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Cytokine-Mediated Programmed Proliferation of Virus-Specific CD8$^+$ Memory T Cells

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SUMMARY

During infection, CD8$^+$ T cells not only respond to antigenic signals through their T cell receptor (TCR) but also incorporate inflammatory signals from cytokines produced in the local infected microenvironment. Transient TCR-mediated stimulation will result in programmed proliferation that continues despite removal of the antigenic stimulus, but it remains unclear whether brief exposure to specific cytokines will elicit similar effects. Here, we have demonstrated that brief stimulation of memory T cells with interleukin-12 (IL-12) and interleukin-18 (IL-18) results in tightly regulated programmed proliferation, in addition to acquisition of enhanced virus-specific cytokine production and cytolytic activity. CD8$^+$ T cells briefly exposed to IL-12 and IL-18 in vitro showed improved antiviral activity in vivo, as demonstrated by increased proliferation and reduced viremia. These results indicate that even transitory exposure to inflammatory cytokines can provide a selective advantage to infiltrating CD8$^+$ T cells by triggering a developmental program that is initiated prior to direct contact with virus-infected cells.

INTRODUCTION

T cell exposure to inflammatory cytokines is common during viral and microbial infections, but little is known about the short-term or long-term consequences that this might have on pre-existing memory T cells or their subsequent antiviral functions. Although the majority of T cells that respond to a given infection are antigen specific (Butz and Bevan, 1998; Miller et al., 2008; Murali-Krishna et al., 1998), heterologous infection might nonetheless trigger a degree of bystander activation and proliferation of memory T cells (Ehl et al., 1997; Kim et al., 2002; Masopust et al., 2007). This might be due to a combination of inflammatory cytokine release (Kim et al., 2002; Tough et al., 1997), as well as potential stimulation by cross-reactive peptide epitopes (Brehm et al., 2002). Further studies have indicated that administration of inflammatory agents, such as lipopolysaccharide (LPS) or poly I:C, will also induce limited CD8$^+$ T cell proliferation in vivo (Kim et al., 2002; Tough et al., 1997), but it is unclear which cytokines are induced under these conditions or how long T cells are exposed to the inflammatory microenvironment. Although it was initially believed that continuous antigenic stimulation was required for maintaining CD8$^+$ T cell proliferation, several studies have demonstrated that T cells might undergo programmed proliferation in the absence of continued stimulation through the TCR (Kaech and Ahmed, 2001; Masopust et al., 2004; Mercado et al., 2000; van Stipdonk et al., 2003; van Stipdonk et al., 2001; Wong and Pamer, 2001, 2004). Bearing this in mind, a number of questions remain. For instance, can cytokines alone trigger programmed proliferation and differentiation of memory T cells after removal of the initial inflammatory signal? Could nonantigenic, cytokine-induced programmed proliferation of CD8$^+$ T cells have a measurable impact on their antiviral functions?

In these studies, we examined several memory T cell characteristics, including proliferative capacity, cytokine production, and cytolytic potential, after a brief (5 hr) exposure to a defined inflammatory microenvironment containing cytokines interleukin-12 (IL-12) and interleukin-18 (IL-18). We found that virus-specific memory T cells did not require further antigenic stimulation in order to initiate programmed proliferation in response to IL-12 and IL-18 and that during the course of programmed proliferation, these cells differentiated into strong effector T cells with enhanced antiviral functions. Moreover, brief exposure to IL-12 and IL-18 provided a proliferative advantage during acute viral infection in vivo and resulted in reduced viremia. The programmed proliferation induced by IL-12 and IL-18 in CD8$^+$ T cells was tightly regulated by CD4$^+$ T cell help in the form of local paracrine interleukin-2 (IL-2) production, and this might be a mechanism for limiting excessive bystander activation of T cells during heterologous infection. On the basis of these results, we propose a model in which memory CD8$^+$ T cells begin to proliferate and differentiate into activated effector T cells upon encountering an inflammatory microenvironment found at the periphery of a site of infection and that this enhances the host CD8$^+$ T cell response by initiating a proliferative program and upregulating antiviral functions (e.g., enhanced cytokine production and cytolytic activity) prior to engagement with virus-infected target cells during migration into the infectious foci.

RESULTS

Programmed Proliferation of Virus-Specific CD8$^+$ T Cells after Exposure to IL-12 and IL-18

IL-12 and IL-18 are well-established activators of CD8$^+$ T cells (Freeman et al., 2012), and exposure to this cytokine

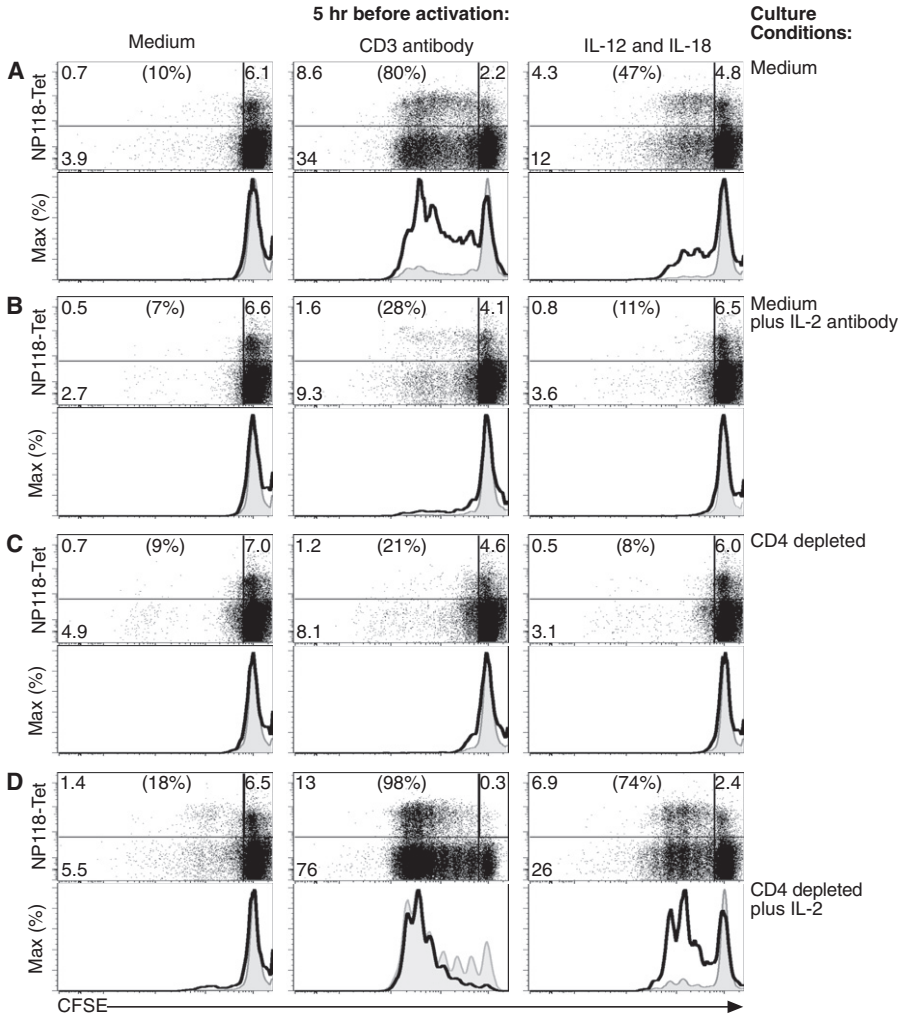

Figure 1. IL-12 and IL-18 Program CD8$^+$ T Cells to Proliferate in the Absence of Stimulation through the TCR

Spleen cells from LCMV-immune mice (>100 days postinfection) were labeled with CFSE and cultured for 5 hr with medium, plate-bound CD3 antibody, or IL-12 and IL-18 (10 ng/ml each). Cells were washed and then cultured for 3 days in (A) medium alone, (B) medium containing neutralizing IL-2 antibody (10 μg/ml), (C) medium after depletion of CD4$^+$ T cells, or (D) medium in which CD4$^+$ T cells were depleted and cultures were supplemented with exogenous IL-2 (1.5 ng/ml). Dot plots were gated on CD8$^+$ T cells, and the associated histograms were gated on NP118-tetramer$^+$ CD8$^+$ T cells (thick solid line) or NP118-tetramer$^-$ CD8$^+$ T cells (thin line, gray fill). Proliferation is indicated by the step-wise loss of CFSE label. After 3 days of in vitro culture, NP118-tetramer$^+$ CD8$^+$ T cell recoveries were 3.9 × 10^4 (IL-12 and IL-18), 3.2 × 10^4 (CD3 antibody), and 3.1 × 10^4 (medium) per well. Data are representative of six experiments.

combination leads to the rapid activation of T cells specific to lymphocytic choriomeningitis virus (LCMV) and the production of interferon-γ (IFN-γ) at concentrations that are comparable to that elicited by stimulation through the TCR (Figure S1A, available online [Beadling and Slifka, 2005; Raué et al., 2004]). This phenomenon is limited to CD8$^+$ T cells with a CD11a^{hi} phenotype because naive T cells with a CD11a^{lo} phenotype are unresponsive to stimulation by this cytokine pair (Raué et al., 2004). In addition, exposure to IL-12 and IL-18 or TCR stimulation by plate-bound CD3 antibody for as little as 5 hr induced upregulation of CD69 (Figure S1B), an early marker of T cell activation and an inhibitor of T cell egress from sites of inflammation (Shiow et al., 2006), as well as upregulation of CD25 (Figure S1C), another important activation marker that also comprises a subunit of the high-affinity IL-2 receptor. These observations indicate that both TCR stimulation and cytokine stimulation are effective at activating CD8$^+$ T cells, but little is known about the downstream outcome on proliferation, cytokine production, or cytolytic activity when measured in parallel. For these studies, spleen cells from mice infected with LCMV were stained with the vital dye carboxyfluorescein diacetate succinimidyl ester (CFSE), which has been used extensively for examining the role of cell division in the differentiation of T cells (Bird et al., 1998; Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000; Murali-Krishna et al., 1999). After 3 days of in vitro culture, LCMV-NP118-tetramer$^+$ CD8$^+$ T cells that had been precultured for 5 hr in medium alone failed to proliferate without exposure to either cytokines or antigenic stimulation through the TCR (Figure 1A). As expected, 5 hr of preactivation with CD3 antibody was sufficient to trigger programmed proliferation by a subset of T cells

after removal of the TCR-mediated stimulus. Similarly, we found that a subpopulation of LCMV-NP118-specific $CD8^+$ T cells that had been preactivated with IL-12 and IL-18 for just 5 hr also reproducibly underwent three to four rounds of cell division in the absence of activation through the TCR (Figure 1A and data not shown). Residual IL-12 and IL-18 were not a factor because >99.999% of these cytokines were removed during the washing steps performed immediately after the initial 5 hr stimulation period (Beadling and Slifka, 2005). To determine the mechanisms underlying the observed programmed proliferation of T cells after either TCR-mediated or cytokine-mediated stimulation, we cultured the preactivated T cells in medium containing a neutralizing IL-2 antibody (10 μg/ml). Blocking IL-2 activity resulted in sharply reduced programmed proliferation (Figure 1B). Autocrine IL-2 production by the brief stimulation of $CD8^+$ T cells (Feau et al., 2011; Spierings et al., 2006) was an unlikely mechanism of action because we found that purified $CD8^+$ T cells (obtained by magnetic activated cell sorting [MACS]) that were preactivated with either CD3 antibody or IL-12 and IL-18 failed to proliferate in vitro unless the cultures were supplemented with exogenous IL-2 (Figure S1D). To determine whether $CD4^+$ T cells were the cellular source of IL-2, we specifically depleted $CD4^+$ T cells by MACS (>90% depletion; data not shown) prior to the 3 day culture period (Figure 1C). Removal of $CD4^+$ T cells resulted in dramatic inhibition of $CD8^+$ T cell proliferation induced by brief exposure to CD3 antibody or IL-12 and IL-18, thus indicating a need for $CD4^+$ T cell help in this process. Culturing purified $CD8^+$ T cells from LCMV-immune mice with naive $CD4^+$ T cells in a mixed splenocyte population was insufficient to rescue cytokine-induced programmed proliferation (Figure S1E), further suggesting the importance of local cross-talk between memory $CD4^+$ and $CD8^+$ T cell populations during the early stages of an inflammatory immune response. Although $CD4^+$ T cells provide help to $CD8^+$ T cells (Matloubian et al., 1994; Shedlock and Shen, 2003; Sun and Bevan, 2003; von Herrath et al., 1996) by a variety of means, including CD40-CD40L interactions (Andreasen et al., 2000; Borrow et al., 1996; Whitmire et al., 1996) or secretion of stimulatory cytokines such as interleukin-21 (Elsaesser et al., 2009; Gagnon et al., 2008; Yi et al., 2009), the fact that we found that specific depletion of IL-2 alone blocked $CD8^+$ T cell proliferation (Figure 1B) indicates that this cytokine is necessary for programmed proliferation to proceed. $CD8^+$ T cells appeared to be highly sensitive to low concentrations of IL-2 given that only a small fraction of $CD4^+$ T cells from LCMV-immune mice spontaneously produced IL-2 directly ex vivo, and this was not augmented by incubation with IL-12 and IL-18 (Figure S2A). To determine whether IL-2 was sufficient to orchestrate programmed proliferation, we supplemented $CD4^+$ T cell-depleted cultures with exogenous IL-2 (1.5 ng/ml) during the 3 day culture period (Figure 1D). Although virus-specific memory T cells cultured in medium alone remained nonresponsive to low-dose IL-2, programmed proliferation by $CD8^+$ T cells stimulated by either CD3 antibody or IL-12 and IL-18 was restored (Figure 1 and Figures S1D and S1E). Together, these results indicate that IL-2 is both necessary and sufficient for programmed proliferation of $CD8^+$ T cells in vitro after stimulation through the TCR or after a brief encounter with inflammatory cytokines IL-12 and IL-18.

Cytokine-Induced Proliferating $CD8^+$ T Cells Demonstrate Enhanced Peptide-Specific Cytokine Production

Previous studies have shown an association between proliferation and enhanced T cell effector function (Auphan-Anezin et al., 2003; Bajénoff et al., 2002; Bird et al., 1998; Murali-Krishna and Ahmed, 2000; Opferman et al., 1999). In the next series of experiments, we examined the link between proliferation and peptide-specific cytokine responses by memory T cells that had been briefly cultured in medium (negative control) or prestimulated for 5 hr with CD3 antibody or the combination of IL-12 and IL-18 prior to 3 days of culture in unsupplemented medium (Figure 2). The majority of memory T cells that were cultured in medium alone remained $CFSE^{hi}$, indicating that they had not proliferated during the 3 day culture period. As expected, their cytokine profile maintained a largely "memory" phenotype (Belz et al., 2001; Slifka and Whitton, 2000), and ~90% of the IFN-γ^+ $CD8^+$ T cells also produced tumor necrosis factor α (TNF-α) after 6 hr of stimulation with LCMV-NP118-coated A20 cells (Figure 2). Parallel analysis using NP118-tetramer staining showed approximately a 1:1 ratio between NP118-$tetramer^+$ $CD8^+$ T cells and IFN-γ^+ $CD8^+$ T cells after peptide stimulation, indicating that there were no overtly dysfunctional T cells observed at 3 days after treatment with medium, CD3 antibody, or IL-12 and IL-18 (data not shown). Antiviral $CD8^+$ T cells that had been previously stimulated with CD3 antibody demonstrated normal or reduced TNF-α production after peptide stimulation: 57% of undivided $CFSE^{hi}$ IFN-γ^+ $CD8^+$ T cells coexpressed TNF-α, and 84% of T cells that had undergone one or more rounds of cell division ($CFSE^{lo/int}$) remained capable of producing both cytokines after antigenic stimulation. Brief pre-exposure to IL-12 and IL-18 at 3 days prior to viral peptide stimulation did not greatly alter the proportion of $CD8^+$ T cells that expressed these cytokines given that 87%–90% of the IFN-γ^+ NP118-specific $CD8^+$ T cells maintained an IFN-γ^+ TNF-α^+ cytokine profile regardless of cell division.

We found that brief prior exposure to CD3-antibody stimulation resulted in a modest defect in IL-2 production after subsequent peptide stimulation (Figure 2B). Although 25%–30% of LCMV-specific $CD8^+$ T cells cultured in medium alone were capable of producing IL-2 after peptide restimulation, T cells that had proliferated after brief TCR-mediated activation showed a reduction in IL-2 production, and <20% of virus-specific $CD8^+$ T cells produced this important cytokine after exposure to cognate peptide. In contrast, antiviral $CD8^+$ T cells that underwent programmed proliferation after transient exposure to IL-12 and IL-18 demonstrated a substantially enhanced capacity for IL-2 production after peptide stimulation; approximately 40%–60% of virus-specific IFN-γ^+ $CD8^+$ T cells were capable of producing IL-2 (Figure 2B and data not shown). This indicates that peptide-specific IL-2 production was approximately three times higher in $CD8^+$ T cells undergoing cytokine-mediated programmed proliferation than in $CD8^+$ T cells undergoing TCR-mediated programmed proliferation ($p = 0.001$).

Not only did brief exposure to inflammatory cytokines (IL-12 and IL-18) prior to antigenic stimulation with cognate peptide 3 days later result in a higher frequency of IFN-γ^+ TNF-α^+ IL-2^+ T cells (Figures 2A and 2B and data not shown), but these activated T cells also produced significantly more IFN-γ and

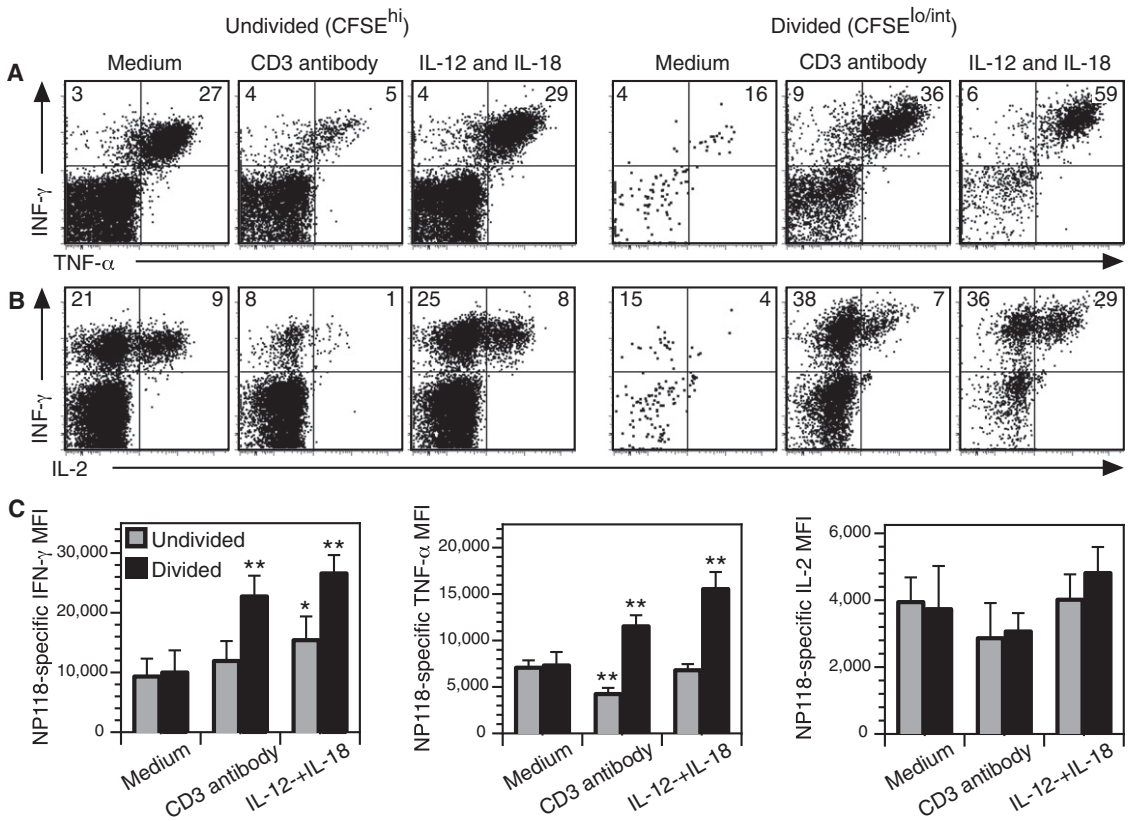

Figure 2. Virus-Specific CD8$^+$ T Cells Preactivated by IL-12 and IL-18 Respond Better to Subsequent Peptide Stimulation

CFSE-labeled cells from LCMV-immune mice were cultured in medium, IL-12 and IL-18, or plate-bound CD3 antibody for 5 hr, washed, and cultured in medium alone for 3 days. Next, cells were stimulated with NP118-peptide-coated A20 cells for 6 hr, and IFN-γ, TNF-α, and IL-2 production was determined by analysis of intracellular-cytokine staining. CD8$^+$ T cells were gated on divided (CFSE$^{lo/int}$) or undivided (CFSE^{hi}) cell populations, and the numbers in the quadrants indicate the percentage of CD8$^+$ T cells producing (A) IFN-γ and TNF-α or (B) IFN-γ and IL-2. In the absence of peptide stimulation, baseline TNF-α and IL-2 responses were $\leq$ 0.5%, and IFN-γ was identified in 0.03%–6% of CD8$^+$ T cells (Figures S2B and S2C).

(C) The mean fluorescence intensity (MFI) of cytokine expression was quantitatively determined, and the amounts of cytokine production by divided (CFSE$^{lo/int}$) CD8$^+$ T cells or undivided (CFSE^{hi}) CD8$^+$ T cells under each condition were compared to the cytokine expression by undivided CFSE^{hi} CD8$^+$ T cells previously cultured in medium alone. A single asterisk (*) indicates $p < 0.02$, and two asterisks (**) indicate $p < 0.001$. Data show the average and SD of three experiments.

TNF-α on a per-cell basis than did peptide-stimulated T cells previously cultured in medium alone (Figure 2C). This indicates that even several days after brief exposure to inflammatory cytokines, virus-specific CD8$^+$ T cells remain poised to elicit strong antiviral cytokine responses after subsequent encounter with virus-infected (or in this case, peptide-coated) target cells.

Brief Exposure to IL-12 and IL-18 Results in Differentiation of Memory T Cells into Cytolytic Effectors

Acquisition of strong cytolytic activity is a key aspect of a successful immune response mediated by CD8$^+$ T cells, and brief exposure to IL-12 and IL-18 for as little as 5 hr is sufficient to program virus-specific memory T cells to differentiate into highly cytolytic effector T cells (Figure 3). Unlike CD8$^+$ T cells cultured in medium alone, prior exposure to stimulation with either CD3 antibody or IL-12 and IL-18 resulted in substantial upregulation of granzyme B expression by virus-specific NP118-tetramer$^+$ CD8$^+$ T cells (Figure 3A). As might be expected, NP118-specific CD8$^+$ T cells that had proliferated (i.e., CFSE$^{lo/int}$) also expressed even higher amounts of granzyme B than did T cells that had not undergone programmed proliferation. To determine whether prior stimulation history would alter direct virus-specific cytolytic activity, we incubated MACS-purified CD8$^+$ T cells with peptide-coated target cells at the indicated NP118-tetramer$^+$ CD8$^+$ T cell effector-to-target (E:T) ratios (Figure 3B). NP118-specific CD8$^+$ T cells that had been cultured in medium alone showed only modest killing of peptide-coated target cells at the highest E:T ratios (<25% specific lysis). In contrast, memory CD8$^+$ T cells that had been stimulated with CD3 antibody or IL-12 and IL-18 at 3 days prior to the CTL assay demonstrated strong and equally efficient lysis of peptide-coated target cells. Collectively, these data show that a brief encounter with inflammatory cytokines, such as IL-12 and IL-18, can induce in virus-specific CD8$^+$ T cells changes that result in programmed proliferation of daughter cells that differentiate into more effective antiviral CD8$^+$ T cells—even days after removal of the initial stimulus.

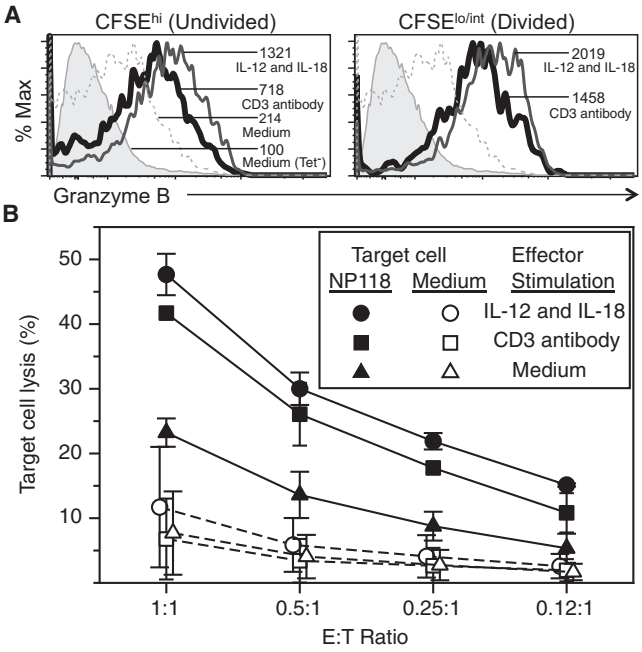

Figure 3. Memory T Cells Efficiently Kill Peptide-Coated Target Cells after Brief Activation by IL-12 and IL-18

Splenocytes containing virus-specific CD8$^+$ T cells from LCMV-immune mice (>120 days postinfection) were cultured in medium or stimulated with either plate-bound CD3 antibody or IL-12 and IL-18 for 5 hr. The cells were then washed and incubated for 3 days without additional stimulation.

(A) For the assessment of granzyme B expression at 3 days after exposure to stimulation with cytokines or CD3 antibody, T cells were segregated into undivided (CFSE^{hi}) and divided (CFSE$^{lo/int}$) groups and were stained for CD8, NP118 tetramer, and intracellular granzyme B. NP118-tetramer$^-$ CD8$^+$ T cells cultured in medium alone (Tet$^-$) served as a staining control. The numbers indicate the MFI of granzyme B in virus-specific T cells grown under the indicated conditions.

(B) At 3 days after brief stimulation with IL-12 and IL-18 or CD3 antibody, CD8$^+$ T cells were purified by MACS and small aliquots of T cells were enumerated by NP118-tetramer staining so that the peptide-specific effector-to-target (E:T) ratio would be the same in each sample during the CTL assay. Solid lines (closed symbols) indicate the percentage of lysis of targets coated with NP118 peptide, and dashed lines (open symbols) represent nonspecific lysis of uncoated targets (A20 cells). Data show the average and SD of two experiments.

Prior Exposure to IL-12 and IL-18 Improves T-Cell-Mediated Protection against Subsequent Viral Infection

Programmed proliferation in vitro might differ from the results observed in vivo (Wong and Pamer, 2004). To determine whether brief exposure to a defined combination of inflammatory cytokines invokes a relevant change in T cell responses in vivo, we cultured CFSE-labeled splenocytes containing approximately 1 × 10^5 LCMV-NP118-specific CD8$^+$ T cells for 5 hr with medium, CD3 antibody, or IL-12 and IL-18 prior to washing steps to remove exogenous cytokines, and we then adoptively transferred them into congenic recipient mice (Figure 4). LCMV-specific CD8$^+$ T cells previously cultured in medium alone showed no proliferation at 60 hr after transfer into uninfected recipients (Figure 4A). Brief CD3-antibody stimulation triggered robust programmed proliferation of virus-specific T cells after transfer into uninfected animals despite no further stimulation through the TCR. In contrast, programmed proliferation by IL-12 and IL-18 was aborted, and proliferation ceased after only one round of proliferation. This suggests that inflammatory-cytokine-induced programmed proliferation is tightly regulated and stops very quickly after T cells exit an inflammatory environment and are dispersed within uninfected tissues.

Exposure to stimulatory cytokines (e.g., IL-2) has been shown to induce or enhance proliferation but might also be involved in activation-induced cell death (AICD) (Waldmann, 2006) and might negatively impact T cell memory (Pipkin et al., 2010). To determine the long-term survival of LCMV-specific memory T cells that have been briefly activated by CD3 antibody or IL-12 and IL-18, we transferred T cells to uninfected recipient mice and measured LCMV-specific donor CD8$^+$ T cell numbers at 7, 28, and 70 days posttransfer (Figure S3A). In the absence of further antigenic or cytokine-mediated stimulation, we observed no substantial difference between the survival rates of CD3-antibody-stimulated or IL-12- and IL-18-stimulated CD8$^+$ T cells and CD8$^+$ T cells that had been precultured in medium alone. To further test the potential effects of inflammatory cytokines on T cell memory, we repeatedly injected LCMV-immune mice with LPS (which triggers IL-12 and IL-18 production by splenocytes in vitro [Raué et al., 2004]) and observed no significant impact on NP118-specific CD8$^+$ T cell memory ($p = 0.38$, Figures S3B and S3C). This indicates that brief exposure to inflammatory cytokines, such as IL-12 and IL-18, induces proliferation and enhanced antiviral T cell activity without causing overt AICD or loss of T cell memory.

To determine whether T cell responsiveness to subsequent viral infection is altered by a recent history of antigenic or inflammatory exposure, we conducted parallel experiments in which an equal number of T cells (1 × 10^5 NP118-specific CD8$^+$ T cells) were transferred into congenic mice that were infected with LCMV on the day of transfer (Figure 4A). Under these conditions, CD8$^+$ T cells that had been cultured in medium alone proliferated rapidly during the first 60 hr after transfer into LCMV-infected mice. Likewise, CD8$^+$ T cells previously stimulated by CD3 antibody also proliferated more in the infected microenvironment than they did after transfer into uninfected hosts. CD8$^+$ T cells that had been stimulated by IL-12 and IL-18 prior to transfer demonstrated the highest amount of proliferation in response to LCMV infection. Because high rates of proliferation might not necessarily indicate that higher protective efficacy is achieved, we also measured viremia after LCMV infection (Figure 4B). After adoptive transfer and challenge with LCMV-Armstrong, CD8$^+$ T cells previously stimulated with CD3 antibody performed similarly to the unstimulated medium-only controls, suggesting that 5 hr of prestimulation through the TCR might be insufficient to elicit enhanced antiviral T cell function. In contrast, mice that received CD8$^+$ T cells that had been exposed to IL-12 and IL-18 for 5 hr prior to transfer showed 65%–85% lower virus titers than did naive mice that received no T cells or naive mice that received CD8$^+$ T cells previously cultured in medium alone ($p = 0.007$ and $p = 0.036$, respectively). This indicates that the enhanced antiviral functions, including increased cytokine production, cytolytic activity, and proliferation, might have combined to provide a protective advantage against viral infection in vivo.

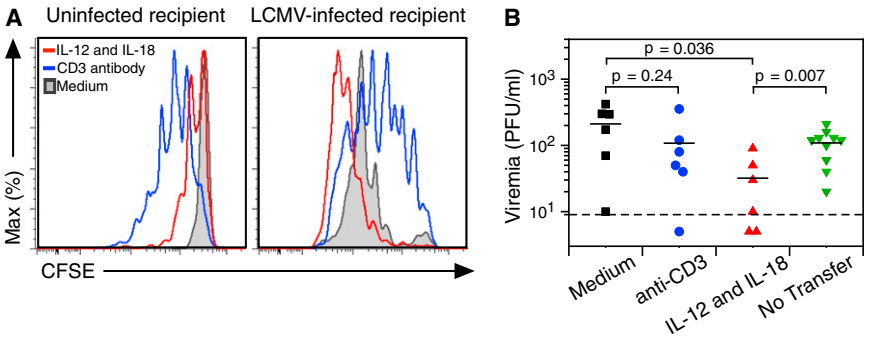

Figure 4. IL-12 and IL-18 Enhance Virus-Specific CD8$^+$ T Cell Proliferation and Result in Decreased Viremia

LCMV-immune spleen cells from BALB/c Thy1.1 mice were labeled with CFSE, stimulated with CD3 antibody (blue) or IL-12 and IL-18 (red) or incubated in medium (gray) for 5 hr, and washed prior to intravenous injection into BALB/c Thy1.2 mice.

(A) Recipient animals were uninfected (left panel) or infected with 2 × 10^5 PFU LCMV-Armstrong on the day of transfer (right panel). Proliferation of donor CD8$^+$ T cells in the spleen was measured at 60–62 hr after adoptive transfer via staining for CFSE, CD8, Thy1.1, and NP118 tetramer. After transfer, the number of NP118-tetramer$^+$ CD8$^+$ T cells recovered per spleen in LCMV-infected recipients was 2.6 × 10^4 (IL-12 and IL-18), 7.4 × 10^3 (CD3 antibody), and 7.9 × 10^3 (medium).

(B) Rapid acquisition of antiviral activity was measured by the quantitation of infectious LCMV in the serum (i.e., viremia) in comparison with mice that received no transferred cells. Data in (A) are representative of three to four mice per group from at least three experiments, and data in (B) show the results of five to six mice per group in four experiments, except for the "no transfer" group, which is based on ten mice from two experiments.

DISCUSSION

In this study, we identified programmed proliferation after transient exposure to inflammatory cytokines IL-12 and IL-18 as a previously unrecognized feature of virus-specific T cell memory. Although naive CD8$^+$ T cells are largely refractory to stimulation by IL-12 and IL-18, it was expected that the combination of IL-12 and IL-18 would activate virus-specific CD8$^+$ T cells (Berg et al., 2002; Berg et al., 2003; Kambayashi et al., 2003; Lertmemongkolchai et al., 2001; Raué et al., 2004; Tough et al., 2001). However, only a brief encounter with these two inflammatory cytokines was sufficient to also induce a developmental program that resulted not only in continued proliferation but also in enhanced antiviral functions that were maintained for several days after removal of the initial stimulus. Programmed proliferation of CD8$^+$ T cells appeared to be regulated by the availability of local CD4$^+$ T cell help, and we speculate that this might be an important factor in reducing unrestricted systemic proliferation, as well as potential T-cell-mediated immunopathology outside of the focal zone of an active infection.

Signaling through either the TCR or the IL-12 and IL-18 receptors results in the activation of a group of transcription factors that partially, but not completely, overlap (Carroll et al., 2008; Smith-Garvin et al., 2009; Verdeil et al., 2006; Watford et al., 2004). TCR ligation and costimulation lead to phosphorylation of p56^{lck} (Lck), zeta-chain-associated protein kinase 70 (ZAP70), and linker for activation of T cells (LAT), ultimately leading to the activation of a variety of transcription factors, including nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), and nuclear factor of activated T cells (NFAT). Binding of IL-12 to its receptor primarily results in the activation of the Janus kinase (Jak) and signal transducer and activator of transcription (STAT) pathway via Jak2 and STAT4, as well as the activation of the mitogen-activated protein kinase (MAPK) cascade, particularly p38. The IL-18 receptor is a member of the interleukin-1 and toll-like receptor family, and it signals via immune-receptor-activated kinases (IRAKs) to activate NF-κB, AP-1, and other transcription factors. Because of the commonalities between TCR- and cytokine-induced transcription factors (e.g., NF-κB activation), stimulation of T cells with peptide or cytokines results in some shared outcomes (e.g., IFN-γ production and CD69 and CD25 upregulation), but not others. For instance, TNF-α and IL-2 are induced by peptide stimulation, but not by IL-12 and IL-18 stimulation (Beadling and Slifka, 2005). Differences between the precise combinations of signaling cascades and transcription factors that are activated by innate versus antigenic signals are likely to contribute to the distinct cellular outcomes observed after exposure to these different stimuli.

Several interrelated factors appear to be involved with the regulation of cytokine-induced programmed proliferation and differentiation. First, exposure to IL-12 and IL-18 initiates antiviral IFN-γ production by LCMV-specific CD8$^+$ memory T cells within just 1–2 hr and IFN-γ expression peaks within 6–8 hr (Beadling and Slifka, 2005; Raué et al., 2004). This is assumed to act as an innate "sentinel response" to a local, yet unidentified microbial infection (Kambayashi et al., 2003; Lertmemongkolchai et al., 2001) prior to direct contact between the infiltrating T cells and infected target cells. On the basis of prior studies (Nguyen and Biron, 1999; Raué et al., 2004), it is likely that the IL-12 and IL-18 concentrations used in this current study for activating CD8$^+$ T cells in vitro are within the range of what might occur in vivo during an inflammatory immune response. For instance, injection of LPS into LCMV-infected mice causes up to one-third of CD8$^+$ T cells to become IFN-γ$^+$ within 4 hr after administration (Nguyen and Biron, 1999), even though LPS does not stimulate CD8$^+$ T cells directly (Raué et al., 2004). Instead, LPS triggers the endogenous production of IL-12 and IL-18 (among other cytokines) that together elicit T cell activation and IFN-γ production. Indeed, exposing splenocytes from LCMV-infected mice to LPS results in rapid IL-12 and IL-18 production, which subsequently triggers IFN-γ production by CD8$^+$ T cells within just 6 hr of in vitro culture (Raué et al., 2004). LPS does not trigger IFN-γ production in purified CD8$^+$ T cells, indicating that de novo production of inflammatory cytokines by accessory splenic cells is required for mediating this effect. Depletion of LPS-induced IL-12 and IL-18 by the addition of neutralizing antibodies to these two specific cytokines resulted in >90% reduction in CD8$^+$ T cell-mediated IFN-γ production, demonstrating that these two cytokines are responsible for the indirect LPS-induced T cell activation and IFN-γ expression by CD8$^+$ T cells. Together with the data from in vivo administration of LPS (Nguyen and Biron, 1999), these

studies show that local accessory cells in the spleen are capable of secreting enough IL-12 and IL-18 within 4–6 hr to elicit T cell activation and cytokine production similar to the results that we observed by direct supplementation of media with defined concentrations of these two cytokines.

In addition to rapidly producing IFN-γ, memory $CD8^+$ T cells also upregulate CD69 and CD25 expression within 5 hr of exposure to IL-12 and IL-18. Upregulation of CD69 inhibits T cell egress from the zone of inflammation, acting as an important mechanism for maintaining the local T cell population on site until the inflammatory insult has abated. Upregulation of CD25 also provides virus-specific memory T cells with the capacity to respond to proliferative signals generated by IL-2. Although the number of $CD4^+$ T cells that spontaneously produce IL-2 in vitro is low, it is possible that IL-12- and IL-18-induced upregulation of high-affinity IL-2 receptor CD25 is key to enhanced sensitivity to local IL-2 production and could be an important mechanism for limiting $CD8^+$ T cell proliferation to sites of acute infection. Regulatory T (Treg) cells are also believed to be major consumers of IL-2, and it is unknown whether exposure to IL-12 and IL-18 alters their regulatory functions or their IL-2 consumption in the context of an infected local microenvironment. Interestingly, although IL-12 and IL-18 trigger strong IFN-γ responses, this interaction does not elicit detectable IL-2 production by $CD8^+$ T cells (Beadling and Slifka, 2005), and this mechanism most likely explains why MACS-purified $CD8^+$ T cells were unable to undergo programmed proliferation on their own. Likewise, purified $CD8^+$ T cells stimulated with CD3 antibody also failed to undergo programmed proliferation, but this might have been due to insufficient activation. Prior studies examining naive $CD8^+$ T cells have shown that insufficient TCR-mediated stimulation results in no further T cell expansion or only abortive clonal expansion (Mercado et al., 2000; van Stipdonk et al., 2003). We anticipated that 5 hr of CD3-antibody stimulation would have been sufficient for full activation of antigen-primed memory T cells, especially given that this approach resulted in strong IFN-γ production, upregulation of CD69 and CD25, and programmed proliferation in vitro and in vivo. However, brief stimulation with CD3 antibody was not sufficient to improve rapid antiviral T cell responses in vivo, and this might be due to a number of factors, including the nature of the stimulus (e.g., plate-bound CD3 antibody) or the short duration of stimulation. Another important question is how long cytokine-mediated enhancement of antiviral $CD8^+$ T cell function will persist. For example, one would not expect permanent upregulation of CD69 and CD25 expression after brief exposure to an inflammatory microenvironment, and it is probable that as T cells revert back to a resting memory state, their antiviral functions also revert back to their baseline characteristics. Understanding the mechanisms underlying these observations and determining how long enhanced protective antiviral activity is maintained will clearly require further studies.

During cytokine-induced programmed proliferation, activated $CD25^+$ $CD8^+$ T cells were primed to proliferate when given the appropriate signals, but this required costimulation in the form of IL-2 production by local $CD4^+$ T cells. This two-signal model of programmed proliferation might explain why increased cytolytic activity (Murali-Krishna et al., 1998) and limited bystander proliferation of non-antigen-specific $CD8^+$ T cells occurs during heterologous infection (Masopust et al., 2007) or inflammation (Kim et al., 2002; Tough et al., 1997) without resulting in overt systemic proliferation of the greater memory T cell compartment. To determine the biological impact of cytokine-induced preconditioning on antiviral T cell responses in vivo, we transferred LCMV-specific $CD8^+$ T cells to recipient mice that were infected with LCMV on the same day and monitored proliferation by CFSE dilution. Cytokine-primed $CD8^+$ T cells proliferated more rapidly in the context of LCMV infection than did T cells previously primed by CD3-antibody stimulation or cultured in medium alone, and they demonstrated substantially improved reduction in viremia within just 60 hr after infection. These results, along with the observation of enhanced peptide-specific cytokine production and cytolytic activity in cytokine-primed $CD8^+$ T cells, provide the basis for our in vivo model. In this model, memory T cells responding to a local inflammatory event undergo different stages of activation, differentiation, and proliferation as they near a site of infection. By secreting IFN-γ at the perimeter of the infection, an innate barrier to viral replication and spread is initiated. As $CD8^+$ and $CD4^+$ T cells accumulate at the edge of an infectious focus, programmed proliferation is triggered, and this results in an increase in local T cell number prior to entry into the site of infection and direct recognition of infected targets. Once the T cells enter the site of infection, the combined effects of cytokine-induced and TCR-driven activation and differentiation synergize to result in an enhanced antiviral immune response.

Together, the studies described here provide an example of how $CD4^+$ T cell help might be important for enhancing antiviral $CD8^+$ T cell function. They also provide further insight into the complex interactions that might occur during homologous or heterologous infection and how this might impact memory $CD8^+$ T cell activation, proliferation, and subsequent antiviral function. Further studies will be needed for determining how long the cytokine-induced enhanced state of antiviral activity is retained by memory T cells after removal of the inflammatory stimulus. It is also unknown whether this phenomenon is common to all memory T cell populations or whether different subpopulations (e.g., effector-memory or central-memory cells) have different rates of responsiveness to inflammatory cytokine-induced programming. Likewise, it is unknown whether programmed proliferation is unique to the combination of IL-12 and IL-18 or whether other cytokines or cytokine combinations (Freeman et al., 2012) are able to elicit a similar enhancement of antiviral T cell function.

EXPERIMENTAL PROCEDURES

Mice, LCMV Infection, and Adoptive Transfer

BALB/cByJ mice were either purchased from The Jackson Laboratory (Bar Harbor, ME, USA) or bred at Oregon Health and Science University (OHSU). BALB/c Thy1.1 mice were bred at OHSU. Mice were used at 6–12 weeks of age. LCMV-immune mice were infected intraperitoneally with 2×10^5 PFU LCMV-Armstrong, and T cell analysis was performed at 28–170 days postinfection. For adoptive-transfer experiments, approximately 1×10^7 spleen cells containing 1×10^5 CFSE-labeled NP118-specific $CD8^+$ $Thy1.1^+$ T cells were transferred into $Thy1.2^+$ mice, and T cell responses and viremia (measured by LCMV plaque assay) were performed at 60–62 hr posttransfer. All experimental procedures were approved by the OHSU Institutional Animal Care and Use Committee.

Reagents

HPLC-purified (>95% pure) LCMV NP118-126 (NP118) peptide (Alpha Diagnostics, San Antonio, TX, USA) was used at 1×10^{-7} M. IL-2 (human IL-2, Amgen Thousand Oaks, CA, USA) was used at 1.5 ng/ml, and IL-12 (R&D systems, Minneapolis, MN, USA) and IL-18 (Medical & Biological Laboratories, Watertown, MA, USA) were used at 10 ng/ml. CD3ε antibody (clone 145-2C11, BD PharMingen, San Diego, CA, USA) was used for coating 24-well plates (20 μg/ml CD3ε antibody in PBS, 37°C for 1–2 hr, and washed before use). IL-2 antibody (clone JES6-1A12, R&D Systems) was used at 10 μg/ml to neutralize IL-2 in culture. H-2L^d NP118 tetramer was obtained from the National Institutes of Health Tetramer Core Facility (Atlanta, GA, USA). CD8a antibody (clone 5H10) and CD25 antibody (clone PC61 5.3) were purchased from InVitrogen (Carlsbad, CA, USA). IFN-γ antibody (clone XMG1.2), IL-2 antibody (clone JES6-5H4), TNFα antibody (clone MP6-XT22), CD69 antibody (clone H1.2F3), granzyme B antibody (clone CB11), and propidium iodine were obtained from BD PharMingen. Thy1.1 antibody (clone His51) was obtained from eBioscience (San Diego, CA, USA), and CFSE was purchased from InVitrogen.

CFSE Proliferation and Intracellular-Cytokine Staining

Single-cell suspensions and in vitro stimulation conditions were performed as previously described (Beadling and Slifka, 2005). CD8$^+$ T cell isolation and CD4$^+$ T cell depletion were performed by MACS with the use of microbeads coated with antibodies against CD8α or CD4 according to protocols supplied by Miltenyi Biotec (Auburn, CA, USA). Post-MACS analysis indicated >90% purity. Cells were labeled with 5 μM CFSE for in vivo experiments and with 1 μM CFSE for in vitro experiments. CFSE-labeled T cells were cultured in medium alone, stimulated with IL-12 and IL-18 (both 10 ng/ml), or stimulated in plates coated with CD3ε antibody in 6% CO_2 at 37°C for 5 hr. Cells were washed extensively and cultured in RPMI 10% fetal bovine serum (FBS) supplemented with 50 mM 2-mercaptoethanol in 24-well plates for 3 days (64–68 hr) for the assessment of proliferation. Antiviral-cytokine production was measured by intracellular-cytokine staining 6 hr after the addition of a 1:1 ratio of A20 cells (a B cell line) coated with 10^{-7} M NP118-126 peptide. All flow cytometry data were analyzed with FlowJo software (Treestar, Ashland, OR, USA).

Nonradioactive Flow-Cytometry-Based CTL Assay

A20 target cells were labeled with 4 μM (CFSE^{hi}) or 1.5 μM (CFSE^{lo}) CFSE. CFSE^{lo} cells were coated with peptide (10^{-7} M NP118). Target cells were washed twice and resuspended in RPMI 10% FBS. CFSE^{hi} and CFSE^{lo} A20 cells were combined 1:1 at 1×10^4 cells/ml (target cell suspension). For the CTL assay, 100 μl target cell suspension was combined with 100 μl effector cell (NP118-specific CD8$^+$ T cells) suspensions at different E:T ratios in round-bottom 96-well plates and incubated in 6% CO_2 at 37°C for 5 hr. For the assessment of CTL activity, cells were washed with PBS and stained with 0.25% propidium iodine in PBS at 4°C for 15 min. Samples were acquired immediately on a FACSCalibur (BD, San Jose, CA, USA), and percent viability was determined by propidium-iodine staining in comparison with positive and negative controls.

Statistical Analysis

A two-tailed Student's t test with unequal variance was used for evaluating statistical significance of differences between groups. A value of $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.09.019.

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