain open to the influence of inflammatory signals, as this could have critical implications for the function of previously established memory populations in patients infected with pathogenic chronic infections.

The specific signals present in chronic inflammation that alter memory differentiation have not yet been elucidated. Multiple factors that are generated during chronic infection have been shown to be critical in determining the functionality of T cells, including IL-10 (183, 184) and IL-21 (186-188). After an acute infection, a ‘third signal’, such as IL-12 or type I IFN, is required for proper

activation and subsequent memory differentiation (116, 117). Other non-cytokine signaling pathways, such as those mediated by MyD88, are also important for the proper differentiation (29). It is not yet clear whether chronic inflammation is actively altering memory differentiation by the presence of a particular signal(s), or whether memory differentiation is not occurring because of the lack of a particular signal in the chronic inflammatory environment. One possible factor that may be lacking is CD4 help. CD4 responses, like CD8 responses, are impaired during chronic viral infections, including LCMV (156, 240, 241), HIV (161, 242-244), and HCV (245, 246). CD4 T cell exhaustion could potentially lead to insufficient CD4 help for CD8 T cells during chronic viral infection, which could contribute to memory defects. Our data shows that the memory differentiation defects induced by chronic inflammation are not unique to LCMV clone 13 infection, as the IL-12-driven inflammatory environment of toxoplasma also leads to similar defects (i.e. CD127 expression, survival) in memory differentiation. This indicates that there may be similarities across multiple different types of chronic infection that induce comparable dysfunction in CD8 T cells, and may be an efficient method of determining the signals are important in the regulation of memory differentiation. In the future, it will be interesting to determine whether other types of chronic inflammation, including Th2- and Th17-driven inflammation, as well as inflammation resulting from autoimmune disease or cancer, also result in functional exhaustion and altered memory differentiation.

In summary, we have identified a role for persistent inflammation in the regulation of memory differentiation during chronic viral infection. Effector CD8 T

cells undergoing memory differentiation do not acquire key memory properties, such as the high expression of homeostatic cytokine receptors and ability to mount robust secondary responses to a challenge. The transition from T_{EM} to T_{CM} is also prevented or delayed by prolonged inflammation. The differentiation state of the CTL as well as the severity of inflammation both determined the extent of dysfunction. Furthermore, our observations were not limited to chronic LCMV infection, as the inflammatory environment of toxoplasma also led to defects in memory differentiation, indicating that this may be a common feature of different types of chronic inflammation.

Chapter 5

Discussion

Overview of results.

Chronic infections are a severe burden on society, limiting quality of life, threatening public health and incurring a great cost for many of the world's economies. Hundreds of millions of people around the world are afflicted with one or more chronic viral infections such as HIV (7), HCV and HBV (8), as well as bacterial infections such as TB (247) and parasitic infections such as malaria (248). While we have made great strides in understanding the pathogenesis of these infections, we still do not have sufficient knowledge of the impact these infections have on the immune system in order to restore natural clearance of the pathogen. CD8 T cell exhaustion is a feature of many different chronic viral infections (14), and likely contributes to the poor control of these diseases. While functional exhaustion has been observed in many different infections and model systems (14), we still do not fully understand how differentiation of exhausted CD8 T cells is regulated or what the underlying molecular mechanisms are. The work in this thesis attempts to further our understanding of functional exhaustion by addressing three different aspects: transcriptional regulation, homeostatic mechanisms and the regulation of the differentiation process by host-pathogen interactions.

While functional exhaustion is known to be a common feature of many chronic infections, the molecular mechanisms underlying this state have not been elucidated. Gene expression studies suggest that functional exhaustion is a differentiation process that is guided by a unique transcriptional program (158). The first chapter focuses on the transcriptional repressor Blimp-1 and the role that it plays in regulating functional exhaustion. We found that Blimp-1 is upregulated in exhausted antigen-specific CD8 T cells as compared to naïve, effector and memory CD8 T cells generated after acute infection, and that Blimp-1 expression correlated with severity of dysfunction. Conditional deletion of Blimp-1 led to decreased expression of inhibitory receptors and upregulation of memory markers such as CD127 and CD62L. However, by controlling the number of alleles that were deleted, we found that some Blimp-1 expression was required for the acquisition and/or maintenance of effector function, especially cytotoxicity. The temporal aspect of Blimp-1 expression also appeared to be crucial, as CD8 T cells lacking Blimp-1 prior to activation had higher viral titers than wt mice earlier during chronic infection. Thus, our study suggested that Blimp-1 may be acting as a molecular rheostat, regulating effector function and memory differentiation at low or intermediate levels, while promoting functional exhaustion at high levels. While we describe an important role for Blimp-1, it is likely that other transcription factors also play a critical role in the regulation of functional exhaustion. Insight into the actions of Blimp-1 as well as other transcription factors in promoting (or repressing) T cell dysfunction could lead to new, potential therapeutic targets in the treatment of chronic infections.

Aside from the loss of effector function and upregulation of inhibitory receptors, dysfunctional CD8 T cells also follow a path of altered memory differentiation. Unlike memory CD8 T cells that differentiate after acute infection, exhausted CD8 T cells express low levels of CD127 and CD122, and they do not use the cytokines IL-7 and IL-15 for their homeostasis. Despite this, however, they persist long-term in chronically infected hosts. In the third chapter we address this apparent paradox by performing adoptive transfers using a variant clone 13 strain that does not present the GP33 peptide. With this variant virus, we found that rather than requiring a cytokine, growth factor or some unknown cell-cell contact, exhausted CD8 T cells require virus-derived antigen for their maintenance. Furthermore, dysfunctional antigen-specific CD8 T cells also undergo a rapid, antigen-driven division that is distinct from the slow, steady, cytokine-driven homeostatic proliferation that is characteristic of memory CD8 T cells. It is interesting to note that the features associated with functional exhaustion, such as loss of effector function and high amounts of inhibitory receptors, become less severe after the control of viremia (155). However, the memory defects of these dysfunctional CD8 T cells remain after virus is controlled (174), though it is not known how long these defects persist. HAART treatment of HIV infected patients can lead to a sharp decrease in viral load, which could compromise the survival signal that HIV-specific CD8 T cells may require (249, 250). It will be important to determine whether any sort of protective immunity can be maintained in after the maintenance signal for exhausted antigen-specific CD8 T cell population is diminished or removed.

While the functional exhaustion and altered memory differentiation often occur simultaneously during chronic viral infection, there are infection models, such as γHV, in which these two states can present separately (179, 234). This suggests that different features of T cell dysfunction may be regulated by distinct signals. Furthermore, co-infections involving pathogens that are not overtly immunosuppressive may repress immune responses to heterologous infections, suggesting that signals other than persistent antigen may be playing a role (220-224). The extrinsic signals that regulate the differentiation of exhausted CD8 T cells are not well known, and in the Chapter 4, we focus on the role of chronic inflammation in regulating T cell dysfunction. We find that inflammation alone leads to the repression of memory differentiation, but does not lead to the upregulation of inhibitory receptor expression. The effector to memory transition was more sensitive to alteration by chronic 'inflammation' than the T_{EM} to T_{CM} transition, and inflammation present at early time points (d8) after infection appeared to have a more severe impact on differentiation than the inflammatory environment at later time points (d30). Furthermore, memory repression induced by persistent inflammation was not limited to our model system of chronic infection, as the inflammatory environment of chronic toxoplasma infection also led to a similar results. In summary, chronic inflammation leads to repression of memory differentiation, but other signals, such as persistent antigen, may be necessary to induce the loss of effector function and upregulation of inhibitory receptors associated with functional exhaustion. Our work could have important implications for the development of cellular responses in the case of co-

infections. Chronic infections could not only impact the differentiation of new memory populations after infection or vaccination, but they could also erode protective immunity established by prior infections or vaccinations. A study by Puissant-Lubrano et al show that in ART-treated HIV patients, memory responses to childhood vaccination against smallpox were lost, but memory was maintained against the BCG vaccine, in which antigen persists (251). While the study did not distinguish between a loss of immunity due to 'inflammation' and direct loss of T cells via HIV infection, it still raises the possibility that protective immunity to heterologous infections may be compromised by ongoing chronic infections.

Regulation of T cell exhaustion and memory differentiation.

After an acute infection, inflammation can affect the antigen-specific CD8 T cell population by regulating both the size of the clonal burst after activation (252) and the differentiation of terminally differentiated effectors vs. memory precursors (60). While antigen load is a strong correlate for the severity of T cell dysfunction during chronic viral infection (89) and may directly cause functional exhaustion (219), it is not clear how prolonged inflammation impacts the regulation of functional exhaustion during chronic viral infection. Our data from Chapter 4 show that inflammation can repress aspects of memory differentiation, but other signals such as antigen may be necessary to induce some of the hallmarks of functional exhaustion, such as loss of effector function and high expression of inhibitory markers. Thus, in order to generate a functional

population of true memory CD8 T cells, rest from both antigen and inflammation appear to be necessary.

The mechanism by which inflammation alters or represses the development of memory is not yet clear. Further work needs to be done to determine the specific signals that are regulating this process, although it is intriguing that expression of two transcription factors, Tbet and Blimp-1, that play an important role in the fate decision between memory precursors and terminally differentiated effector CD8 T cells during acute infection can be mediated by inflammatory signals. Tbet expression can be regulated by IL-12 in CD8 T cells (60), and the differential expression of Tbet between memory precursors and terminally differentiated effector CD8 T cells can be controlled by both strength of TCR and severity of inflammation (60, 61). Likewise, Blimp-1 expression in T cells is regulated by cytokine signals such as IL-2 and IL-4 (197, 198), and in B cells can be regulated by a variety of factors, including cytokines and TLR ligands (253). Furthermore, we show in Chapter 2 that Blimp-1 plays an important role in repressing memory differentiation in exhausted CD8 T cells during chronic viral infection (Fig 29). It will be important to determine whether different types of persistent inflammation (i.e. Th2, Th17, cancer microenvironments, etc.) can also alter memory CD8 T cell differentiation, and whether there are changes to the transcriptional profiles of the antigen-specific CD8 T cells that are common to different inflammatory settings. By using this approach, it may be possible to identify common factors within different settings of prolonged inflammation that can regulate the expression of certain

transcription factors such as Blimp-1, and thus alter memory differentiation. Determining the specific signal(s) that may regulate memory CD8 T cell differentiation during chronic infection could provide new targets for future therapies.

Heterogeneity of the exhausted CD8 T cell population.

After an acute infection, memory CD8 T cells form a heterogeneous population composed of different subsets. While the lineage relationship between the T_{EM} and T_{CM} subsets remains under contention, it is clear that they are delineated by cells with differences in phenotype (134, 137), function, such as the ability to mount secondary responses and produce IL-2 (135, 140, 142), and tissue migration (134, 135, 138) Another aspect of memory heterogeneity comes from a stem cell model of self-renewal (130-132), where only a subset of memory CD8 T cells would potentially be recruited to homeostatically proliferate and thus maintain the entire population. The work from Chapter 3 suggests that heterogeneity may also exist in the long-term exhausted CD8 T cell population. Our data show that the antigen-driven 'homeostatic' mechanism of rapid division gives rise to two distinct populations – one that divides to antigen, and one that does not (Fig 19). It is possible that all exhausted antigen-specific CD8 T cells do eventually divide, but it is also possible that this profile indicates differences in the properties of the cells themselves, including proliferative potential or tissue migration. The divided vs. undivided profile may reflect subpopulations that are 'tuned' to antigen differently, and thus possess different proliferative capabilities.

During chronic viral infection, immunodominance hierarchies amongst epitope specificities can change (89, 217, 218). With an antigen-driven method of maintenance, immunodominance shifts could reflect differences in proliferative capacity between TCR specificities. In addition, as the virus that drives this division post-viremia likely resides in tissue reservoirs such as the kidney and brain (89), those cells that divide may represent a subpopulation that can more efficiently migrate to peripheral tissues, in parallel to the different homing abilities of T_{EM} and T_{CM} cells (134, 138). Subpopulations of cells with different functional capacities have been described within the total exhausted CD8 T cell population, such as the PD-1^{hi} and PD-1^{int} subsets (163), or those delineated by the expression of different numbers of inhibitory receptors (Fig 2 and 8) (160). It will be interesting to examine whether these subsets correlate with cells that are capable of dividing upon a 'homeostatic' antigen encounter (Fig 29). In the future, it will be important to determine whether this potential heterogeneity correlates to differences in the ability to provide protective immunity and how it may be manipulated to improve therapeutic strategies.

As a whole, exhausted antigen-specific CD8 T cell populations do not require IL-7 or IL-15 for their maintenance during chronic viral infections (174, 178, 179). However, it is difficult to rule out the possibility that a small subset of exhausted CD8 T cells may be able to utilize IL-7 and IL-15 rather than antigen for their homeostasis. In Fig 18, a small population of donor cells persists in the absence of cognate antigen up to 10 weeks after adoptive transfer. In human chronic infections such as HIV, HBV, HCV, CMV and EBV, antigen-specific CD8

T cells express low levels of IL-7Rα compared to cells specific for influenza or RSV (14, 180). Furthermore, IL-7 and IL-15 signaling appears to be impaired in CMV-specific CD8 T cells . However, treatment of HIV infected patients with IL-7 leads to a transient increase in cell cycle entry by HIV-specific CD8 T cells (254). This suggests that despite low CD127 expression, some HIV-specific CD8 T cells were able to respond to IL-7, though not as efficiently as CD8 T cells specific for acute infections such as flu (254). Also, CD127 expression on antigen-specific CD8 T cells during chronic LCMV infection can increase over time after the control of viremia (data not shown), and prolonged treatment of HIV patients with HAART can also lead to the restoration of CD8 T cell populations with high CD127 expression (177, 255). Due to the potential for decreased protective immunity with an antigen-based mechanism of homeostasis after treatment of infections such as HIV, it will be important to examine whether there are small subsets of antigen-specific CD8 T cells that can be maintained by cytokine and whether exhausted CD8 T cells can ever regain their ability to use IL-7 and IL-15 for homeostasis.

Reversal of T cell dysfunction.

One of the ultimate goals of studying functional exhaustion is to uncover pathways that will permanently restore productive immune responses during chronic infections, and thus mediate natural clearance of a pathogen and provide optimal protective immunity. This goal relies on the assumption that functional exhaustion can be reversed, and that the state of T cell dysfunction is not a

terminally differentiated one. Considerable evidence suggests that manipulation of multiple pathways, including inhibitory receptors (159-162, 171, 256) and cytokines (183, 184), can lead to the 'revival' of established exhausted CD8 T cells at late time points during chronic viral infection. It is not known, however, whether the 'revival' of dysfunctional T cells is transient or permanent. Simply resting established, exhausted CD8 T cells (post d15 p.i.) from antigen and inflammation is not sufficient to restore proper function (S. Blackburn data not shown) (174). However, data from our lab show that removing antigen-specific CD8 T cells from a chronically infected environment at earlier time points (d8 p.i.) could rescue the cells from becoming functionally exhausted (S. Blackburn data not shown). Also, only early HAART treatment of HIV patients seems to be effective at establishing of $CD127^{hi}$ HIV-specific CD8 T cells (177). Together, the data suggests that functional exhaustion is progressive, and that it may become more 'permanent' or more difficult to reverse over time. One possible reason for this may be that gradual changes in gene expression occur as exhausted CD8 T cells progress in their differentiation (158). In many other cell types, chromatin remodeling plays a key role in limiting the plasticity of a particular lineage, whether during development of hematopoeisis (239) or within the Th1/Th2 paradigm (37). As shown in Chapter 2, while expression of the transcriptional repressor Blimp-1 in effector CD8 T cells (d8 p.i.) is comparable between acute and chronic infection, by d15 p.i. expression during chronic infection is much higher than during acute infection (Fig 1). This sharp upregulation correlates with the onset of other markers of functional exhaustion,

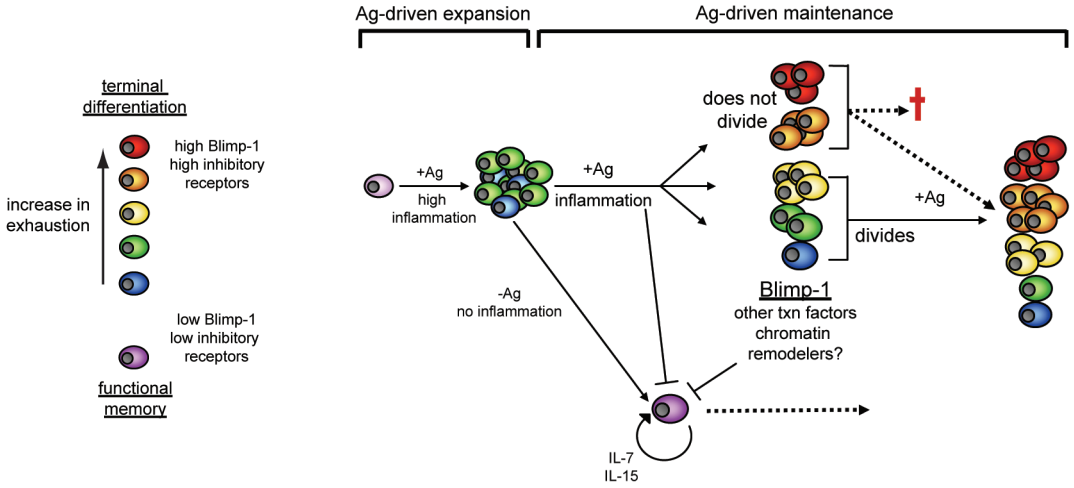

Figure 28. Progression of functional exhaustion and population heterogeneity during chronic viral infection. Model of data. Upon chronic infection, naive CD8 T cells expand into a pool of effector CD8 T cells that still have the capacity to differentiate into functional memory CD8 T cells if rested from antigen and inflammation. After the effector phase persistent antigen and inflammation regulate changes in the gene expression pattern of antigen-specific CD8 T cells, including the upregulaton of Blimp-1, as well as the high expression of inhibitory receptors. Inflammation alone also prevents memory differentiation through an unknown mechanism. By altering the transcriptional and epigenetic landscape, the functionally exhausted fate may become 'imprinted' into virus-specific CD8 T cells, rendering them resistant to memory differentiation without extrinsic manipulation (i.e. IL-10/IL-10R or PD-1/PD-L blockades). Heterogeneity in the exhausted CD8 T cell pool leads to a subpopulation of virus-specific CD8 T cells that undergoes rapid, antigen-driven proliferation during the maintenance phase and a subpopulation that does not. It is unclear whether the 'undivided' subset is capable of eventually dividing, or whether it dies. The model above assumes that those cells that are more exhausted will compose the population that is 'undivided'. It is also unclear whether a very small population of cytokine-dependent memory CD8 T cells is generated during chronic viral infection.

such as the high expression of inhibitory receptors (158, 162). Blimp-1 is known to exert its repressive role by epigenetic modification through the recruitment of co-repressors (257), HDACs (258) and methyltransferases (259, 260). Thus, it is possible that the accumulation of effector and memory defects as well as the 'imprinting' of the exhausted cell fate during chronic viral infection may occur through chromatin remodeling mediated by differential expression of transcription factors such as Blimp-1 (Fig 29). It will be important to determine whether modification of Blimp-1 expression or other transcription factors after onset of exhaustion can lead to a lasting reversal of the T cell dysfunction that is present during chronic viral infection. It will also be important to determine whether current methods to improve exhausted CD8 T cell function, such as blockade of the PD-1/PD-L pathway which leads to selective expansion rather than redifferentiation (163), can induce molecular changes that lead to a permanently improved population of antigen-specific CD8 T cells.

In sum, the work in this thesis has attempted to elucidate different pathways by which functional exhaustion is regulated during chronic viral infection, and to further our knowledge of the biology of these cells. We hope that by understanding how cell-extrinsic and –intrinsic factors regulate the process, we can provide new, potential therapeutic targets and design strategies that can address different aspects of T cell dysfunction. Also, by understanding the behavior of functionally exhausted CD8 T cells, we can better manipulate

them in order to lead to a productive immune response that will ultimately control infection and provide optimal protective immunity.

References

1. Thucydides, C. R., Finley JH. 1951. *The complete writings of Thucydides: The Peloponnesian War*. New York: Modern Library.

2. Plotkin, S. A. 2005. Vaccines: past, present and future. *Nat Med*.

3. Riedel, S. 2005. Edward Jenner and the history of smallpox and vaccination. *BUMC Proceedings* 18:21-25.

4. Pasteur, L. 1881. On the germ theory. *Science* 2:420-422.

5. Masopust, D., V. Vezys, E. J. Wherry, and R. Ahmed. 2007. A brief history of CD8 T cells. *Eur J Immunol* 37:S103-S110.

6. Burnet, F. M. 1962. *Natural History of Infectious Disease*. University Press, Cambridge.

7. UNAIDS/WHO. 2007. Global summary of the AIDS epidemic, December 2007.

8. Rehermann, B., and M. Nascimbeni. 2005. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* 5:215-229.

9. Douek, D. C., M. Roederer, and R. A. Koup. 2009. Emerging Concepts in the Immunopathogenesis of AIDS*. *Annual Review of Medicine* 60:471.

10. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283:857-860.

11. Lechner, F., D. K. H. Wong, P. R. Dunbar, R. Chapman, R. T. Chung, P. Dohrenwend, G. Robbins, R. Phillips, P. Klenerman, and B. D. Walker. 2000. Analysis of Successful Immune Responses in Persons Infected with Hepatitis C Virus. *J. Exp. Med.* 191:1499-1512.

12. Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho. 1999. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* 189:991-998.

13. Seder, R. A., P. A. Darrah, and M. Roederer. 2008. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* 8:247-258.

14. Shin, H., and E. J. Wherry. 2007. CD8 T cell dysfunction during chronic viral infection. *Current Opinion in Immunology* 19:408-415.

15. Davis, M. M., and Y.-H. Chien. 2008. T Cell Antigen Receptors. In *Fundamental Immunology*, 6th ed. W. E. Paul, ed. Lippincott Williams & Wilkins.

16. McHeyzer-Williams, M. 2008. B Lymphocyte Biology. In *Fundamental Immunology*, 6th ed. W. E. Paul, ed. Lippincott Williams & Wilkins.

17. Ahmed, R., and D. Gray. 1996. Immunological Memory and Protective Immunity: Understanding Their Relation. *Science* 272:54-60.

18. Bhandoola, A., H. von Boehmer, H. T. Petrie, and J. C. Zuniga-Pflucker. 2007. Commitment and Developmental Potential of Extrathymic and Intrathymic T Cell Precursors: Plenty to Choose from. *Immunity* 26:678-689.

19. von Boehmer, H., A. Iannis, G. Fotini, A. Orly, H. Loralee, A. Irina, J. Elmar, G. Fabio, and K. Ludger. 2003. Thymic selection revisited: how essential is it? *Immunological Reviews* 191:62-78.

20. Singer, A., S. Adoro, and J.-H. Park. 2008. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat Rev Immunol* 8:788-801.

21. Alena, M. G., and J. B. Michael. 2006. Central tolerance: good but imperfect. *Immunological Reviews* 209:290-296.

22. Surh, C. D., and J. Sprent. 2008. Homeostasis of Naive and Memory T Cells. *Immunity* 29:848-862.

23. Crawford, A., and E. J. Wherry. 2009. The diversity of costimulatory and inhibitory receptor pathways and the regulation of antiviral T cell responses. *Current Opinion in Immunology* 21:179-186.

24. Curtsinger, J. M., C. S. Schmidt, A. Mondino, D. C. Lins, R. M. Kedl, M. K. Jenkins, and M. F. Mescher. 1999. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J Immunol* 162:3256-3262.

25. Kolumam, G. A., S. Thomas, L. J. Thompson, J. Sprent, and K. Murali-Krishna. 2005. Type I interferons act directly on CD8 T cells to allow clonal

expansion and memory formation in response to viral infection. *J Exp Med* 202:637-650.

26. Mescher, M. F., J. M. Curtsinger, P. Agarwal, K. A. Casey, M. Gerner, C. D. Hammerbeck, F. Popescu, and Z. Xiao. 2006. Signals required for programming effector and memory development by CD8+ T cells. *Immunological Reviews* 211:81-92.

27. Murphy, K. M., and S. L. Reiner. 2002. The lineage decisions of helper T cells. *Nat Rev Immunol* 2:933-944.

28. Zhou, L., M. M. W. Chong, and D. R. Littman. 2009. Plasticity of CD4+ T Cell Lineage Differentiation. *Immunity* 30:646-655.

29. Rahman, A. H., W. Cui, D. F. Larosa, D. K. Taylor, J. Zhang, D. R. Goldstein, E. J. Wherry, S. M. Kaech, and L. A. Turka. 2008. MyD88 plays a critical T cell-intrinsic role in supporting CD8 T cell expansion during acute lymphocytic choriomeningitis virus infection. *J Immunol* 181:3804-3810.

30. Gelman, A. E., D. F. LaRosa, J. Zhang, P. T. Walsh, Y. Choi, J. O. Sunyer, and L. A. Turka. 2006. The Adaptor Molecule MyD88 Activates PI-3 Kinase Signaling in CD4+ T Cells and Enables CpG Oligodeoxynucleotide-Mediated Costimulation. *Immunity* 25:783-793.

31. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136:2348-2357.

32. Scott, P., P. Natovitz, R. L. Coffman, E. Pearce, and A. Sher. 1988. Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J Exp Med* 168:1675-1684.

33. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201:233-240.

34. Harrington, L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L. Murphy, K. M. Murphy, and C. T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6:1123-1132.

35. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y.-H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.

36. Ouyang, W., J. K. Kolls, and Y. Zheng. 2008. The Biological Functions of T Helper 17 Cell Effector Cytokines in Inflammation. *Immunity* 28:454-467.

37. Wilson, C. B., E. Rowell, and M. Sekimata. 2009. Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol* 9:91-105.

38. Yang, X. O., R. Nurieva, G. J. Martinez, H. S. Kang, Y. Chung, B. P. Pappu, B. Shah, S. H. Chang, K. S. Schluns, S. S. Watowich, X.-H. Feng, A. M. Jetten, and C. Dong. 2008. Molecular Antagonism and Plasticity of Regulatory and Inflammatory T Cell Programs. *Immunity* 29:44-56.

39. Bennett, S. R., F. R. Carbone, F. Karamalis, R. A. Flavell, J. F. Miller, and W. R. Heath. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393:478-480.

40. Schoenberger, S. P., R. E. M. Toes, E. I. H. van der Voort, R. Offringa, and C. J. M. Melief. 1998. T-cell help for cytotoxic T Lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393:480-483.

41. Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T- helper and a T-killer cell. *Nature* 393:474-478.

42. Vinuesa, C. G., S. G. Tangye, B. Moser, and C. R. Mackay. 2005. Follicular B helper T cells in antibody responses and autoimmunity. *Nat Rev Immunol* 5:853-865.

43. Williams, M. A., and M. J. Bevan. 2007. Effector and Memory CTL Differentiation. *Annual Review of Immunology* 25:171.

44. Woodland, D. L., and R. W. Dutton. 2003. Heterogeneity of CD4(+) and CD8(+) T cells. *Curr Opin Immunol* 15:336-342.

45. Mosmann, T., and S. Sad. 1997. Functions of CD8 T-cell subsets secreting different cytokine patterns. *Seminars in Immunology* 9:87-92.

46. Anichini, A., R. Mortarini, L. Romagnoli, P. Baldassari, A. Cabras, C. Carlo-Stella, A. M. Gianni, and M. Di Nicola. 2006. Skewed T-cell differentiation in patients with indolent non-Hodgkin lymphoma reversed by ex vivo T-cell culture with {gamma}c cytokines. *Blood* 107:602-609.

47. Maggi, E., M. G. Giudizi, R. Biagiotti, F. Annunziato, R. Manetti, M. P. Piccinni, P. Parronchi, S. Sampognaro, L. Giannarini, and G. Zuccati. 1994. Th2-like CD8+ T cells showing B cell helper function and reduced cytolytic activity in human immunodeficiency virus type 1 infection. *J. Exp. Med.* 180:489-495.

48. Accapezzato, D., V. Francavilla, M. Paroli, M. Casciaro, L. V. Chircu, A. Cividini, S. Abrignani, M. U. Mondelli, and V. Barnaba. 2004. Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. *J Clin Invest* 113:963-972.

49. Iezzi, G., A. Boni, E. Degl'Innocenti, M. Grioni, M. T. S. Bertilaccio, and M. Bellone. 2006. Type 2 Cytotoxic T Lymphocytes Modulate the Activity of Dendritic Cells Toward Type 2 Immune Responses. *J Immunol* 177:2131-2137.

50. Moretta, A. 1997. Molecular Mechanisms in Cell-Mediated Cytotoxicity. *Cell* 90:13-18.

51. Ye, Z., C. Tan, S. Xu, B. Zhan, X. Zhang, T. Moyana, J. Yang, and J. Xiang. 2007. Type CD8+ T cells are superior to type 2 CD8+ T cells in Tumor Immunotherapy due to their efficient cytotoxicity, prolonged survival and type I immune modulation. *Cellular & Molecular Immunology* 4:277-285.

52. Hamada, H., M. d. l. L. Garcia-Hernandez, J. B. Reome, S. K. Misra, T. M. Strutt, K. K. McKinstry, A. M. Cooper, S. L. Swain, and R. W. Dutton. 2009. Tc17, a Unique Subset of CD8 T Cells That Can Protect against Lethal Influenza Challenge. *J Immunol* 182:3469-3481.

53. Kondo, T., H. Takata, F. Matsuki, and M. Takiguchi. 2009. Cutting Edge: Phenotypic Characterization and Differentiation of Human CD8+ T Cells Producing IL-17. *J Immunol* 182:1794-1798.

54. Intlekofer, A. M., A. Banerjee, N. Takemoto, S. M. Gordon, C. S. DeJong, H. Shin, C. A. Hunter, E. J. Wherry, T. Lindsten, and S. L. Reiner. 2008. Anomalous Type 17 Response to Viral Infection by CD8+ T Cells Lacking T-bet and Eomesodermin. *Science* 321:408-411.

55. Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 8:177-187.

56. Butz, E. A., and M. J. Bevan. 1998. Massive Expansion of Antigen-Specific CD8+ T Cells during an Acute Virus Infection. *Immunity* 8:167-175.

57. Blattman, J. N., Antia, R., Soudive, D.J.D., Wang, X., Kaech, S.M., Murali-Krishan, K., Altman, J.D., Ahmed, R. 2002. Estimating the Precursor Frequency of Naive Antigen-specific CD8 T Cells. *J Exp Med* 195:657-664.

58. Kaech, S. M., and R. Ahmed. 2001. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat. Immunol.* 2:415-422.

59. van Stipdonk, M. J. B., E. E. Lemmens, and S. P. Schoenberger. 2001. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol* 2:423-429.

60. Joshi, N. S., W. Cui, A. Chandele, H. K. Lee, D. R. Urso, J. Hagman, L. Gapin, and S. M. Kaech. 2007. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27:281-295.

61. Sarkar, S., V. Kalia, W. N. Haining, B. T. Konieczny, S. Subramaniam, and R. Ahmed. 2008. Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J Exp Med* 205:625-640.

62. Huster, K. M., V. Busch, M. Schiemann, K. Linkemann, K. M. Kerksiek, H. Wagner, and D. H. Busch. 2004. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proc Natl Acad Sci U S A* 101:5610-5615.

63. Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh, and R. Ahmed. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 4:1191-1198.

64. Kaech, S. M., S. Hemby, E. Kersh, and R. Ahmed. 2002. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell* 111:837-851.

65. Rubinstein, M. P., N. A. Lind, J. F. Purton, P. Filippou, J. A. Best, P. A. McGhee, C. D. Surh, and A. W. Goldrath. 2008. IL-7 and IL-15 differentially regulate CD8+ T-cell subsets during contraction of the immune response. *Blood* 112:3704-3712.

66. Kaech, S. M., and E. J. Wherry. 2007. Heterogeneity and Cell-Fate Decisions in Effector and Memory CD8+ T Cell Differentiation during Viral Infection. *Immunity* 27:393-405.

67. Manjunath, N., P. Shankar, J. Wan, W. Weninger, M. A. Crowley, K. Hieshima, T. A. Springer, X. Fan, H. Shen, J. Lieberman, and U. H. von Andrian. 2001. Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. *J. Clin. Invest.* 108:871-878.

68. Badovinac, V. P., K. A. N. Messingham, A. Jabbari, J. S. Haring, and J. T. Harty. 2005. Accelerated CD8+ T-cell memory and prime-boost response after dendritic-cell vaccination. *Nat Med* 11:748-756.

69. Kamimura, D., and M. J. Bevan. 2007. Naive CD8+ T cells differentiate into protective memory-like cells after IL-2 anti IL-2 complex treatment in vivo. *J. Exp. Med.* 204:1803-1812.

70. Stemberger, C., K. M. Huster, M. Koffler, F. Anderl, M. Schiemann, H. Wagner, and D. H. Busch. 2007. A Single Naive CD8+ T Cell Precursor Can Develop into Diverse Effector and Memory Subsets. *Immunity* 27:985-997.

71. Peixoto, A., C. Evaristo, I. Munitic, M. Monteiro, A. Charbit, B. Rocha, and H. Veiga-Fernandes. 2007. CD8 single-cell gene coexpression reveals three different effector types present at distinct phases of the immune response. *J. Exp. Med.* 204:1193-1205.

72. Chang, J. T., V. R. Palanivel, I. Kinjyo, F. Schambach, A. M. Intlekofer, A. Banerjee, S. A. Longworth, K. E. Vinup, P. Mrass, J. Oliaro, N. Killeen, J. S. Orange, S. M. Russell, W. Weninger, and S. L. Reiner. 2007. Asymmetric T Lymphocyte Division in the Initiation of Adaptive Immune Responses. *Science* 315:1687-1691.

73. Wherry, E. J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. *J Virol* 78:5535-5545.

74. Traub, E. 1935. A filterable virus recovered from white mice. . *Science* 81:298-299.

75. Barton, L. L., and N. J. Hyndman. 2000. Lymphocytic Choriomeningitis Virus: Reemerging Central Nervous System Pathogen. *Pediatrics* 105:e35-.

76. Jurg, B., H. Hans, M. Z. Rolf, and A. C. Gerald. 1986. Induction or prevention of immunopathological disease by cloned cytotoxic T cell lines specific for lymphocytic choriomeningitis virus. *Eur J Immunol* 16:387-393.

77. Jamieson, B. D., L. D. Butler, and R. Ahmed. 1987. Effective clearance of a persistent viral infection requires cooperation between virus-specific Lyt2+ T cells and nonspecific bone marrow-derived cells. *J Virol* 61:3930-3937.

78. Ahmed, R., B. D. Jamieson, and D. D. Porter. 1987. Immune therapy of a persistent and disseminated viral infection. *J Virol* 61:3920-3929.

79. Matloubian, M., R. J. Concepcion, and R. Ahmed. 1994. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol* 68:8056-8063.

80. Asano, M. S., and R. Ahmed. 1996. CD8 T cell memory in B cell-deficient mice. *J Exp Med* 183:2165-2174.

81. Masopust, D., K. Murali-Krishna, and R. Ahmed. 2007. Quantitating the Magnitude of the Lymphocytic Choriomeningitis Virus-Specific CD8 T-Cell Response: It Is Even Bigger than We Thought. *J. Virol.* 81:2002-2011.

82. Kotturi, M. F., B. Peters, F. Buendia-Laysa, Jr., J. Sidney, C. Oseroff, J. Botten, H. Grey, M. J. Buchmeier, and A. Sette. 2007. The CD8+ T-Cell Response to Lymphocytic Choriomeningitis Virus Involves the L Antigen: Uncovering New Tricks for an Old Virus. *J. Virol.* 81:4928-4940.

83. van der Most, R., K. Murali-Krishna, L. Whitton, C. Oseroff, J. Alexander, S. Southwood, J. Sidney, R. Chesnut, A. Sette, and R. Ahmed. 1998. Identification of D^b and K^b-Restricted Subdominant Cytotoxic T-Cell Responses in Lymphocytic Choriomeningitis Virus-Infected Mice. *Vir* 240:158-167.

84. Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. *J Exp Med* 160:521-540.

85. Matloubian, M., S. R. Kolhekar, T. Somasundaram, and R. Ahmed. 1993. Molecular determinants of macrophage tropism and viral persistence: importance of single amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus. *J Virol* 67:7340-7349.

86. Matloubian, M., T. Somasundaram, S. R. Kolhekar, R. Selvakumar, and R. Ahmed. 1990. Genetic basis of viral persistence: single amino acid change in the viral glycoprotein affects ability of lymphocytic choriomeningitis virus to persist in adult mice. *J Exp Med* 172:1043-1048.

87. Sevilla, N., S. Kunz, A. Holz, H. Lewicki, D. Homann, H. Yamada, K. P. Campbell, J. C. de la Torre, and M. B. A. Oldstone. 2000. Immunosuppression and Resultant Viral Persistence by Specific Viral Targeting of Dendritic Cells. *J. Exp. Med.* 192:1249-1260.

88. Sevilla, N., D. B. McGavern, C. Teng, S. Kunz, and M. B. Oldstone. 2004. Viral targeting of hematopoietic progenitors and inhibition of DC maturation as a dual strategy for immune subversion. *J Clin Invest* 113:737-745.

89. Wherry, E. J., J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77:4911-4927.

90. Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed. 1998. Viral immune evasion due to persistence of activated T cells without effector function. *J. Exp. Med.* 188:2205-2213.

91. Zhou, S., R. Ou, L. Huang, and D. Moskophidis. 2002. Critical role for perforin-, Fas/FasL-, and TNFR1-mediated cytotoxic pathways in down-regulation of antigen-specific T cells during persistent viral infection. *J Virol* 76:829-840.

92. Ou, R., S. Zhou, L. Huang, and D. Moskophidis. 2001. Critical role for alpha/beta and gamma interferons in persistence of lymphocytic choriomeningitis virus by clonal exhaustion of cytotoxic T cells. *J Virol* 75:8407-8423.

93. Hughes, P. D., G. T. Belz, K. A. Fortner, R. C. Budd, A. Strasser, and P. Bouillet. 2008. Apoptosis Regulators Fas and Bim Cooperate in Shutdown of Chronic Immune Responses and Prevention of Autoimmunity. *Immunity* 28:197-205.

94. Weant, A. E., R. D. Michalek, I. U. Khan, B. C. Holbrook, M. C. Willingham, and J. M. Grayson. 2008. Apoptosis Regulators Bim and Fas Function Concurrently to Control Autoimmunity and CD8+ T Cell Contraction. *Immunity* 28:218-230.

95. Hand, T. W., M. Morre, and S. M. Kaech. 2007. Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. *Proc Natl Acad Sci U S A* 104:11730-11735.

96. Barber, D. L., E. J. Wherry, and R. Ahmed. 2003. Cutting edge: rapid in vivo killing by memory CD8 T cells. *J Immunol* 171:27-31.

97. Cho, B. K., C. Wang, S. Sugawa, H. N. Eisen, and J. Chen. 1999. Functional differences between memory and naive CD8 T cells. *Proc Natl Acad Sci U S A* 96:2976-2981.

98. Lalvani, A., R. Brookes, S. Hambleton, W. J. Britton, A. V. Hill, and A. J. McMichael. 1997. Rapid effector function in CD8+ memory T cells. *J Exp Med* 186:859-865.

99. Liu, F., and J. L. Whitton. 2005. Cutting edge: re-evaluating the in vivo cytokine responses of CD8+ T cells during primary and secondary viral infections. *J Immunol* 174:5936-5940.

100. Selin, L. K., M. Y. Lin, K. A. Kraemer, D. M. Pardoll, J. P. Schneck, S. M. Varga, P. A. Santolucito, A. K. Pinto, and R. M. Welsh. 1999. Attrition of T Cell Memory: Selective Loss of LCMV Epitope Specific Memory CD8 T Cells following Infections with Heterologous Viruses. *Immunity* 11:733-742.

101. Selin, L. K., K. Vergilis, R. M. Welsh, and S. R. Nahill. 1996. Reduction of otherwise remarkably stable virus-specific cytotoxic T lymphocyte memory by heterologous viral infections. *J. Exp. Med.* 183:2489-2499.

102. Vezys, V., A. Yates, K. A. Casey, G. Lanier, R. Ahmed, R. Antia, and D. Masopust. 2009. Memory CD8 T-cell compartment grows in size with immunological experience. *Nature* 457:196-199.

103. Khanolkar, A., M. J. Fuller, and A. J. Zajac. 2004. CD4 T Cell-Dependent CD8 T Cell Maturation. *J Immunol* 172:2834-2844.

104. Sun, J. C., and M. J. Bevan. 2003. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* 300:339-342.

105. Sun, J. C., M. A. Williams, and M. J. Bevan. 2004. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat Immunol* 5:927-933.

106. Bourgeois, C., B. Rocha, and C. Tanchot. 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science* 297:2060-2063.

107. Janssen, E. M., N. M. Droin, E. E. Lemmens, M. J. Pinkoski, S. J. Bensinger, B. D. Ehst, T. S. Griffith, D. R. Green, and S. P. Schoenberger. 2005. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature* 434:88-93.

108. Williams, M. A., A. J. Tyznik, and M. J. Bevan. 2006. Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* 441:890-893.

109. Badovinac, V. P., K. A. N. Messingham, T. S. Griffith, and J. T. Harty. 2006. TRAIL Deficiency Delays, but Does Not Prevent, Erosion in the Quality of "Helpless" Memory CD8 T Cells. *J Immunol* 177:999-1006.

110. Sacks, J. A., and M. J. Bevan. 2008. TRAIL Deficiency Does Not Rescue Impaired CD8+ T Cell Memory Generated in the Absence of CD4+ T Cell Help. *J Immunol* 180:4570-4576.

111. Bachmann, M. F., K. Schwarz, P. Wolint, E. Meijerink, S. Martin, V. Manolova, and A. Oxenius. 2004. Cutting Edge: Distinct Roles for T Help and CD40/CD40 Ligand in Regulating Differentiation of Proliferation-Competent Memory CD8+ T Cells. *J Immunol* 173:2217-2221.

112. Marzo, A. L., V. Vezys, K. D. Klonowski, S.-J. Lee, G. Muralimohan, M. Moore, D. F. Tough, and L. Lefrancois. 2004. Fully Functional Memory CD8 T Cells in the Absence of CD4 T Cells. *J Immunol* 173:969-975.

113. Shedlock, D. J., and H. Shen. 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 300:337-339.

114. Northrop, J. K., R. M. Thomas, A. D. Wells, and H. Shen. 2006. Epigenetic Remodeling of the IL-2 and IFN-{gamma} Loci in Memory CD8 T Cells Is Influenced by CD4 T Cells. *J Immunol* 177:1062-1069.

115. Northrop, J. K., A. D. Wells, and H. Shen. 2008. Cutting Edge: Chromatin Remodeling as a Molecular Basis for the Enhanced Functionality of Memory CD8 T Cells. *J Immunol* 181:865-868.

116. Xiao, Z., K. A. Casey, S. C. Jameson, J. M. Curtsinger, and M. F. Mescher. 2009. Programming for CD8 T Cell Memory Development Requires IL-12 or Type I IFN. *J Immunol* 182:2786-2794.

117. Pearce, E. L., and H. Shen. 2007. Generation of CD8 T Cell Memory Is Regulated by IL-12. *J Immunol* 179:2074-2081.

118. Tanchot, C., F. Lemonnier, B. Perarnau, A. Freitas, and B. Rocha. 1997. Differential requirements for survival and proliferation of CD8 naive or memory cells. *Science* 276:2057-2062.

119. Lau, L. L., B. D. Jamieson, T. Somasundaram, and R. Ahmed. 1994. Cytotoxic T-cell memory without antigen. *Nature* 369:648-652.

120. Murali-Krishna, K., L. L. Lau, S. Sambhara, F. Lemonnier, J. Altman, and R. Ahmed. 1999. Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* 286:1377-1381.

121. Tough, D. F., P. Borrow, and J. Sprent. 1996. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science* 272:1947-1950.

122. Zhang, X., S. Sun, I. Hwang, D. F. Tough, and J. Sprent. 1998. Potent and Selective Stimulation of Memory-Phenotype CD8+ T Cells In Vivo by IL-15. *Immunity* 8:591-599.

123. Schluns, K. S., and L. Lefrancois. 2003. Cytokine control of memory T-cell development and survival. *Nat Rev Immunol* 3:269-279.

124. Ku, C. C., M. Murakami, A. Sakamoto, J. Kappler, and P. Marrack. 2000. Control of homeostasis of CD8+ memory T cells by opposing cytokines. *Science* 288:675-678.

125. Schluns, K. S., W. C. Kieper, S. C. Jameson, and L. Lefrancois. 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat. Immunol.* 1:426-432.

126. Grayson, J. M., A. J. Zajac, J. D. Altman, and R. Ahmed. 2000. Cutting Edge: Increased Expression of Bcl-2 in Antigen-Specific Memory CD8+ T Cells. *J Immunol* 164:3950-3954.

127. Goldrath, A. W., P. V. Sivakumar, M. Glaccum, M. K. Kennedy, M. J. Bevan, C. Benoist, D. Mathis, and E. A. Butz. 2002. Cytokine requirements for acute and basal homeostatic proliferation of naive and memory CD8+ T cells. *J. Exp. Med.* 195:1515-1522.

128. Becker, T. C., E. J. Wherry, D. Boone, K. Murali-Krishna, R. Antia, A. Ma, and R. Ahmed. 2002. Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J. Exp. Med.* 195:1541-1548.

129. Becker, T. C., S. M. Coley, E. J. Wherry, and R. Ahmed. 2005. Bone marrow is a preferred site for homeostatic proliferation of memory CD8 T cells. *J Immunol* 174:1269-1273.

130. Zhang, Y., G. Joe, E. Hexner, J. Zhu, and S. G. Emerson. 2005. Host-reactive CD8+ memory stem cells in graft-versus-host disease. *Nat Med* 11:1299-1305.

131. Gattinoni, L., X.-S. Zhong, D. C. Palmer, Y. Ji, C. S. Hinrichs, Z. Yu, C. Wrzesinski, A. Boni, L. Cassard, L. M. Garvin, C. M. Paulos, P. Muranski,

and N. P. Restifo. 2009. Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nat Med* 15:808-813.

132. Luckey, C. J., D. Bhattacharya, A. W. Goldrath, I. L. Weissman, C. Benoist, and D. Mathis. 2006. Memory T and memory B cells share a transcriptional program of self-renewal with long-term hematopoietic stem cells. *Proc Natl Acad Sci U S A* 103:3304-3309.

133. Heffner, M., and D. T. Fearon. 2007. Loss of T cell receptor-induced Bmi-1 in the KLRG1(+) senescent CD8(+) T lymphocyte. *Proc Natl Acad Sci U S A* 104:13414-13419.

134. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.

135. Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 4:225-234.

136. Marzo, A. L., K. D. Klonowski, A. Le Bon, P. Borrow, D. F. Tough, and L. Lefrancois. 2005. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat Immunol* 6:793-799.

137. Hikono, H., J. E. Kohlmeier, S. Takamura, S. T. Wittmer, A. D. Roberts, and D. L. Woodland. 2007. Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. *J. Exp. Med.* 204:1625-1636.

138. Masopust, D., V. Vezys, A. L. Marzo, and L. Lefrancois. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 291:2413-2417.

139. Bachmann, M. F., P. Wolint, K. Schwarz, P. Jager, and A. Oxenius. 2005. Functional Properties and Lineage Relationship of CD8+ T Cell Subsets Identified by Expression of IL-7 Receptor {alpha} and CD62L. *J Immunol* 175:4686-4696.

140. Bachmann, M. F., P. Wolint, K. Schwarz, and A. Oxenius. 2005. Recall Proliferation Potential of Memory CD8+ T Cells and Antiviral Protection. *J Immunol* 175:4677-4685.

141. Klonowski, K. D., A. L. Marzo, K. J. Williams, S.-J. Lee, Q.-M. Pham, and L. Lefrancois. 2006. CD8 T Cell Recall Responses Are Regulated by the

Tissue Tropism of the Memory Cell and Pathogen. *J Immunol* 177:6738-6746.

142. Roberts, A. D., K. H. Ely, and D. L. Woodland. 2005. Differential contributions of central and effector memory T cells to recall responses. *J Exp Med* 202:123-133.

143. Sarkar, S., V. Teichgraber, V. Kalia, A. Polley, D. Masopust, L. E. Harrington, R. Ahmed, and E. J. Wherry. 2007. Strength of Stimulus and Clonal Competition Impact the Rate of Memory CD8 T Cell Differentiation. *J Immunol* 179:6704-6714.

144. Bouneaud, C., Z. Garcia, P. Kourilsky, and C. Pannetier. 2005. Lineage relationships, homeostasis, and recall capacities of central- and effector-memory CD8 T cells in vivo. *J Exp Med* 201:579-590.

145. Obar, J. J., K. M. Khanna, and L. Lefrancois. 2008. Endogenous Naive CD8+ T Cell Precursor Frequency Regulates Primary and Memory Responses to Infection. *Immunity* 28:859-869.

146. Sallusto, F., and A. Lanzavecchia. 2001. Exploring pathways for memory T cell generation. *J Clin Invest* 108:805-806.

147. Wirth, T. C., N.-L. L. Pham, J. T. Harty, and V. P. Badovinac. 2009. High initial frequency of TCR-transgenic CD8 T cells alters inflammation and pathogen clearance without affecting memory T cell function. *Molecular Immunology* In Press, Corrected Proof.

148. Intlekofer, A. M., N. Takemoto, E. J. Wherry, S. A. Longworth, J. T. Northrup, V. R. Palanivel, A. C. Mullen, C. R. Gasink, S. M. Kaech, J. D. Miller, L. Gapin, K. Ryan, A. P. Russ, T. Lindsten, J. S. Orange, A. W. Goldrath, R. Ahmed, and S. L. Reiner. 2005. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol* 6:1236-1244.

149. Intlekofer, A. M., N. Takemoto, C. Kao, A. Banerjee, F. Schambach, J. K. Northrop, H. Shen, E. J. Wherry, and S. L. Reiner. 2007. Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells. *J. Exp. Med.* 204:2015-2021.

150. Kallies, A., E. D. Hawkins, G. T. Belz, D. Metcalf, M. Hommel, L. M. Corcoran, P. D. Hodgkin, and S. L. Nutt. 2006. Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance. *Nat Immunol* 7:466-474.

151. Martins, G. A., L. Cimmino, M. Shapiro-Shelef, M. Szabolcs, A. Herron, E. Magnusdottir, and K. Calame. 2006. Transcriptional repressor Blimp-1 regulates T cell homeostasis and function. *Nat Immunol* 7:457-465.

152. Ichii, H., A. Sakamoto, Y. Kuroda, and T. Tokuhisa. 2004. Bcl6 acts as an amplifier for the generation and proliferative capacity of central memory CD8+ T cells. *J Immunol* 173:883-891.

153. Manders, P. M., P. J. Hunter, A. I. Telaranta, J. M. Carr, J. L. Marshall, M. Carrasco, Y. Murakami, M. J. Palmowski, V. Cerundolo, S. M. Kaech, R. Ahmed, and D. T. Fearon. 2005. BCL6b mediates the enhanced magnitude of the secondary response of memory CD8+ T lymphocytes. *Proc Natl Acad Sci U S A* 102:7418-7425.

154. Cannarile, M. A., N. A. Lind, R. Rivera, A. D. Sheridan, K. A. Camfield, B. B. Wu, K. P. Cheung, Z. Ding, and A. W. Goldrath. 2006. Transcriptional regulator Id2 mediates CD8+ T cell immunity. *Nat Immunol* 7:1317-1325.

155. Fuller, M. J., A. Khanolkar, A. E. Tebo, and A. J. Zajac. 2004. Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. *J Immunol* 172:4204-4214.

156. Fuller, M. J., and A. J. Zajac. 2003. Ablation of CD8 and CD4 T cell responses by high viral loads. *J Immunol* 170:477-486.

157. Moskophidis, D., F. Lechner, H. Pircher, and R. M. Zinkernagel. 1993. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells [published erratum appears in Nature 1993 Jul 15;364(6434):262] [see comments]. *Nature* 362:758-761.

158. Wherry, E. J., S.-J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed. 2007. Molecular Signature of CD8+ T Cell Exhaustion during Chronic Viral Infection. *Immunity* 27:670-684.

159. Nakamoto, N., H. Cho, A. Shaked, K. Olthoff, M. E. Valiga, M. Kaminski, E. Gostick, D. A. Price, G. J. Freeman, E. J. Wherry, and K.-M. Chang. 2009. Synergistic Reversal of Intrahepatic HCV-Specific CD8 T Cell Exhaustion by Combined PD-1/CTLA-4 Blockade. *PLoS Pathog* 5:e1000313.

160. Blackburn, S. D., H. Shin, W. N. Haining, T. Zou, C. J. Workman, A. Polley, M. R. Betts, G. J. Freeman, D. A. A. Vignali, and E. J. Wherry. 2009. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 10:29-37.

161. Kaufmann, D. E., D. G. Kavanagh, F. Pereyra, J. J. Zaunders, E. W. Mackey, T. Miura, S. Palmer, M. Brockman, A. Rathod, A. Piechocka-Trocha, B. Baker, B. Zhu, S. Le Gall, M. T. Waring, R. Ahern, K. Moss, A. D. Kelleher, J. M. Coffin, G. J. Freeman, E. S. Rosenberg, and B. D. Walker. 2007. Upregulation of CTLA-4 by HIV-specific CD4+ T cells correlates with disease progression and defines a reversible immune dysfunction. *Nat Immunol* 8:1246-1254.

162. Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439:682-687.

163. Blackburn, S. D., H. Shin, G. J. Freeman, and E. J. Wherry. 2008. Selective expansion of a subset of exhausted CD8 T cells by aPD-L1 blockade. *Proc Natl Acad Sci U S A* 105:15016-15021.

164. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. R. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443:350-354.

165. Sharpe, A. H., E. J. Wherry, R. Ahmed, and G. J. Freeman. 2007. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 8:239-245.

166. Urbani, S., B. Amadei, D. Tola, M. Massari, S. Schivazappa, G. Missale, and C. Ferrari. 2006. PD-1 Expression in Acute Hepatitis C Virus (HCV) Infection Is Associated with HCV-Specific CD8 Exhaustion. *J. Virol.* 80:11398-11403.

167. Boni, C., P. Fisicaro, C. Valdatta, B. Amadei, P. Di Vincenzo, T. Giuberti, D. Laccabue, A. Zerbini, A. Cavalli, G. Missale, A. Bertoletti, and C. Ferrari. 2007. Characterization of Hepatitis B Virus (HBV)-Specific T-Cell Dysfunction in Chronic HBV Infection. *J. Virol.* 81:4215-4225.

168. Velu, V., S. Kannanganat, C. Ibegbu, L. Chennareddi, F. Villinger, G. J. Freeman, R. Ahmed, and R. R. Amara. 2007. Elevated Expression Levels of Inhibitory Receptor Programmed Death 1 on Simian Immunodeficiency Virus-Specific CD8 T Cells during Chronic Infection but Not after Vaccination. *J. Virol.* 81:5819-5828.

169. Nakamoto, N., D. E. Kaplan, J. Coleclough, Y. Li, M. E. Valiga, M. Kaminski, A. Shaked, K. Olthoff, E. Gostick, D. A. Price, G. J. Freeman, E. J. Wherry, and K.-M. Chang. 2008. Functional Restoration of HCV-

Specific CD8 T Cells by PD-1 Blockade Is Defined by PD-1 Expression and Compartmentalization. *Gastroenterology* 134:1927-1937.e1922.

170. Radziewicz, H., C. C. Ibegbu, M. L. Fernandez, K. A. Workowski, K. Obideen, M. Wehbi, H. L. Hanson, J. P. Steinberg, D. Masopust, E. J. Wherry, J. D. Altman, B. T. Rouse, G. J. Freeman, R. Ahmed, and A. Grakoui. 2007. Liver-Infiltrating Lymphocytes in Chronic Human Hepatitis C Virus Infection Display an Exhausted Phenotype with High Levels of PD-1 and Low Levels of CD127 Expression. *J. Virol.* 81:2545-2553.

171. Velu, V., K. Titanji, B. Zhu, S. Husain, A. Pladevega, L. Lai, T. H. Vanderford, L. Chennareddi, G. Silvestri, G. J. Freeman, R. Ahmed, and R. R. Amara. 2009. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature* 458:206-210.

172. Petrovas, C., J. P. Casazza, J. M. Brenchley, D. A. Price, E. Gostick, W. C. Adams, M. L. Precopio, T. Schacker, M. Roederer, D. C. Douek, and R. A. Koup. 2006. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J. Exp. Med.* 203:2281-2292.

173. Jones, R. B., L. C. Ndhlovu, J. D. Barbour, P. M. Sheth, A. R. Jha, B. R. Long, J. C. Wong, M. Satkunarajah, M. Schweneker, J. M. Chapman, G. Gyenes, B. Vali, M. D. Hyrcza, F. Y. Yue, C. Kovacs, A. Sassi, M. Loutfy, R. Halpenny, D. Persad, G. Spotts, F. M. Hecht, T.-W. Chun, J. M. McCune, R. Kaul, J. M. Rini, D. F. Nixon, and M. A. Ostrowski. 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J. Exp. Med.* 205:2763-2779.

174. Wherry, E. J., D. L. Barber, S. M. Kaech, J. N. Blattman, and R. Ahmed. 2004. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci U S A* 101:16004-16009.

175. Lang, K. S., M. Recher, A. A. Navarini, N. L. Harris, M. Lohning, T. Junt, H. C. Probst, H. Hengartner, and R. M. Zinkernagel. 2005. Inverse correlation between IL-7 receptor expression and CD8 T cell exhaustion during persistent antigen stimulation. *Eur J Immunol* 35:738-745.

176. Fuller, M. J., D. A. Hildeman, S. Sabbaj, D. E. Gaddis, A. E. Tebo, L. Shang, P. A. Goepfert, and A. J. Zajac. 2005. Cutting edge: emergence of CD127high functionally competent memory T cells is compromised by high viral loads and inadequate T cell help. *J Immunol* 174:5926-5930.

177. Sabbaj, S., S. L. Heath, A. Bansal, S. Vohra, J. M. Kilby, A. J. Zajac, and P. A. Goepfert. 2007. Functionally Competent Antigen-Specific CD127hi Memory CD8+ T Cells Are Preserved Only in HIV-Infected Individuals

Receiving Early Treatment. *The Journal of Infectious Diseases* 195:108-117.

178. Shin, H., S. D. Blackburn, J. N. Blattman, and E. J. Wherry. 2007. Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. *J. Exp. Med.* 204:941-949.

179. Obar, J. J., S. G. Crist, E. K. Leung, and E. J. Usherwood. 2004. IL-15-independent proliferative renewal of memory CD8+ T cells in latent gammaherpesvirus infection. *J Immunol* 173:2705-2714.

180. van Leeuwen, E. M. M., G. J. de Bree, E. B. M. Remmerswaal, S.-L. Yong, K. Tesselaar, I. J. M. t. Berge, and R. A. W. van Lier. 2005. IL-7 receptor {alpha} chain expression distinguishes functional subsets of virus-specific human CD8+ T cells. *Blood* 106:2091-2098.

181. Miller, N. E., J. R. Bonczyk, Y. Nakayama, and M. Suresh. 2005. Role of thymic output in regulating CD8 T-cell homeostasis during acute and chronic viral infection. *J Virol* 79:9419-9429.

182. Vezys, V., D. Masopust, C. C. Kemball, D. L. Barber, L. A. O'Mara, C. P. Larsen, T. C. Pearson, R. Ahmed, and A. E. Lukacher. 2006. Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *J. Exp. Med.* 203:2263-2269.

183. Brooks, D. G., M. J. Trifilo, K. H. Edelmann, L. Teyton, D. B. McGavern, and M. B. A. Oldstone. 2006. Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med* 12:1301-1309.

184. Ejrnaes, M., C. M. Filippi, M. M. Martinic, E. M. Ling, L. M. Togher, S. Crotty, and M. G. von Herrath. 2006. Resolution of a chronic viral infection after interleukin-10 receptor blockade. *J. Exp. Med.* 203:2461-2472.

185. Brockman, M. A., D. S. Kwon, D. P. Tighe, D. F. Pavlik, P. C. Rosato, J. Sela, F. Porichis, S. Le Gall, M. T. Waring, K. Moss, H. Jessen, F. Pereyra, D. G. Kavanagh, B. D. Walker, and D. E. Kaufmann. 2009. IL-10 is upregulated in multiple cell types during viremic HIV infection and reversibly inhibits virus-specific T cells. *Blood*:blood-2008-2012-191296.

186. Elsaesser, H., K. Sauer, and D. G. Brooks. 2009. IL-21 Is Required to Control Chronic Viral Infection. *Science* 324:1569-1572.

187. Frohlich, A., J. Kisielow, I. Schmitz, S. Freigang, A. T. Shamshiev, J. Weber, B. J. Marsland, A. Oxenius, and M. Kopf. 2009. IL-21R on T Cells Is Critical for Sustained Functionality and Control of Chronic Viral Infection. *Science* 324:1576-1580.

188. Yi, J. S., M. Du, and A. J. Zajac. 2009. A Vital Role for Interleukin-21 in the Control of a Chronic Viral Infection. *Science* 324:1572-1576.

189. Calame, K. 2006. Transcription factors that regulate memory in humoral responses. *Immunol Rev* 211:269-279.

190. Shaffer, A. L., K. I. Lin, T. C. Kuo, X. Yu, E. M. Hurt, A. Rosenwald, J. M. Giltnane, L. Yang, H. Zhao, K. Calame, and L. M. Staudt. 2002. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 17:51-62.

191. Shapiro-Shelef, M., K.-I. Lin, L. J. McHeyzer-Williams, J. Liao, M. G. McHeyzer-Williams, and K. Calame. 2003. Blimp-1 Is Required for the Formation of Immunoglobulin Secreting Plasma Cells and Pre-Plasma Memory B Cells. *Immunity* 19:607-620.

192. Hayashi, K., S. M. C. de Sousa Lopes, and M. A. Surani. 2007. Germ Cell Specification in Mice. *Science* 316:394-396.

193. Ohinata, Y., B. Payer, D. O'Carroll, K. Ancelin, Y. Ono, M. Sano, S. C. Barton, T. Obukhanych, M. Nussenzweig, A. Tarakhovsky, M. Saitou, and M. A. Surani. 2005. Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* 436:207-213.

194. Horsley, V., D. O'Carroll, R. Tooze, Y. Ohinata, M. Saitou, T. Obukhanych, M. Nussenzweig, A. Tarakhovsky, and E. Fuchs. 2006. Blimp1 Defines a Progenitor Population that Governs Cellular Input to the Sebaceous Gland. *Cell* 126:597-609.

195. Cimmino, L., G. A. Martins, J. Liao, E. Magnusdottir, G. Grunig, R. K. Perez, and K. L. Calame. 2008. Blimp-1 Attenuates Th1 Differentiation by Repression of ifng, tbx21, and bcl6 Gene Expression. *J Immunol* 181:2338-2347.

196. Martins, G. A., L. Cimmino, J. Liao, E. Magnusdottir, and K. Calame. 2008. Blimp-1 directly represses Il2 and the Il2 activator Fos, attenuating T cell proliferation and survival. *J. Exp. Med.* 205:1959-1965.

197. Gong, D., and T. R. Malek. 2007. Cytokine-Dependent Blimp-1 Expression in Activated T Cells Inhibits IL-2 Production. *J Immunol* 178:242-252.

198. Wang, L., N. van Panhuys, J. Hu-Li, S. Kim, G. Le Gros, and B. Min. 2008. Blimp-1 Induced by IL-4 Plays a Critical Role in Suppressing IL-2 Production in Activated CD4 T Cells. *J Immunol* 181:5249-5256.

199. Betts, M. R., J. M. Brenchley, D. A. Price, S. C. De Rosa, D. C. Douek, M. Roederer, and R. A. Koup. 2003. Sensitive and viable identification of

antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *Journal of Immunological Methods* 281:65-78.

200. Hermans, I. F., J. D. Silk, J. Yang, M. J. Palmowski, U. Gileadi, C. McCarthy, M. Salio, F. Ronchese, and V. Cerundolo. 2004. The VITAL assay: a versatile fluorometric technique for assessing CTL- and NKT-mediated cytotoxicity against multiple targets in vitro and in vivo. *Journal of Immunological Methods* 285:25-40.

201. Jacob, J., and D. Baltimore. 1999. Modelling T-cell memory by genetic marking of memory T cells in vivo. *Nature* 399:593-597.

202. Lin, F.-R., H.-K. Kuo, H.-Y. Ying, F.-H. Yang, and K.-I. Lin. 2007. Induction of Apoptosis in Plasma Cells by B Lymphocyte Induced Maturation Protein-1 Knockdown. *Cancer Res* 67:11914-11923.

203. Koch, M. A., G. Tucker-Heard, N. R. Perdue, J. R. Killebrew, K. B. Urdahl, and D. J. Campbell. 2009. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* 10:595-602.

204. Zheng, Y., A. Chaudhry, A. Kas, P. deRoos, J. M. Kim, T. T. Chu, L. Corcoran, P. Treuting, U. Klein, and A. Y. Rudensky. 2009. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* 458:351-356.

205. Louis, M., and A. Becskei. 2002. Binary and graded responses in gene networks. *Sci STKE* 2002:PE33.

206. Niwa, H., J. Miyazaki, and A. G. Smith. 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 24:372-376.

207. Rizzino, A. 2008. Transcription factors that behave as master regulators during mammalian embryogenesis function as molecular rheostats. *Biochem J* 411:e5-7.

208. Laslo, P., C. J. Spooner, A. Warmflash, D. W. Lancki, H. J. Lee, R. Sciammas, B. N. Gantner, A. R. Dinner, and H. Singh. 2006. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell* 126:755-766.

209. Trabattoni, D., S. Piconi, M. Biasin, G. Rizzardini, M. Migliorino, E. Seminari, A. Boasso, L. Piacentini, M. L. Villa, R. Maserati, and M. Clerici. 2004. Granule-dependent mechanisms of lysis are defective in CD8 T

cells of HIV-infected, antiretroviral therapy-treated individuals. *Aids* 18:859-869.

210. Migueles, S. A., A. C. Laborico, W. L. Shupert, M. S. Sabbaghian, R. Rabin, C. W. Hallahan, D. Van Baarle, S. Kostense, F. Miedema, M. McLaughlin, L. Ehler, J. Metcalf, S. Liu, and M. Connors. 2002. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat. Immunol.* 3:1061-1068.

211. Migueles, S. A., C. M. Osborne, C. Royce, A. A. Compton, R. P. Joshi, K. A. Weeks, J. E. Rood, A. M. Berkley, J. B. Sacha, N. A. Cogliano-Shutta, M. Lloyd, G. Roby, R. Kwan, M. McLaughlin, S. Stallings, C. Rehm, M. A. O'Shea, J. Mican, B. Z. Packard, A. Komoriya, S. Palmer, A. P. Wiegand, F. Maldarelli, J. M. Coffin, J. W. Mellors, C. W. Hallahan, D. A. Follman, and M. Connors. 2008. Lytic Granule Loading of CD8+ T Cells Is Required for HIV-Infected Cell Elimination Associated with Immune Control. *Immunity* 29:1009-1021.

212. Appay, V., D. F. Nixon, S. M. Donahoe, G. M. Gillespie, T. Dong, A. King, G. S. Ogg, H. M. Spiegel, C. Conlon, C. A. Spina, D. V. Havlir, D. D. Richman, A. Waters, P. Easterbrook, A. J. McMichael, and S. L. Rowland-Jones. 2000. HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J Exp Med* 192:63-75.

213. Agnellini, P., P. Wolint, M. Rehr, J. Cahenzli, U. Karrer, and A. Oxenius. 2007. Impaired NFAT nuclear translocation results in split exhaustion of virus-specific CD8+ T cell functions during chronic viral infection. *Proc Natl Acad Sci U S A* 104:4565-4570.

214. Puglielli, M. T., A. J. Zajac, R. G. van der Most, J. L. Dzuris, A. Sette, J. D. Altman, and R. Ahmed. 2001. In vivo selection of a lymphocytic choriomeningitis virus variant that affects recognition of the GP33-43 epitope by H-2Db but not H-2Kb. *J Virol* 75:5099-5107.

215. Kondrack, R. M., J. Harbertson, J. T. Tan, M. E. McBreen, C. D. Surh, and L. M. Bradley. 2003. Interleukin 7 Regulates the Survival and Generation of Memory CD4 Cells. *J. Exp. Med.* 198:1797-1806.

216. Sudo, T., S. Nishikawa, N. Ohno, N. Akiyama, M. Tamakoshi, H. Yoshida, and S. Nishikawa. 1993. Expression and function of the interleukin 7 receptor in murine lymphocytes. *Proc Natl Acad Sci U S A* 90:9125-9129.

217. van der Most, R. G., K. Murali-Krishna, J. G. Lanier, E. J. Wherry, M. T. Puglielli, J. N. Blattman, A. Sette, and R. Ahmed. 2003. Changing immunodominance patterns in antiviral CD8 T-cell responses after loss of epitope presentation or chronic antigenic stimulation. *Virology* 315:93-102.

218. Karrer, U., S. Sierro, M. Wagner, A. Oxenius, H. Hengel, U. H. Koszinowski, R. E. Phillips, and P. Klenerman. 2003. Memory inflation: continuous accumulation of antiviral CD8+ T cells over time. *J Immunol* 170:2022-2029.

219. Mueller, S. N., and R. Ahmed. 2009. High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proceedings of the National Academy of Sciences* 106:8623-8628.

220. Kublin, J. G., P. Patnaik, C. S. Jere, W. C. Miller, I. F. Hoffman, N. Chimbiya, R. Pendame, T. E. Taylor, and M. E. Molyneux. 2005. Effect of Plasmodium falciparum malaria on concentration of HIV-1-RNA in the blood of adults in rural Malawi: a prospective cohort study. *The Lancet* 365:233-240.

221. Abu-Raddad, L. J., P. Patnaik, and J. G. Kublin. 2006. Dual Infection with HIV and Malaria Fuels the Spread of Both Diseases in Sub-Saharan Africa. *Science* 314:1603-1606.

222. Secor, W. E., and J. B. Sundstrom. 2007. Below the belt: new insights into potential complications of HIV-1/schistosome coinfections. *Current Opinion in Infectious Diseases* 20:519-523.

223. Slifka, M. K., D. Homann, A. Tishon, R. Pagarigan, and M. B. Oldstone. 2003. Measles virus infection results in suppression of both innate and adaptive immune responses to secondary bacterial infection. *J Clin Invest* 111:805-810.

224. Beadling, C., and M. K. Slifka. 2004. How do viral infections predispose patients to bacterial infections? *Current Opinion in Infectious Diseases* 17:185-191.

225. Smith, D. K., R. Dudani, J. A. Pedras-Vasconcelos, Y. Chapdelaine, H. van Faassen, and S. Sad. 2002. Cross-Reactive Antigen Is Required to Prevent Erosion of Established T Cell Memory and Tumor Immunity: A Heterologous Bacterial Model of Attrition. *J Immunol* 169:1197-1206.

226. Villegas, E. N., M. M. Elloso, G. Reichmann, R. Peach, and C. A. Hunter. 1999. Role of CD28 in the Generation of Effector and Memory Responses Required for Resistance to Toxoplasma gondii. *J Immunol* 163:3344-3353.

227. Barton, E. S., D. W. White, J. S. Cathelyn, K. A. Brett-McClellan, M. Engle, M. S. Diamond, V. L. Miller, and H. W. Virgin. 2007. Herpesvirus latency confers symbiotic protection from bacterial infection. *Nature* 447:326-329.

228. Kinter, A. L., E. J. Godbout, J. P. McNally, I. Sereti, G. A. Roby, M. A. O'Shea, and A. S. Fauci. 2008. The Common {gamma}-Chain Cytokines IL-2, IL-7, IL-15, and IL-21 Induce the Expression of Programmed Death-1 and Its Ligands. *J Immunol* 181:6738-6746.

229. Muller, U., U. Steinhoff, L. F. L. Reis, S. Hemmi, J. Pavlovic, R. M. Zinkernagel, and M. Aguet. 1994. Functional Role of Type I and Type II Interferons in Antiviral Defense. *Science* 264:1918-1921.

230. Sher, A., C. Collazzo, C. Scanga, D. Jankovic, G. Yap, and J. Aliberti. 2003. Induction and regulation of IL-12-dependent host resistance to Toxoplasma gondii. *Immunologic Research* 27:521-527.

231. Gazzinelli, R. T., S. Hieny, T. A. Wynn, S. Wolf, and A. Sher. 1993. Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. *Proc Natl Acad Sci U S A* 90:6115-6119.

232. Ely, K. H., T. Cookenham, A. D. Roberts, and D. L. Woodland. 2006. Memory T cell populations in the lung airways are maintained by continual recruitment. *J Immunol* 176:537-543.

233. Masopust, D., V. Vezys, E. J. Wherry, D. L. Barber, and R. Ahmed. 2006. Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population. *J Immunol* 176:2079-2083.

234. Obar, J. J., S. Fuse, E. K. Leung, S. C. Bellfy, and E. J. Usherwood. 2006. Gammaherpesvirus persistence alters key CD8 T-cell memory characteristics and enhances antiviral protection. *J Virol* 80:8303-8315.

235. Swainson, L., E. Verhoeyen, F.-L. Cosset, and N. Taylor. 2006. IL-7R{alpha} Gene Expression Is Inversely Correlated with Cell Cycle Progression in IL-7-Stimulated T Lymphocytes. *J Immunol* 176:6702-6708.

236. Park, J. H., Q. Yu, B. Erman, J. S. Appelbaum, D. Montoya-Durango, H. L. Grimes, and A. Singer. 2004. Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. *Immunity* 21:289-302.

237. Alves, N. L., E. M. M. van Leeuwen, I. A. M. Derks, and R. A. W. van Lier. 2008. Differential Regulation of Human IL-7 Receptor {alpha} Expression by IL-7 and TCR Signaling. *J Immunol* 180:5201-5210.

238. Smale, S. T. 2003. The establishment and maintenance of lymphocyte identity through gene silencing. *Nat Immunol* 4:607-615.

239. Mohn, F., and D. Schubeler. 2009. Genetics and epigenetics: stability and plasticity during cellular differentiation. *Trends in Genetics* 25:129-136.

240. Mothe, B. R., B. S. Stewart, C. Oseroff, H.-H. Bui, S. Stogiera, Z. Garcia, C. Dow, M. P. Rodriguez-Carreno, M. Kotturi, V. Pasquetto, J. Botten, S. Crotty, E. Janssen, M. J. Buchmeier, and A. Sette. 2007. Chronic Lymphocytic Choriomeningitis Virus Infection Actively Down-Regulates CD4+ T Cell Responses Directed against a Broad Range of Epitopes. *J Immunol* 179:1058-1067.

241. Brooks, D. G., L. Teyton, M. B. A. Oldstone, and D. B. McGavern. 2005. Intrinsic Functional Dysregulation of CD4 T Cells Occurs Rapidly following Persistent Viral Infection. *J. Virol.* 79:10514-10527.

242. D'Souza, M., A. P. Fontenot, D. G. Mack, C. Lozupone, S. Dillon, A. Meditz, C. C. Wilson, E. Connick, and B. E. Palmer. 2007. Programmed Death 1 Expression on HIV-Specific CD4+ T Cells Is Driven by Viral Replication and Associated with T Cell Dysfunction. *J Immunol* 179:1979-1987.

243. McNeil, A. C., W. L. Shupert, C. A. Iyasere, C. W. Hallahan, J. A. Mican, R. T. Davey, Jr., and M. Connors. 2001. High-level HIV-1 viremia suppresses viral antigen-specific CD4(+) T cell proliferation. *Proc Natl Acad Sci U S A* 98:13878-13883.

244. Younes, S. A., B. Yassine-Diab, A. R. Dumont, M. R. Boulassel, Z. Grossman, J. P. Routy, and R. P. Sekaly. 2003. HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. *J Exp Med* 198:1909-1922.

245. Kasprowicz, V., J. Schulze zur Wiesch, T. Kuntzen, B. E. Nolan, S. Longworth, A. Berical, J. Blum, C. McMahon, L. L. Reyor, N. Elias, W. W. Kwok, B. G. McGovern, G. Freeman, R. T. Chung, P. Klenerman, L. Lewis-Ximenez, B. D. Walker, T. M. Allen, A. Y. Kim, and G. M. Lauer. 2008. High Level of PD-1 Expression on Hepatitis C Virus (HCV)-Specific CD8+ and CD4+ T Cells during Acute HCV Infection, Irrespective of Clinical Outcome. *J. Virol.* 82:3154-3160.

246. Ulsenheimer, A., J. T. Gerlach, N. H. Gruener, M. C. Jung, C. A. Schirren, W. Schraut, R. Zachoval, G. R. Pape, and H. M. Diepolder. 2003. Detection of functionally altered hepatitis C virus-specific CD4 T cells in acute and chronic hepatitis C. *Hepatology* 37:1189-1198.

247. WHO. 2009. Global tuberculosis control. WHO.

248. WHO. 2009. Malaria fact sheet.

249. Alter, G., G. Hatzakis, C. M. Tsoukas, K. Pelley, D. Rouleau, R. LeBlanc, J. G. Baril, H. Dion, E. Lefebvre, R. Thomas, P. Cote, N. Lapointe, J. P. Routy, R. P. Sekaly, B. Conway, and N. F. Bernard. 2003. Longitudinal assessment of changes in HIV-specific effector activity in HIV-infected patients starting highly active antiretroviral therapy in primary infection. *J Immunol* 171:477-488.

250. Casazza, J. P., M. R. Betts, L. J. Picker, and R. A. Koup. 2001. Decay kinetics of human immunodeficiency virus-specific CD8+ T cells in peripheral blood after initiation of highly active antiretroviral therapy. *J Virol* 75:6508-6516.

251. Puissant-Lubrano, B., B. CombadiËre, D. Duffy, N. Wincker, M.-J. Frachette, H. Ait-Mohand, B. Verrier, C. Katlama, and B. Autran. 2009. Influence of antigen exposure on the loss of long-term memory to childhood vaccines in HIV-infected patients. *Vaccine* 27:3576-3583.

252. Mescher, M. F., P. Agarwal, K. A. Casey, C. D. Hammerbeck, Z. Xiao, and J. M. Curtsinger. 2007. Molecular basis for checkpoints in the CD8 T cell response: Tolerance versus activation. *Seminars in Immunology* 19:153-161.

253. Calame, K. 2008. Activation-dependent induction of Blimp-1. *Current Opinion in Immunology* 20:259-264.

254. Sereti, I., R. M. Dunham, J. Spritzler, E. Aga, M. A. Proschan, K. Medvik, C. A. Battaglia, A. L. Landay, S. Pahwa, M. A. Fischl, D. M. Asmuth, A. R. Tenorio, J. D. Altman, L. Fox, S. Moir, A. Malaspina, M. Morre, R. Buffet, G. Silvestri, M. M. Lederman, and A. S. T. for the. 2009. IL-7 administration drives T cell-cycle entry and expansion in HIV-1 infection. *Blood* 113:6304-6314.

255. Colle, J. H., J. L. Moreau, A. Fontanet, O. Lambotte, M. Joussemet, J. F. Delfraissy, and J. ThËze. 2006. CD127 expression and regulation are altered in the memory CD8 T cells of HIV-infected patients - reversal by highly active anti-retroviral therapy (HAART). *Clinical & Experimental Immunology* 143:398-403.

256. Freeman, G. J., E. J. Wherry, R. Ahmed, and A. H. Sharpe. 2006. Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *J. Exp. Med.* 203:2223-2227.

257. Ren, B., K. J. Chee, T. H. Kim, and T. Maniatis. 1999. PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. *Genes & Development* 13:125-137.

258. Yu, J., C. Angelin-Duclos, J. Greenwood, J. Liao, and K. Calame. 2000. Transcriptional Repression by Blimp-1 (PRDI-BF1) Involves Recruitment of Histone Deacetylase. *Mol. Cell. Biol.* 20:2592-2603.

259. Ancelin, K., U. C. Lange, P. Hajkova, R. Schneider, A. J. Bannister, T. Kouzarides, and M. A. Surani. 2006. Blimp1 associates with Prmt5 and directs histone arginine methylation in mouse germ cells. *Nat Cell Biol* 8:623-630.

260. Gyory, I., J. Wu, G. Fejer, E. Seto, and K. L. Wright. 2004. PRDI-BF1 recruits the histone H3 methyltransferase G9a in transcriptional silencing. *Nat Immunol* 5:299-308.