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Exhaustion Renders CD8 T Cells Unresponsive to Antigen-Independent Activation and Is Characterized by IL-18 Receptor Downregulation

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EXHAUSTION RENDERS CD8 T CELLS UNRESPONSIVE TO ANTIGEN-INDEPENDENT ACTIVATION AND IS CHARACTERIZED BY IL-18 RECEPTOR DOWNREGULATION

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BIOLOGY

ABSRACT

During many chronic infections virus-specific CD8 T cells succumb to exhaustion as they lose their ability to respond to antigenic activation. Combinations of interleukin (IL)-12, IL-18, and IL-21 have been shown to induce the antigen-independent production of IFN-γ by effector and memory CD8 T cells. In this study we investigated whether exhausted CD8 T cells are sensitive to activation by these cytokines. We show that effector and memory, but not exhausted, CD8 T cells produce interferon-gamma (IFN-γ) and upregulate CD25 following exposure to certain combinations of IL-12, IL-18, and IL-21. This unresponsiveness was associated with downregulation of the IL-18 receptor (R) on exhausted T cells. Although IL-18R expression is connected with the ability of memory CD8 T cells to self-renew and efflux rhodamine 123, the IL-18R-low exhausted cells remained capable of secreting this dye. To further evaluate the consequences of IL-18R downregulation, we tracked the fate of IL-18R-deficient CD8 T cells in chronically infected mixed bone marrow chimeras and discovered that IL-18R$^{-/-}$ cells were preferentially lost during the contraction phase of the response. The antigen-independent responsiveness of exhausted CD8 T cells was also investigated following co-infection with *Listeria monocytogenes*, which induces the expression of IL-12 and IL-18. Although IL-18R-high memory cells upregulated CD25 and produced IFN-γ, the IL-18R-low exhausted cells failed to respond. Collectively, these findings indicate that as

exhausted T cells adjust to the chronically infected environment, they lose their susceptibility to antigen-independent activation by cytokines, which compromises their ability to detect bacterial coinfections.

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INTRODUCTION

EXHAUSTED CD8 T CELLS DOWNREGULATE THE IL-18 RECEPTOR AND BECOME UNRESPONSIVE TO INFLAMMATORY CYTOKINES AND BACTERIAL COINFECTIONS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
APC	Antigen presenting cell
ARM	Armstrong
B6	C57BL/6J
BFA	Brefeldin A
Cl 13	Clone 13
CsA	Cyclosporin A
GP	Glycoprotein
HSC	Hematopoietic stem cells
IFN-γ	Interferon-gamma
IL-12	Interleukin-12
IL-12R	Interleukin-12 receptor
IL-18	Interleukin-18
IL-18R	Interleukin-18 receptor

IL-21	Interleukin-21
IL-21R	Interleukin-21 receptor
LCMV	Lymphocytic choriomeningitis virus
LM	Listeria monocytogenes
MHC	Major histocompatibility complex
MFI	Mean fluorescence intensity
PAMPs	Pathogen-associated activation patterns
Rh123	Rhodamine 123
SD	Standard deviation
SEM	Standard error of the mean
TCR	T cell receptor
TNFα	Tumor necrosis factor-alpha

INTRODUCTION

T cell Responses to Infection

Upon infection, the innate immune system initiates the first wave of defenses for the host. Cells of the innate immune system become activated by detecting pathogen-associated activation patterns (PAMPs) such as lipopolysaccharide (LPS) or viral RNA, resulting in phagocytosis and the production of cytokines such as IL-12 and IL-18 [1]. Macrophages and dendritic cells comprise a subset of the innate system that, in addition to directly killing infected cells by phagocytosis, is responsible for activating cells of the adaptive immune system. Expression of major histocompatibility complexes (MHC) and costimulatory molecules (CD80 and CD86) on these cells are required for the effective activation of T cells. CD4 T cells recognize exogenous peptide antigen attached to MHC class II molecules, and CD8 T cells typically recognize peptide antigen derived from endogenous proteins non-covalently associated with MHC class I molecules. CD8 T cells are able to efficiently target and kill any infected cell in the host because MHC class I is expressed on virtually every cell in the body. Thus, when a cell becomes infected, pathogen-derived peptides are presented on MHC class I molecules, leading to CD8 T cell recognition and killing of the target cell.

Naïve CD4 and CD8 T cells require three signals from professional antigen presenting cells for proper activation: TCR stimulation, costimulation through CD28, and inflammatory cytokine signaling. Without all three signals fully functional effector cells

do not develop. In addition to these priming signals, CD8 T cells require help from CD4 T cells in order to transition into fully functional memory cells [2-4]. Some of the proposed mechanisms of help are activation of APCs through CD40 (APC licensing), IL-21 production, and IL-2 production. CD4 T cells are the main providers of IL-2 for CD8 T cells, which is important for the differentiation and expansion of effector T cells [5-7]. CD25 upregulation on CD8 T cells is essential to receive IL-2 signals. Upon activation, CD8 T cells upregulate IL-2Rα (CD25) and IL-2Rβ (CD122) to become highly sensitive to IL-2 signals. IL-2 binds these two chains of the IL-2R and recruits the common gamma chain cytokine receptor (CD132). During the primary response IL-2 signals enhance the expansion and differentiation of effector cells, but neither IL-2 nor CD4 T cell help is strictly required for control of certain pathogens, including LCMV-Arm and *Listeria monocytogenes*. A lack of CD4 T cell help or IL-2 signals during the primary response does, however, compromise the functional capacity of the memory T cell pool [8-10].

With activation CD8 T cells change their surface marker expression, rapidly proliferate, produce effector cytokines, and gain the ability to directly kill infected cells. These cytokines can activate antiviral pathways and amplify immune responses while the CD8 T cells directly kill via perforin and granzyme. Once the infection has been resolved, approximately 90% of the effector CD8 T cells die due to apoptosis leaving a small population of long-lived memory cells. These memory CD8 T cells have high proliferative capabilities, reduced perforin and granzyme, higher IL-2 production, and often higher IL-7R (CD127) expression. These cells are rapidly activated by TCR

stimulation and proliferate to form a large secondary effector population that functions to swiftly resolve the infection [11, 12].

The maintenance of memory T cells is dependent on the cytokines IL-15 and IL-7 which allow for homeostatic proliferation and survival [13]. Memory cells also share the self-renewing properties of toxin removal and proliferative capabilities with hematopoietic stem cells (HSCs), and these populations allow for infection-free recovery from lymphocytopenia following chemotherapy [11, 14]. These cells are protected from the toxic effects of chemotherapy due to their overexpression of ABC superfamily multidrug efflux proteins. These proteins provide protection by pumping toxins from the cell, a process that can be visualized using the fluorescent marker rhodamine 123 (Rh123). This dye is preferentially removed through ABCB1-mediated efflux, and efficient efflux of Rh123 has been associated with cells that posses high self-renewal capabilities [14].

Chronic Viral Infections

Infections can progress in different ways. They can become resolved, kill the host, or establish a persistent infection. Whether the infection is resolved or becomes persistent depends on the virulence of the virus and also on the strength of the host's immune system. To establish a chronic persistent infection during which the virus continually replicates, the virus must evade the immune responses and modulate the immune system to reduce inflammation [15]. These chronic infections therefore can result in persistently high viremia as in human immunodeficiency virus (HIV), hepatitis C virus (HCV), and lymphocytic choriomeningitis virus (LCMV), or persist at a much

lower level as in the case with cytomegalovirus (CMV), Epstein-Barr Virus (EBV), and murine gammaherpesvirus-68 (MHV-68). Antigen-specific activation normally results in the release of inflammatory cytokines that can damage host tissue causing immunopathology. In order to protect the host, regulatory mechanisms suppress potentially immunopathogenic immune responses. During chronic infections this is often achieved by downregulating the anti-viral CD8 T cell response [15-17].

CD8 T cell Exhaustion

Whereas following acute infections effector CD8 T cells contract and the remaining cells differentiate into a memory population, in chronic infections these cells instead undergo a process termed exhaustion in which they gradually become unresponsive. CD8 T cell exhaustion is characterized by the stepwise loss of effector functions, beginning with IL-2 production and proliferative capacity, then TNFα production. In cases of severe exhaustion, the ability to produce IFN-γ will be lost, and eventually the virus-specific cells will be deleted. Exhaustion occurs in many chronic infections and is most severe in those with high viral loads (e.g. HIV, LCMV); however, if the viral load is reduced, effector functions can be restored [16, 18-20]. Exhausted CD8 T cells have been shown to have a unique molecular signature that includes elevated levels of inhibitory receptors. Several studies have proven that by blocking certain inhibitory receptors, some function can be restored to these cells. PD-1 is the best understood inhibitory receptor, and blockade of PD-1/PD-L1 signaling leads to improved cytokine production, proliferation, and reduced viral loads [21]. Dual blockade with TIM-3 or LAG-3 has also been shown to enhance the effects of PD-1 blockade [22-24].

Exhausted cells express high levels of CD43 (1B11), CD69, and various inhibitory receptors (e.g. PD-1, TIM-3, LAG-3). Elevated levels of immunosuppressive cytokines (e.g. IL-10 and TGFβ) have also been shown in these chronic infections. IL-10 has been shown to impede the function of T cells, and TGFβ restricts the size of the antigen-specific population, therefore increased levels lead to the reduced expansion and deletion of cells in this needed population [19, 25]. Exhausted cells also express low levels of CD62L and CD127, which is indicative of an effector T cell phenotype, and unlike memory populations cannot be maintained by IL-7 and IL-15 signals [26, 27].

The development of exhaustion depends on cytokine levels, antigen presence, and CD4 T cell help. CD4 T cells are important in activating professional antigen presenting cells that are needed in order to activate other cells, secreting cytokines in order to guide other T cells, and producing cytokines such as IL-2 and IL-21, which act directly to promote differentiation and maintenance of polyfunctional CD8 T cells [28-30]. During chronic infections with intact CD4 populations, IL-21 production is increased as IL-2 production is decreased, and this possibly maintains some level of CD8 function leading to the gradual control of the viral load. In the absence of CD4 T cells, exhaustion becomes extremely severe resulting in the loss of virus-specific CD8 T cells [5]. During chronic LCMV infection initial treatment with recombinant IL-21 has been shown to prevent the full exhaustion of the virus-specific CD8 T cells. This restored the cytokine production and lowered viral loads, yet caused severe immunopathology thus illustrating the delicate balance that must be maintained [31, 32].

Lymphocytic Choriomeningitis Virus

LCMV infection of mice has been well characterized and widely used to study T cell responses to viral infection. Depending on the strain of LCMV and the immunological status of the mice, an acute, protracted, or chronic infection can be elicited. For example, the Armstrong strain of LCMV can be used to initiate an acute infection, but the cl 13 strain of LCMV can establish a protracted or chronic infection, depending on the mouse strain infected. The more severe infection from the cl 13 strain is due to two amino acid mutations that separate it from the Armstrong strain. The amino acid substitution of F to L at residue 260 of the glycoprotein (GP) allows for the cl 13 strain to more easily infect macrophages and dendritic cells, while a K to Q mutation at residue 1079 of the polymerase protein allows for enhanced viral replication. Together these mutations account for the increased severity of cl 13 infections [33, 34].

Infection of immunocompetent C57BL/6J (B6) mice with the Armstrong (Arm) strain of LCMV initiates an acute infection. This infection primes a large CD8 T cell response that effectively eradicates the virus within several weeks. The T cell response to LCMV-Arm is characterized by the development of a massive effector response and establishment of a long-lived functional memory T cell pool. These memory cells respond vigorously to reinfection and exhibit polyfunctional capacity for cytokine production. In contrast to the acute LCMV-Arm infection, intravenous (i.v.) injection of B6 mice with the clone 13 strain of LCMV leads to a protracted infection in which effector responses are initially elicited, but fail to adequately control viremia and succumb to exhaustion. Over time, these suboptimal effector responses are able to bring the viral levels under control, and the T cells will partially recover their function.

Infection of mice with the clone 13 strain of LCMV results in a chronic infection that is never controlled for the life of the animal. As seen in protracted infections, cl 13 infected IL-21$^{-/-}$ or CD4$^{-/-}$ mice initially mount an effector response, but these responses are short-lived as the virus-specific cells become exhausted. Without CD4 T cell help, these cells become severely exhausted and will lose their effector functions completely and eventually become deleted from the host. The T cells never recover and the mice will remain chronically infected (Fig. 1) [18].

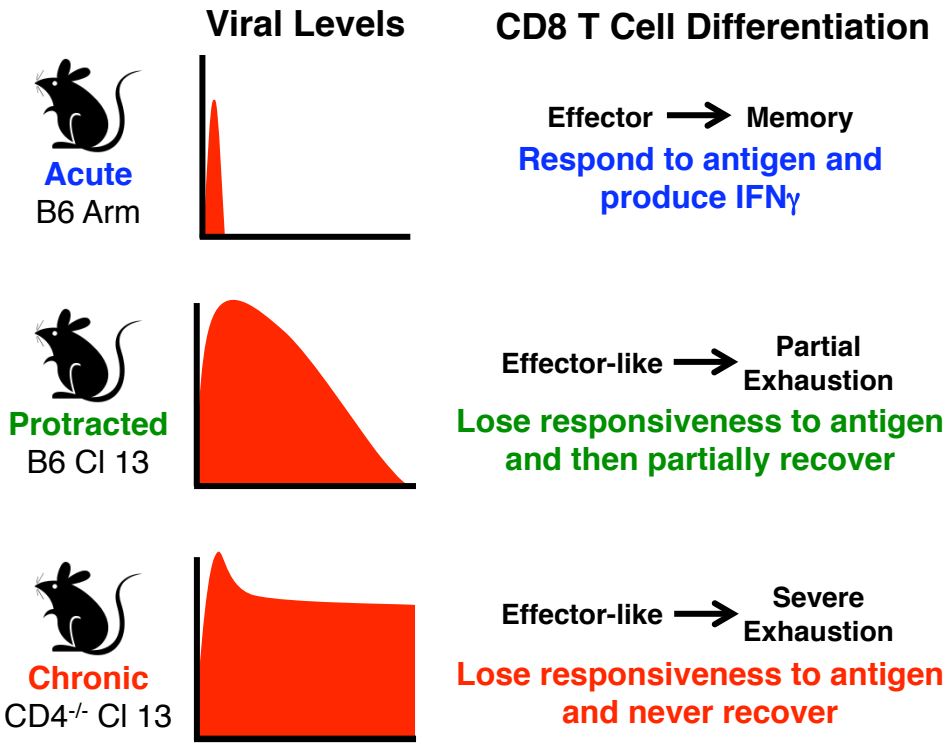

Figure 1. Acute, protracted, and chronic lymphocytic choriomeningitis virus infections

Bystander Activation

T cells can be activated through two different activation pathways. Activation can be initiated through a TCR-dependent pathway in which the T cell recognizes a

peptide epitope of the invading pathogen that is presented by the infected or antigen-presenting cell. Alternatively, T cells can also be activated in a TCR-independent fashion. This occurs only in previously activated effector and memory cells, as naïve T cells do not respond to TCR-independent stimulation. This is referred to as bystander activation, because the cytokines that activate the T cells are produced by innate cells in response to activation through toll-like receptors (TLRs) that recognize microbial PAMPs. The innate cytokines that are produced in turn activate T cells in an antigen-independent manner, priming all memory cells to react regardless of antigenic specificity [35-37]. This bystander activation may serve to limit the initial pathogen load during infection and enhance activation and differentiation of pathogen-specific cells. Bystander activation can be elicited through various bacterial (e.g. *Listeria monocytogenes*) and protozoan (e.g. *Leishmania major*) infections [38, 39]. Interleukin (IL)-12 and IL-18 have been extensively studied as cytokines that are produced and induce IFN-γ production from CD8 T cells. Alone, they do not elicit strong IFN-γ production, but they do synergistically incite a very strong response during the early stages of infection and also during in vitro stimulation of effector and memory CD8 T cell populations [35, 40].

IL-12, IL-18, and IL-21

Combinations of cytokines have been shown to synergistically activate memory T cells to produce IFN-γ. IL-12 and IL-18 have been broadly studied in their ability to induce strong responses from CD8 T cells. This synergism has also been reported with IFNα and IL-18 by effector and memory CD8 T cells and with IL-21 and IL-15 or IL-18 in human NK and T cells [36, 41, 42].

IL-18 is a member of the IL-1 cytokine family and is constitutively expressed by macrophages, dendritic cells, and epithelial cells as pro-IL-18. Caspase 1 is required to cleave pro-IL-18 to produce active IL-18. IL-18 is activated in response to microbial products and induces IFN-γ production and Th1 responses by T cells. IL-18 binds the IL-18Rα subunit and then recruits the accessory protein-like IL-18Rβ subunit, which initiates signaling. IL-18R signaling requires the MyD88 adaptor protein and signaling through AP-1 and NF-kB to induce IFN-γ production. IL-18Rbp is a soluble protein that regulates the activity of IL-18 in response to IFN-γ production in a negative feedback loop. It binds IL-18 and prevents binding the cell surface IL-18R, but in instances of inflammation there will be excess circulating IL-18 [43-45].

Like IL-18, IL-12 is produced in response to microbial stimulation. IL-12 is a proinflammatory cytokine mainly produced by phagocytes and dendritic cells and also induces Th1 responses and IFN-γ production. IL-12 signals act through the IL-12R, which consists of two subunits, IL-12Rβ1 and IL-12Rβ2. Responsiveness in T cells to IL-12 signals is dependent upon expression of the IL-12Rβ2 chain as IL-12Rβ1 also binds IL-23. Together IL-12Rβ1 and IL-12Rβ2 bind to IL-2 and results in STAT4 phosphorylation and induction of IFN-γ mRNA [46, 47].

IL-21, unlike IL-12 and IL-18, is not an innate cytokine but has been shown to act directly on CD8 T cells. IL-21 is a member of the common gamma chain family of cytokines and is primarily produced by CD4 T cells. The production of IL-21 has been shown to enhance the third signal to activate CD8 T cells, and may also slow effector differentiation while favoring the development of memory cells. IL-21 activity is required in the control of chronic viral infections to preserve the polyfunctionality of

CD8 T cells and promote their maintenance [31, 32, 48, 49]. It has also been implicated in acute viral infections not for the generation of memory cells, but for the elaboration of strong recall responses [50].

Because exhausted CD8 T cells are unable to respond to TCR activation signals, we hypothesized that by stimulating these cells with IL-12, IL-18, and IL-21 we could identify whether this unresponsiveness reflected a defect in TCR signaling or rather a global ablation of this effector response. Cytokine stimulation is known to activate memory cells; therefore it was plausible that the exhausted cells could be activated via an alternative IFN-γ inducing pathway.

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ABSTRACT

During many chronic infections virus-specific CD8 T cells succumb to exhaustion as they lose their ability to respond to antigenic activation. Combinations of interleukin (IL)-12, IL-18, and IL-21 have been shown to induce the antigen-independent production of IFN-γ by effector and memory CD8 T cells. In this study we investigated whether exhausted CD8 T cells are sensitive to activation by these cytokines. We show that effector and memory, but not exhausted, CD8 T cells produce interferon-gamma (IFN-γ) and upregulate CD25 following exposure to certain combinations of IL-12, IL-18, and IL-21. This unresponsiveness was associated with downregulation of the IL-18R on exhausted T cells. Although IL-18R expression is connected with the ability of memory CD8 T cells to self-renew and efflux rhodamine 123, the IL-18R-low exhausted cells remained capable of secreting this dye. To further evaluate the consequences of IL-18R downregulation, we tracked the fate of IL-18R-deficient CD8 T cells in chronically infected mixed bone marrow chimeras and discovered that IL-18R$^{-/-}$ cells were preferentially lost during the contraction phase of the response. The antigen-independent responsiveness of exhausted CD8 T cells was also investigated following co-infection with *Listeria monocytogenes*, which induces the expression of IL-12 and IL-18. Although IL-18R-high memory cells upregulated CD25 and produced IFN-γ, the IL-18R-low exhausted cells failed to respond. Collectively, these findings indicate that as exhausted T cells adjust to the chronically infected environment, they lose their susceptibility to antigen-independent activation by cytokines, which compromises their ability to detect bacterial co-infections.

INTRODUCTION

Memory CD8 T cells typically develop following a short period of antigenic activation, which occurs during acute infections with intracellular pathogens. A hallmark of these memory T cells is their ability to rapidly respond following re-exposure to their original inducing antigen. This recall response includes the production of cytokines such as IFN-γ, the elaboration of cytotoxic effector activities, proliferation, and changes in the expression of cytokine receptors and adhesion molecules [1, 2]. In addition to their vigorous ability to respond to antigenic activation, memory CD8 T cells have also been shown to possess an innate-like ability to respond to certain sets of cytokines in the absence of exposure to antigen. Most notably, a combination of the proinflammatory cytokines IL-12 and IL-18 causes the pronounced production of IFN-γ by memory T cells. Other cytokine combinations including IL-18 and IL-21 as well as type I IFN have been shown to have similar activating effects [3-6]. This sensitivity to cytokine stimulation endows memory T cells with the capacity to respond in an antigen- and TCR-independent manner to certain infections that induce inflammation such as *Listeria monocytogenes* (LM) and *Burkholderia pseudomallei* [7, 8]. Thus, pre-existing memory CD8 T cells can potentially contribute to the control of a broad set of infections due to their ability to detect changes in the inflammatory cytokine milieu.

During chronic viral infections CD8 T cell responses are usually elaborated; however, the development of prototypic memory CD8 T cells is disrupted. Instead, the responding virus-specific CD8 T cells undergo a differentiation process that results in their exhaustion. The most severely exhausted T cells develop under conditions of high viral load and ineffective CD4 T cell help [9-12]. Although severely exhausted CD8 T

cells retain expression of IFN-γ mRNA, they fail to produce IFN-γ protein after exposure to their cognate antigen [13]. In addition, exhausted cells exhibit altered maintenance requirements, losing the self-renewal properties associated with normal memory T cells, and therefore may become deleted overtime. It is possible that exhaustion has evolved to allow antigen-specific T cells to become tuned to an environment of persisting antigen. Thus their loss of responsiveness to antigenic activation may serve as a safety mechanism that limits pronounced and sustained effector activities that could be immunopathogenic. It is less clear, however, whether exhaustion alters the ability of T cells to mount antigen-independent responses to inflammatory cytokines.

Although T cell exhaustion has been described during several chronic viral infections including HIV and hepatitis C virus infections, it is most well characterized in mice persistently infected with lymphocytic choriomeningitis virus (LCMV). The LCMV system is particularly informative as different durations of infection can be established depending upon the isolate of virus and strain of mice used. In the current study we have used LCMV infection of mice to investigate whether the development of T cell exhaustion alters the ability of virus-specific CD8 T cells to perceive and respond to antigen-independent activation with combinations of IL-12, IL-18, and IL-21. The findings show that unlike effector and memory CD8 T cells, exhausted cells are not activated by these cytokines, and this correlates with differential expression of the IL-18-receptor (IL-18R). The decrease of IL-18R expression on exhausted CD8 T cells is consequential as it renders these cells more prone to deletion during the initial phase of persistent LCMV infection. In addition, lower IL-18R expression is associated with the

failure of exhausted CD8 T cells to respond to bacterial coinfection by upregulating CD25 and producing IFN-γ.

RESULTS

Effector and memory, but not exhausted, CD8 T cells respond to TCR-independent activation.

Previous studies have shown that various combinations of IL-12, IL-18, and IL-21 activate primed CD8 T cells to produce IFN-γ in the absence of antigenic stimulation [5, 14, 15]. We confirmed and extended these findings using LCMV-specific effector CD8 T cells and observed qualitative and quantitative differences in the ability of the cytokine mixtures to stimulate IFN-γ production (Fig. 1). IL-12, IL-18, and IL-21 alone caused at best minimal IFN-γ production by LCMV GP33-specific effector CD8 T cells (Fig. 1A). IL-12+IL-18 with or without IL-21 stimulated IFN-γ production by 68±8% (SD) of the cells; however, IL-18+IL-21 had a more modest effect, activating 33±17% of these CD8 T cells, and the levels of IFN-γ production were also lower based upon the fluorescence intensity of the IFN-γ^+ population (Fig. 1B). IL-12+IL-21 was less effective at stimulating IFN-γ production (Fig. 1).

Since severely exhausted CD8 T cells fail to produce IFN-γ in response to stimulation with their cognate antigen, we next investigated whether effector, memory, and exhausted LCMV-specific CD8 T cells were susceptible to activation with the various cytokine combinations. To address this, acute (LCMV-Arm), protracted (LCMV-cl13 infection of B6 mice), and chronic (LCMV-cl13 infection of $CD4^{-/-}$ mice) infections were established. During the effector phase of the CD8 T cell response, at day

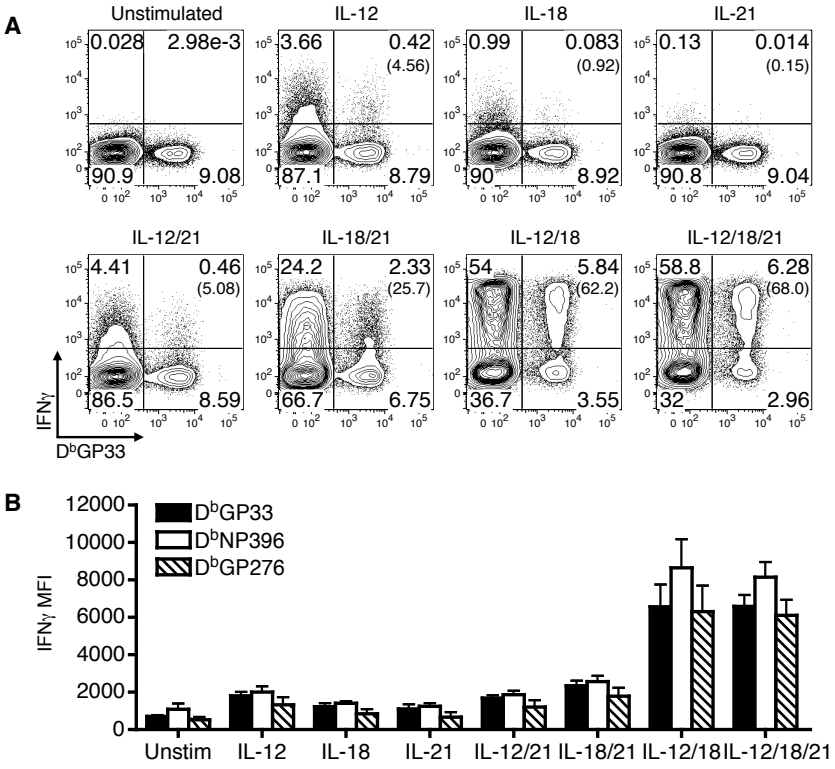

Figure 1. Combinations of IL-12, IL-18, and IL-21 qualitatively and quantitatively differ in their ability to stimulate IFN-γ production by LCMV-specific CD8 T cells.

Splenocytes from LCMV-Arm infected B6 mice (acute infection) were prepared at 9 days post-infection and stimulated with the indicated cytokines. (A) Representative flow cytometry plots show IFN-γ production by gated CD8 T cells following stimulation with the indicated cytokines. The numbers in parentheses indicate the percentage of IFN-γ-producing D^bGP33^+ cells. (B) The MFI of IFN-γ production by gated IFN-γ^+ tetramer$^+$ CD8 T cells is shown in response to stimulation with the various cytokines. Error bars are SD. Representative or composite results are shown from five experiments analyzing a total of 11-16 mice.

9 following infection, LCMV GP33-specific CD8 T cells from all of the cohorts analyzed produced IFN-γ in response to a brief (5.5h) exposure to IL-12+IL-18 and IL-12+IL-18+IL-21 (Fig. 2 and Fig. 3). The other cytokine combinations and the single cytokines alone had minimal to modest stimulatory effects. By day 35 post-infection, the memory CD8 T cells that developed in acutely infected hosts retained the ability to respond to the cytokine combinations (Fig. 2 and Fig. 3). By contrast, the development of exhaustion (day 35 post-infection of cl13 infected mice) was associated with the inability to produce IFN-γ in response to antigen-independent cytokine activation.

We also checked whether exposure to IL-12, IL-18, and IL-21 either alone or in combination activated effector, memory, or exhausted CD8 T cells to upregulate expression of the IL-2 receptor-α chain, CD25. Changes in CD25 expression by both LCMV-GP33 and LCMV-GP276 epitope-specific CD8 T cells were assessed following stimulation with the various cytokine combinations. The levels of CD25 increased on memory CD8 T cells from B6 LCMV-Arm infected mice (Fig. 4 and Fig. 5). The magnitude of upregulation paralleled the trends observed for IFN-γ production with the most marked expression induced by IL-12+IL-18 and IL-12+IL-18+IL-21, while IL-18+IL-21 had less potent activating abilities (Fig. 4). LCMV-specific CD8 T cells from protracted and chronically infected mice failed to increase CD25 expression in response to stimulation with the cytokine panels. Collectively these findings show the divergence between memory and exhausted CD8 T cells and demonstrate that persistent viral infections corrupt the ability of virus-specific CD8 T cells to respond to antigen-independent stimuli.

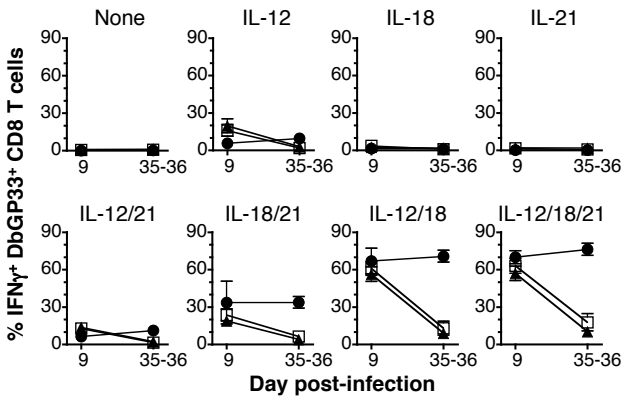

Figure 2. Exhausted CD8 T cells do not produce IFN-γ in response to antigen-independent stimulation with combinations of IL-12, IL-18, and/or IL-21.

IFN-γ production by LCMV GP33-specific CD8 T cells was analyzed at days 9 or 35-36 following acute (LCMV-Arm infected B6 mice; black circles), protracted (LCMV-cl 13 infected B6 mice; white squares) and chronic (LCMV-cl 13 infected $CD4^{-/-}$ mice; black triangles) infections. Splenocytes were stimulated for 5.5hr with the indicated cytokines and IFN-γ production by D^{b}GP33 tetramer-binding cells was determined by intracellular cytokine analysis. Composite results are shown from five independent day 9 experiments with a total of 14-19 mice per group and three independent day 35-36 experiments with a total of 8-9 mice per group.

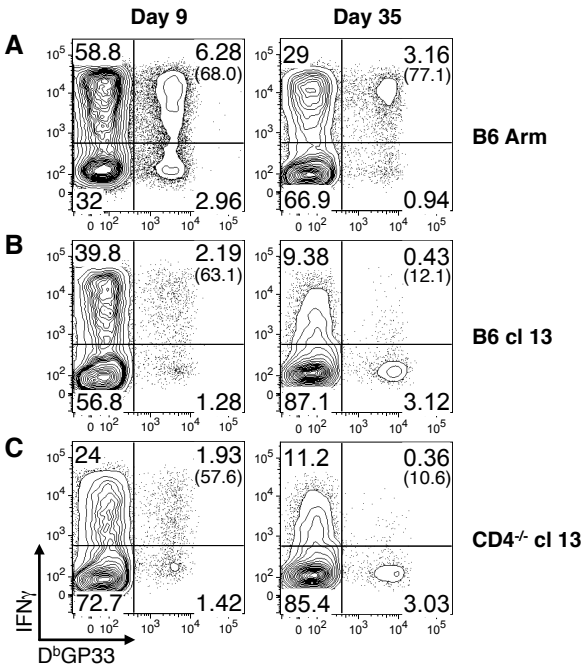

Figure 3. Exhausted CD8 T cells fail to produce IFN-γ in response to antigen-independent stimulation with IL-12, IL-18, and IL-21.

Representative flow cytometry plots are shown for (A) acute, (B) protracted, or (C) chronic infections analyzed at day 9 (effector) and day 35 (memory/exhausted) timepoints for IFN-γ production in response to stimulation with IL-12, IL-18, and IL-21. Numbers in parentheses indicate the percentage of IFN-γ-producing GP33-specific CD8 T cells. Results are representative of five independent day 9 experiments with a total of 14-19 mice per group and three independent day 35-36 experiments with a total of 8-9 mice per group.

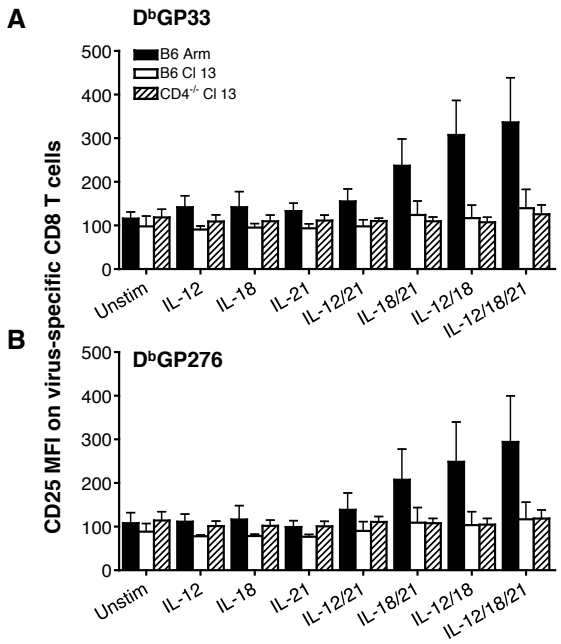

Figure 4. Memory, but not exhausted, CD8 T cells upregulate CD25 expression following exposure to combinations of IL-12, IL-18, and/or IL-21.

(A) LCMV D^bGP33- and (B) D^bGP276-specific CD8 T cells were analyzed for their ability to upregulate CD25 expression in response to the indicated sets of cytokines. Splenic responses were analyzed between days 38-232 following acute (LCMV-Arm; black bars), protracted (LCMV-cl 13 infected B6 mice; white bars), and chronic (LCMV-cl 13 infected CD4-/- mice; striped bars) infections in the absence of BFA. Composite results are shown from six independent experiments analyzing a total of 7-24 mice per group. Error bars are S.D.

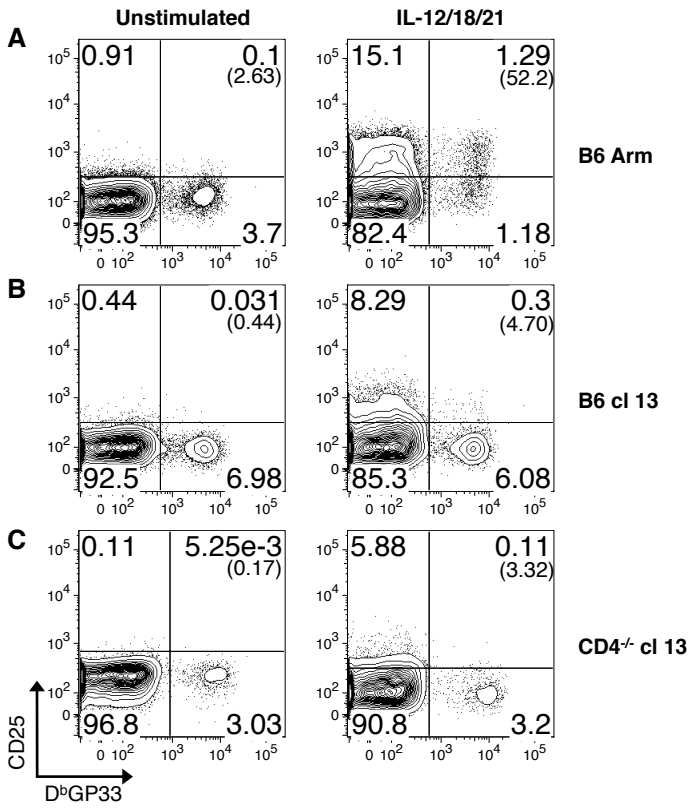

Figure 5. CD25 is not upregulated on exhausted cells in response to IL-12, IL-18, and IL-21.

CD25 upregulation was analyzed after a 5.5 hour stimulation with or without IL-12, IL-18, and IL-21. Representative flow cytometry plots show the CD25 and D^bGP33 tetramer staining profiles of gated CD8 T cells from mice undergoing (A) acute, (B) protracted, and (C) chronic infections. Numbers in parentheses indicate the percentage of $CD25^+$ GP33-specific CD8 T cells. Data are representative of six independent experiments at 38-232 days post-infection with a total of 7-24 mice per group.

Exhausted CD8 T cells downregulate IL-18R.

To investigate why exhausted cells lose responsiveness to cytokine stimulation, the expression of the cognate cytokine receptors on virus-specific CD8 T cells was evaluated. Whereas the expression of IL-12Rβ2 was similar on LCMV GP33-specific CD8 T cells from mice undergoing acute, protracted, and chronic LCMV infections (Fig. 6A and Fig. 7A), the levels of the IL-21R tended to be higher in the protracted and chronically infected cohorts (Fig. 6B and Fig. 7B). The IL-18Rα was clearly expressed at high levels during the effector phase of the response (day 9) and was maintained on memory CD8 T cells (day 35, B6 Arm); however, the levels of expression became downregulated on the exhausted CD8 T cells that developed in the protracted and chronically infected groups (day 35, B6 cl13 and $CD4^{-/-}$ cl13) (Fig. 6C and Fig. 7C).

Since severely exhausted CD8 T cells are not always maintained over time and express only low levels of the IL-18R, we next evaluated whether these cells were capable of effluxing the fluorescent dye rhodamine 123 (Rh123). A previous study has shown that IL-18R expression is associated with populations of human memory T cells which have self-renewing capabilities and, by comparison with IL-18R-low CD8 T cells, the IL-18R-high cells preferentially effluxed Rh123 [16]. Consistent with these findings, IL-18R-high CD8 T cells from acutely infected mice (Fig. 8A), which encompass the virus-specific memory T cell population (Fig. 8B), efficiently effluxed Rh123 during a one hour period. This loss of Rh123 was blocked by either CsA (Fig. 8 A and B) or vinblastine (Fig. 8B), which inhibit ABCB1 transporters required for the removal of Rh123. By contrast, virus-specific CD8 T cells from protracted and chronically infected mice, which have downregulated the IL-18R, effluxed Rh123 at least as efficiently as

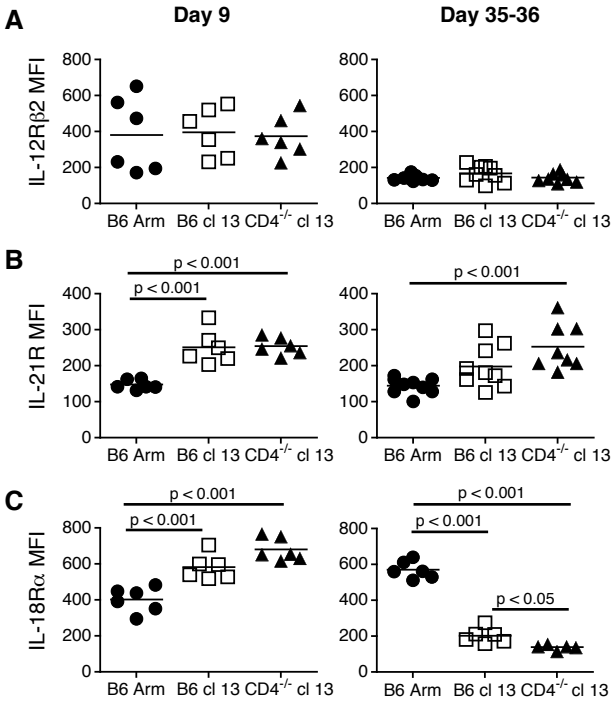

Figure 6. IL-18R is downregulated on exhausted CD8 T cells.

Splenic LCMV D^bGP33-specific CD8 T cells were analyzed at days 9 or 35-36 following acute (LCMV-Arm; black circles), protracted (LCMV-cl 13 infected B6 mice; white squares) and chronic (LCMV-cl 13 infected $CD4^{-/-}$ mice; black triangles) infections for expression of (A) IL-12Rβ2, (B) IL-21R, and (C) IL-18Rα. The results are shown from a total of 6-9 individual mice per group, derived from 2-3 independent experiments, and the horizontal bar indicates mean values.

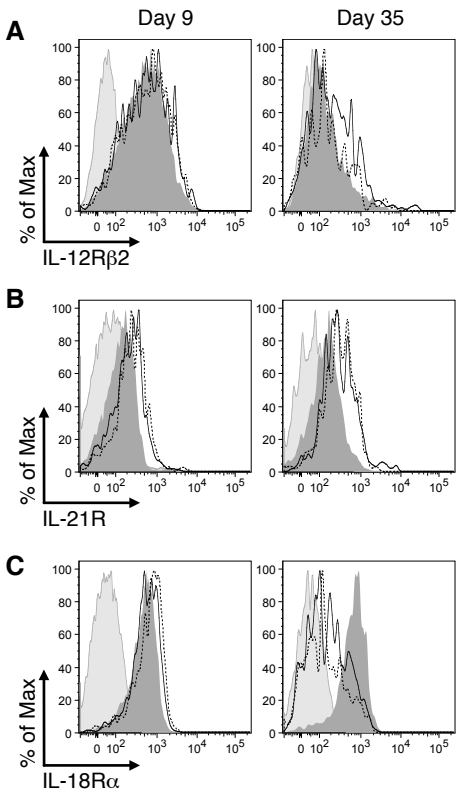

Figure 7. IL-18Rα downregulation on exhausted cells.

Representative histograms show the level of (A) IL-12Rβ2, (B) IL-18Rα, and (C) IL-21R expression on GP33-specific CD8 T cells at either day 9 or day 35 post-infection. Light shaded peak: isotype control, dark shaded peak: B6 Arm, solid line: B6 cl 13, dotted line: $CD4^{-/-}$ cl 13.

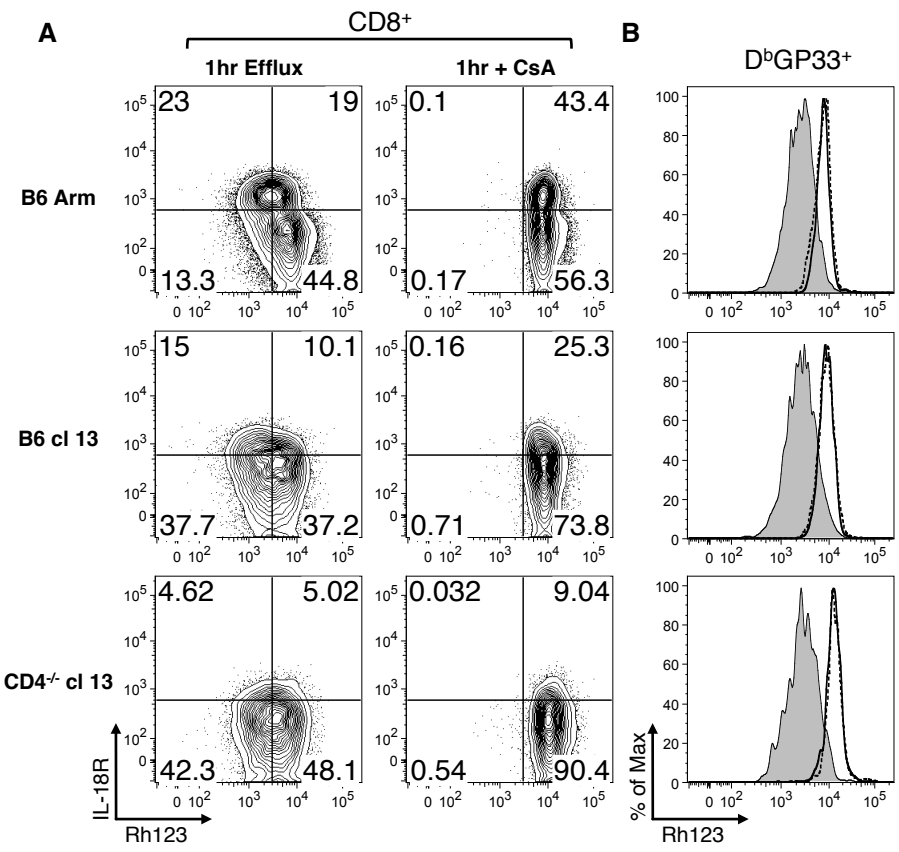

Figure 8. Rh123 efflux in memory and exhausted CD8 T cells.

Splenocytes prepared from mice at day 38 following acute, protracted, and chronic infections were loaded with Rh123 and then treated or not treated with efflux inhibitors before assessing the cells' efflux abilities. (A) The Rh123 efflux profiles of gated CD8 T cells is shown in conjunction with IL-18Rα staining. (B) The Rh123 efflux of gated LCMV GP33-specific CD8 T cells. Shaded line: without inhibitor, solid line: with cyclosporine A, dashed line: with vinblastine. Data are representative of four independent experiments with a total of 15-16 mice analyzed per group.

their memory counterparts from acutely infected mice. Thus, even though exhausted CD8 T cells have been shown to have altered maintenance requirements and proliferative properties, in chronic LCMV infection, the levels of IL-18R on virus-specific CD8 T cells do not correlate with their ability to efflux Rh123.

IL-18R expression influences the maintenance of CD8 T cells during the contraction phase.

To further examine the consequences of decreased IL-18R expression on virus-specific CD8 T cell responses, we analyzed mixed bone marrow chimeras generated by reconstituting lethally irradiated mice with a mixture of CD45.1 IL-18R$^{+/+}$ cells and either CD45.2 IL-18R$^{-/-}$ (experimental) or CD45.2 IL-18R$^{+/+}$ (control) cells. By 8 weeks following reconstitution, the mean proportion of CD8 T cells that were IL-18R$^{-/-}$ (CD45.2) was 59% with a range of 58-64% in the experimental chimeras, and in the control chimeras the fraction of CD8 T cells that were CD45.2 (IL18R$^{+/+}$) was 42% with a range of 35-46%.

Following infection with LCMV cl13, virus-specific CD45.2^{+} CD8 T cell responses were detectable in both the experimental and control chimeras. Between days 7-16 following infection, the virus-specific IL-18R$^{-/-}$ (CD45.2) cells preferentially contracted in the experimental chimeras, as the fraction of these cells decreased during this period; however, the proportion of virus-specific CD8 T cells that were CD45.2 (IL-18R$^{+/+}$) in the control chimeras remained stable (Fig. 9A). Although the IL-18R$^{-/-}$ CD8 T cells appeared to be outcompeted during the contraction phase, this disproportionate loss stabilized between days 16-26 post infection (Fig. 9 A and B). This stabilization was

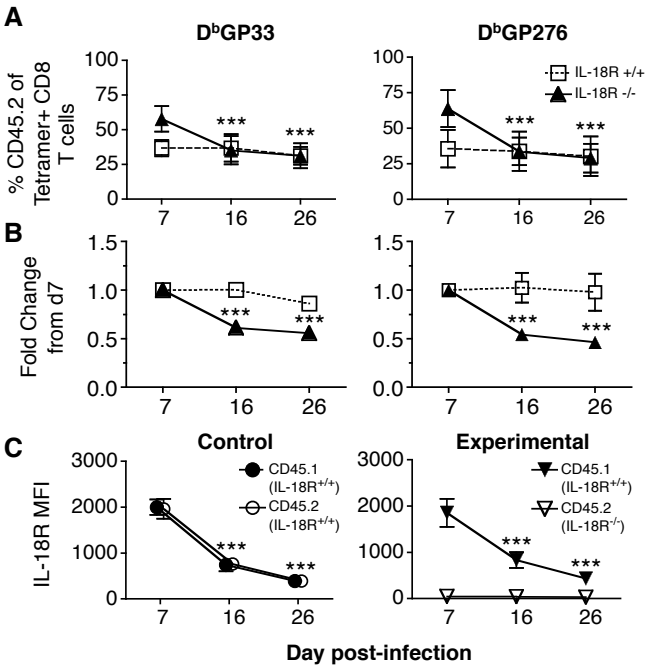

Figure 9. LCMV-specific IL-18R$^{-/-}$ cells are preferentially lost between days 7 and 16.

The maintenance of IL-18R$^{+/+}$ and IL-18R$^{-/-}$ LCMV GP33-specific T cells was monitored in mixed bone marrow chimeras following LCMV-cl 13 infection. Mice from control (CD45.1, IL-18R$^{+/+}$ and CD45.2, IL-18R$^{+/+}$) and experimental (CD45.1, IL-18R$^{+/+}$ and CD45.2, IL-18R$^{-/-}$) cohorts were bled and analyzed at the indicated days post-infection. (A) The percentage of CD45.2 LCMV GP33-specific CD8 T cells over time in the control (IL-18R$^{+/+}$, open squares) and experimental (IL-18R$^{-/-}$, filled triangles) cohorts and (B) the fold change in the CD45.2^{+}GP33^{+} population, normalized to the levels detected at day 7 post infection. (C) The MFI of IL-18R expression on CD45.1^{+} and CD45.2^{+} GP33-specific cells in the control and experimental cohorts. Data are shown from two independent experiments with (A and B) 8-11 mice per group and (C) 3-10 mice per group at each time-point; *** $p < 0.001$. Error bars are SD (A and C) or SEM (B).

concurrent with the downregulation of the IL-18R on the IL-18R$^{+/+}$ CD8 T cells present in the control and experimental chimeras (Fig. 9C). Therefore, IL-18R expression on CD8 T cells is transiently associated with a greater maintenance during the early phase of persistent LCMV infection. Nevertheless, this beneficial effect is overcome as the levels of IL-18R become downregulated on the virus-specific CD8 T cells as the exhausted state is established.

Memory but not exhausted CD8 T cells respond to bystander bacterial infection.

Given the marked downregulation of IL-18R on exhausted CD8 T cells, we evaluated whether this would compromise their ability to respond in an antigen-independent manner to a bacterial coinfection. To address this, cohorts of acute, protracted, or chronically infected mice were challenged with wild-type LM, which is known to induce the production of IL-12 and IL-18 but does not encode any know LCMV epitopes. The impact of this coinfection on the "bystander" LCMV-specific CD8 T cells was checked 20 hours later (Fig. 10A). In all cases, the CD8 T cells which became IFN-γ^+ following LM challenge were IL-18R-high, indicating that the ability to sense this proinflammatory cytokine was critical for the response (Fig. 10 B and C). LM infection caused between 66-91%, of LCMV GP33-specific memory CD8 T cells to produce IFN-γ. This response was severely curtailed in hosts undergoing protracted or chronic LCMV infections as only 11-35%, and 0.9-9%, respectively, of the virus-specific CD8 T cells detected became IFN-γ positive following LM coinfection (Fig. 11A). We further checked whether LM infection also resulted in increased CD25 expression on the LCMV-specific CD8 T cells. Again, although the memory CD8 T cells did upregulate

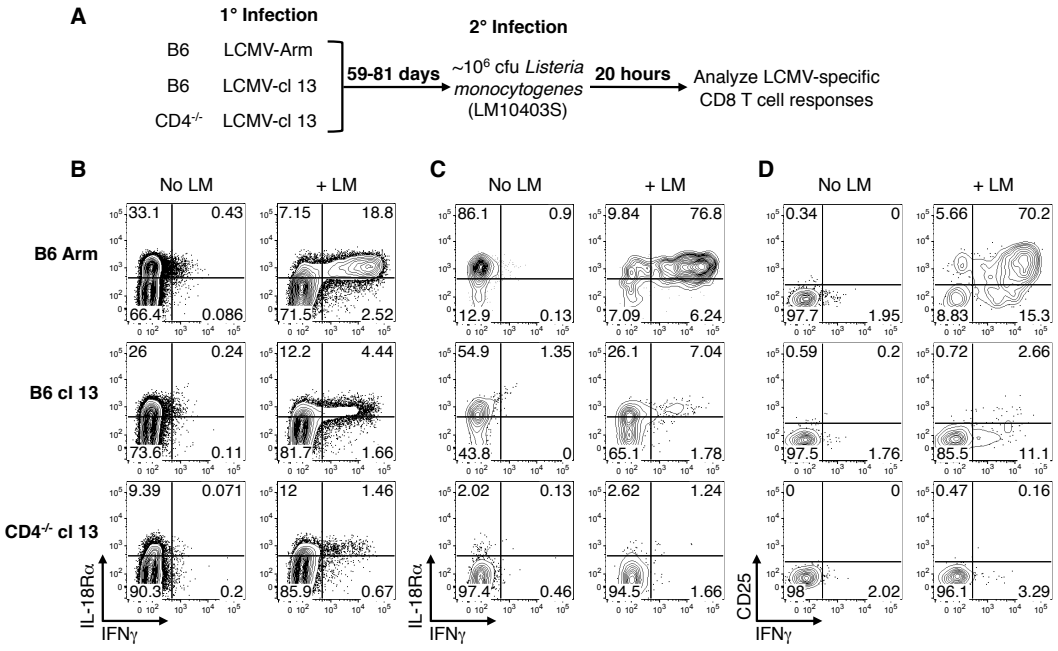

Figure 10. IL-18R-high memory CD8 T cells, but not IL-18R-low exhausted CD8 T cells, respond in an innate-like manner to LM coinfection.

Mice with acute, protracted, and chronic infections were challenged with LM, and the bystander response of their LCMV-specific CD8 T cells was analyzed 20h later. (A) Overview of the experimental design. (B) The levels of IL-18Rα expression and IFN-γ production by gated CD8 T cells are shown with and without LM infection. The levels of IL-18Rα (C) and CD25 (D) staining in conjunction with IFN-γ production is shown for gated D^bGP33^+ CD8 T cells. Flow cytometry plots shown are representative of data from three independent experiments analyzing a total of 11-12 LM-challenged mice per group.

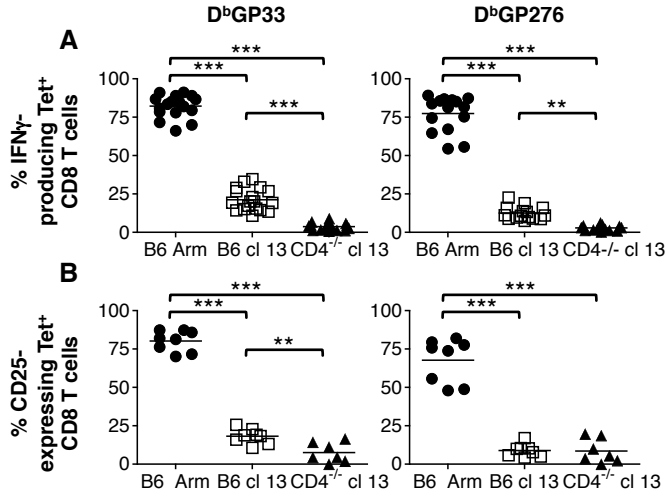

Figure 11. Memory, but not exhausted, LCMV-specific CD8 T cells produce IFN-γ and upregulate CD25 in response to bystander LM infection.

Cohorts of mice were challenged with wild-type LM between 59-81 days following acute (LCMV-Arm; black circles), protracted (LCMV-cl 13 infected B6 mice; white squares) and chronic (LCMV-cl 13 infected $CD4^{-/-}$ mice; black triangles) infections. Splenocytes were prepared 20h after the bacterial infection and then cultured in the presence of BFA for 3 hours prior to surface MHC tetramer staining and intracellular staining for IFN-γ accumulation. The percentages of IFN-γ-producing (A) and CD25-expressing (B) LCMV epitope-specific CD8 T cells are shown for individual mice, and mean values are indicated. The results are from 4-5 experiments with 15-18 mice per group in panel A or from two experiments with 6-8 mice per group in panel B; *** $p < 0.001$, ** $p < 0.01$.

CD25, the responsiveness of the exhausted cells was significantly diminished, which was consistent with the observations of IFN-γ production (Fig. 10D and Fig. 11B). Thus, whereas memory CD8 T cells can vigorously respond to sets of inflammatory cytokines and certain bacterial infections, the development of exhaustion is associated with the inability to respond to both the underlying viral infection and the inflammatory cytokine milieu.

DISCUSSION

Our investigation of the sensitivity of virus-specific CD8 T cell populations to stimulation with combinations of IL-12, IL-18, and IL-21 highlights the developmental divergence between memory and exhausted T cells. Although exhausted and memory CD8 T cell populations are identifiable by their signature levels of IL-18R, the effector populations which develop during the earlier stages of acute, protracted, and chronic LCMV infection are more similar, and in all cases these effector populations express the IL-18R and produced IFN-γ in response to cytokine stimulations. Distinct differences manifest as constituents of the effector pool transition into the memory compartment following acute infection, and as the exhausted state progressively develops if the infection persists. The downregulation of IL-18R by exhausted T cells correlates with their loss of reactivity to antigen-independent activation, whereas memory cells are clearly distinguished by the maintenance of IL-18R expression and sustained responsiveness to the cytokine combinations tested. These observations provide further insights into how virus-specific T cells adjust to sustained presence of antigen and the unique environmental conditions that occur in the persistently infected host.

The pattern of receptors expressed by exhausted CD8 T cells is quite distinct from that of prototypical memory T cells as exhausted cell express upregulated levels of certain inhibitory receptors including PD-1 but downregulate the IL-18R. Prototypic memory CD8 T cells are maintained at remarkably stable levels, principally due to the common-γ chain family members IL-7 and IL-15. The cognate receptors for these homeostatic cytokines are downregulated on exhausted CD8 T cells. Consequently, exhausted CD8 T cells lack the self-renewal properties of memory T cells and exhibit altered maintenance requirements [17]. The continued presence of viral antigen appears to allow exhausted CD8 T cell to bypass the normal requirements for IL-7 and IL-15, thereby preserving this exhausted population. Interestingly, in humans expression of the IL-18R is linked with the ability of memory phenotype cells to reconstitute and "self-renew" following chemotherapy, and this property is also associated with the capacity of these cells to efflux Rh123 [16]. We confirmed this finding in acutely infected mice as the ability to efflux Rh123 was primarily detected in the IL-18R-high subset of cells. These findings and the preferential deletion of IL-18R$^{-/-}$ cells in the mixed bone marrow chimeras suggests that IL-18 signaling may be involved in allowing functional cells to be retained through the contraction phage to form highly functional memory cells.

Because exhausted CD8 T cells have significantly lower IL-18R expression than memory cells, responses to antigen-independent cytokine stimulation were diminished. In chronically infected individuals, this unresponsiveness may account for increased susceptibility to opportunistic bacterial infections. Memory cells are able to rapidly respond to the presence of inflammatory cytokines to eradicate the infection; however, exhausted cells remain ineffectual in elaborating this antigen-independent response, thus

allowing the bacteria to become established. The correlation between low IL-18R expression and unresponsiveness presents a mechanism for the inability of exhausted cells to respond to cytokine activation, but it still remains unclear as to the cause of this downregulation.

MATERIALS AND METHODS

Mice and infections

C57BL/6J (B6), C57BL/6-Cd4^{tm1Mak}/J (CD4$^{-/-}$), B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ (CD45.1), B6.129S7-Rag1^{tm1Mom}/J (Rag-1$^{-/-}$), and B6.129P2-Il18r1^{tm1Aki}/J (IL-18R$^{-/-}$) mice were originally purchased from Jackson Laboratory (Bar Harbor, ME). All mice were bred and/or maintained in fully accredited facilities at the University of Alabama at Birmingham. For acute infections mice were infected by i.p. injection with 2x10^{5} PFU LCMV-Armstrong (Arm). Protracted and chronic infections were established by i.v. inoculation with 2-4x10^{6} PFU LCMV-clone 13 (cl 13) into B6 and CD4$^{-/-}$ mice, respectively. In certain experiments 0.96-3.3x10^{6} CFU of the 10403S strain of *Listeria monocytogenes* (LM) (kindly provided by Dr. D. Portnoy, University of California, Berkeley, CA) was administered by i.v injection into mice that had been infected with LCMV 59-81 days previously.

Cell preparation

Splenocytes and blood samples were processed as previously described [10]. For LM coinfection studies splenic samples were collected, prepared, and cultured for 3 hours at

37°C in medium without antibiotics but containing 10μg/ml Brefeldin A (Sigma-Aldrich, St. Louis, MO) prior to staining and flow cytometric analyses.

In vitro stimulations

Splenocytes were cultured for 5.5 hours at 37°C in the presence or absence of recombinant murine IL-12 (Biosource/Invitrogen, Camarillo, CA or Peprotech, Rocky Hill, NJ), IL-18 (Biosource/Invitrogen, Camarillo, CA), IL-21 (R&D Systems, Minneapolis, MN or Peprotech, Rocky Hill, NJ), or various combinations of the three cytokines. All cytokines were used at a final concentration of 20ng/ml. Brefeldin A (Golgi Plug, BD Biosciences, San Jose, CA) was added for the last 1.5 hours of culture to facilitate the intracellular accumulation of IFN-γ.

Cell staining and flow cytometry

Surface and intracellular staining was performed essentially as previously described [10]. All samples were pre-treated with anti-CD16/CD32 mAb (clone 2.4G2) (UAB Immunoreagent Core) prior to staining. Surface staining was performed using various combinations of anti-CD44 (clone IM7, BD Biosciences), anti-CD25 (clone 3C7 or PC61, Biolegend, San Diego, CA), anti-IL-12Rβ2 (clone 305719, R&D Systems), anti-IL-18Rα (clone 112614, R&D Systems), and anti-IL-21R (clone 4A9, Biolegend) mAbs together with anti-CD8α clone 53-6.7 (eBioscience), and PE or allophycocyanin conjugated MHC class I tetramers. MHC tetramers were produced in house or obtained from the National Institute of Allergy and Infectious Diseases tetramer core facility, Atlanta, GA. For intracellular staining the anti-IFN-γ antibody XMG1.2 was used

(eBioscience). All samples were acquired on an LSRII flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Rhodamine 123 efflux

Splenocytes were loaded with Rhodamine 123 (Rh123) (Invitrogen; Sigma-Aldrich) in RPMI-1640 supplemented with 10% FCS, 50μM β-mercaptoethanol, 100U/ml penicillin, and 100μg/ml streptomycin for 30 minutes on ice. Samples were then washed and cultured for 1 hour at 37°C in the absence or presence of either 0.1μg/ml Cyclosporine A (Sigma-Aldrich) or 5ug/ml Vinblastine (Sigma-Aldrich). After incubation cells were washed and stained for CD8α, IL-18Rα together with PE-conjugated D^b(GP33) MHC tetramers, as described above. Samples were resuspended in 0.1% BSA and 0.01% NaN_3in PBS prior to flow cytometric analyses, and Rh123 fluorescence was detected using a 530/30 bandpass filter.

Mixed bone marrow chimeras

Chimeras were generated essentially as previously described [18]. Bone marrow from CD45.1 (IL-18$R^{+/+}$), CD45.2 IL-18$R^{-/-}$, and CD45.2 B6 (IL-18$R^{+/+}$) mice was T cell depleted using anti-CD5 (Ly-1) microbeads (Miltenyi Biotec, Auburn, CA). Recipient RAG-$1^{-/-}$ mice were administered a split dose of radiation to give a total exposure of ~1000 rads. These recipient mice were then reconstituted by i.v. injection with an approximate 50:50 ratio of either CD45.1 (IL-18$R^{+/+}$): CD45.2 (IL-18$R^{+/+}$) bone marrow, to generate a control cohort, or CD45.1 (IL-18$R^{+/+}$): CD45.2 (IL-18$R^{-/-}$) bone marrow, to

generate an experimental cohort. The mice were infected with $4x10^6$ PFU cl 13 at 9 or 11 weeks after reconstitution.

Statistical analysis

One-way ANOVA was used to determine statistical significance. P values were calculated using Prism software (Graph Pad, San Diego, CA). Statistical significance is defined as *p<0.05, **p<0.01, ***p<0.001.

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CONCLUSIONS

The impaired ability of exhausted CD8 T cells to respond to antigen and cytokine stimulation is significant, as the unresponsive cells are found in many chronic infections and are associated with the failure to eradicate the virus. As the viral load in the host increases, the level of exhaustion increases to a point where the virus-specific cells are eventually deleted. Although these exhausted cells are ineffective at controlling the infection, the decreased responsiveness of these cells protects the host from lethal immunopathology. Because exhausted cells also fail to respond to antigen-independent stimulation by inflammatory cytokines, this potentially renders the host more susceptible to opportunistic infections. Thus, by studying the characteristics and mechanisms of exhaustion, it may be provide therapeutic targets for the treatment or prevention of chronic viral infections.

In this study, we have analyzed the responses of exhausted cells to antigen-independent cytokine stimulation. Although exhausted cells are known to be refractory to TCR stimulation, their capacity to respond to non-specific stimulation with cytokines has never been explored. By determining whether or not exhausted cells could respond to TCR-independent activation, we hoped to identify either a method to improve responsiveness during chronic infection or to better define the mechanism driving unresponsiveness in these cells. We found that stimulating with IL-12, IL-18, and IL-21 alone or in combination did not elicit robust IFN-γ production in exhausted CD8 T cells.

Along with this unresponsiveness we also showed that the IL-18Rα is significantly lower in the exhausted cells, which we believe is the reason these cells cannot respond to the cytokine stimulation. We did not detect significant differences in the expression of the other cognate receptors, IL-12Rβ2 and IL-21R. Following challenge with LM, memory cells were susceptible to bystander activation, but exhausted cells were unable to produce IFN-γ.

In analyzing further qualities of the exhausted CD8 T cells, we found that the cells producing IFN-γ following cytokine stimulation and LM challenge also greatly upregulate CD25. Because CD25 is the high affinity IL-2R chain, it is possible that the failure of the exhausted cells to upregulate CD25 greatly impairs the ability of these cells to respond to IL-2 signals and proliferate. This could contribute to the deterioration in virus-specific responses that we observe over time as it has been shown that treatment of chronically infected mice with exogenous IL-2 can boost virus-specific T cell numbers.

In addition to identifying dysregulation of the IL-18R and CD25 on exhausted cells, we also analyzed the maintenance of IL-18R$^{-/-}$ cells in a competitive environment. From the mixed bone marrow chimeras, we concluded that the decrease in percentage of these cells between days 7 and 16 might indicate the need for these signals during the contraction phase.

In order to address the possibility of IL-18R expressing cells being able to have better self-renewing capabilities indicated by Rh123 efflux, we determined these abilities in acute, protracted, and chronic LCMV infection. Because we found that the IL-18R-low cells of the acute versus chronic infections behaved differently, we concluded that this is not a full indication of self-renewal populations, but perhaps in an environment

with both IL-18R-high and IL-18R-low cells, the receptor expressing cells preferentially efflux. These differences could indicate that perhaps with exhaustion, there is another distinction other than IL-18R to be made to indicate which of the effluxing cells are most beneficial for self-renewal.

Collectively, these studies indicate that exhausted CD8 T cells are unable to respond to innate signals that for normal memory cells would provide a reinforcing method of infection control. As some infections are rapidly resolved simply by bystander activation before full activation of a specific memory response can be elicited, the unresponsiveness of exhausted cells to TCR stimulation and bystander activation thus decreases the overall response in the infected hosts. Decreased responsiveness on this scale allows for the susceptibility to opportunistic infections that would otherwise be quickly cleared.

Along with investigating the defectiveness of these responses, we have identified a couple of dysregulated surface markers. Defective CD25 upregulation indicates that the IL-2 signals necessary for functional memory formation and proliferation are not being received. Also, the IL-18R downregulation during the contraction phase correlates with the decreased maintenance found in cells that lack the IL-18R, and IL-18R expression may not be a marker for self-renewing cells based on Rh123 efflux.

These studies identify a possible mechanism for the unresponsiveness of exhausted cells to antigen-independent activation; however, additional experiments will need to be conducted in order to determine the exact defect(s) that exist in their inability to respond. It would be beneficial to first determine and compare the levels of cytokine expression in the chronically infected mice to those in the acutely infected. Perhaps the

downregulation of the IL-18R is a direct result of elevated levels of IL-18, and the unresponsiveness is due to a defect in the signaling pathway. Because IL-18 is also bound by IL-18Rbp, the levels of this soluble receptor should be determined. If these experiments show no abnormality in the level of cytokine or receptor, then defects in the signaling pathway may be explored. Because exhausted cells from chronically infected mice exhibit multiple changes in their regulation of transcription factors and receptor expression, multiple factors may affect the responses to TCR-independent activation. In preliminary experiments, we have found that expression of the inhibitory receptor CD200 is elevated on exhausted cells. Because some inhibitory receptor blockades have restored partial function in exhausted cells, it is possible that these treatments can restore the levels of IL-18R expression. It would be of interest to determine if the inhibitory receptor blocking treatments would restore the ability of exhausted cells to respond to cytokine activation in addition to antigenic stimulation.

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APPENDIX

IACUC APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: February 9, 2010

TO: Zajac, Allan
BBRB 446 2170
975-5644

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: IL-21 and Immune Mediated Viral Control
Sponsor: NIH
Animal Project Number: 100208741

On February 9, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	C	3025
Mice	A	1295

Animal use is scheduled for review one year from February 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100208741 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: March 15, 2010

TO: Zajac, Allan
BBRB 446 2170
975-5644

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Regulation of T Cell Activity During Chronic Infections
Sponsor: NIH
Animal Project Number: 100307945

On March 15, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	C	1893
Mice	A	130

Animal use is scheduled for review one year from March 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100307945 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: March 16, 2010

TO: Zajac, Allan
BBRB 446 2170
975-5644

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Helper-Dependent CD8 T Cell Memory
Sponsor: NIH
Animal Project Number: 100307972

On March 16, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	C	3049
Mice	A	911

Animal use is scheduled for review one year from March 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100307972 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.