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CD8+ Memory T Cells Appear Exhausted within Hours of Acute Virus Infection

Martin P. Hosking,* Claudia T. Flynn,* Jason Botten,† and J. Lindsay Whitton*

CD8+ memory T cells are abundant and are activated in a near-synchronous manner by infection, thereby providing a unique opportunity to evaluate the coordinate functional and phenotypic changes that occur in vivo within hours of viral challenge. Using two disparate virus challenges of mice, we show that splenic CD8+ memory T cells rapidly produced IFN-γ in vivo; however, within 18–24 h, IFN-γ synthesis was terminated and remained undetectable for ≥48 h. A similar on/off response was observed in CD8+ memory T cells in the peritoneal cavity. Cessation of IFN-γ production in vivo occurred despite the continued presence of immunostimulatory viral Ag, indicating that the initial IFN-γ response had been actively downregulated and that the cells had been rendered refractory to subsequent in vivo Ag contact. Downregulation of IFN-γ synthesis was accompanied by the upregulation of inhibitory receptor expression on the T cells, and ex vivo analyses using synthetic peptides revealed a concurrent hierarchical loss of cytokine responsiveness (IL-2, then TNF, then IFN-γ) taking place during the first 24 h following Ag contact. Thus, within hours of virus challenge, CD8+ memory T cells display the standard hallmarks of T cell exhaustion, a phenotype that previously was associated only with chronic diseases and that is generally viewed as a gradually developing and pathological change in T cell function. Our data suggest that, instead, the “exhaustion” phenotype is a rapid and normal physiological T cell response. The Journal of Immunology, 2013, 191: 4211–4222.

The successful resolution of an acute viral infection is accompanied by the establishment of a pool of memory T cells that is maintained for the lifetime of the host. Together with Abs, these cells protect the host from disease upon re-encounter with infectious pathogens. Memory cells differ from their naive counterparts in several ways. They are more abundant (often, ~1000-fold), they are triggered by lower levels of Ag (1, 2), and they are more capable of entering nonlymphoid tissues, allowing for effective surveillance and antiviral function in the periphery (3, 4). In response to Ag, CD8+ memory T cells rapidly express a wide range of effector functions, including the secretion of multiple cytokines (5) and the cytolyis of target cells following re-encounter with their cognate Ag. These effector functions are expressed before the onset of memory T cell division, which begins only after a "lag phase" ≥ 24–48 h (5, 6), and perhaps as long as ~72 h (7). One would predict that an incoming pathogen would be most vulnerable to an educated immune system within the first few hours postinfection (p.i.), before dissemination, when the agent is at low abundance. Thus, if memory T cells play a part in controlling the infection at a very early stage, they must do so prior to dividing and presumably by rapidly imposing their antiviral effector functions upon the newly infected host cells. In this study, we sought to better analyze the expression, antiviral effects, and subsequent regulation of CD8+ memory T cell effector responses that occur within a few hours of challenge in vivo. We found that, following infection with either an arenavirus or a poxvirus (both of which cause acute, not chronic, infections), virus-specific CD8+ memory T cells quickly produced IFN-γ, recapitulating previous findings. However, remarkably, in both cases the number of T cells actively producing IFN-γ in the spleen rapidly declined within 18 h p.i., and the cells remained cytokine-negative in vivo for a prolonged period. This downregulation occurred despite the availability of virus or immunostimulatory viral Ag and was accompanied by an in vivo upregulation of inhibitory receptors and by a reduced ability to produce multiple cytokines when exposed to exogenous peptide ex vivo. Therefore, within hours of responding to infection, CD8+ memory T cells appear to enter a state that is phenotypically reminiscent of T cell exhaustion, a process that, until now, has been associated with chronic virus infections.

Materials and Methods

Ethics statement

All animal experiments were approved by The Scripps Research Institute’s Institutional Animal Care and Use Committee and were carried out in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

Mice and viruses

P14-transgenic mice (C57BL/6J-Thy1.1* or C57BL/6J-Thy1.1*GFP+) specific for the H-2b LCMV epitope GP3-41 (8) and congenic Ly5a (CD45.1) were used, together with lymphocytic choriomeningitis virus (LCMV) strain Armstrong (LCMV-Arm). Vaccinia virus (VV) encoding LCMV strain glycoprotein (VV-GP) was described previously (9). To generate LCMV-immune mice, 2 × 10⁷ PFU LCMV-Arm were injected i.p. into 5–6-wk-old male mice. For secondary challenge, mice were inoculated i.p. 5–8 wk later with 2 × 10⁶ PFU PFU LCMV-Arm or with 5 × 10⁶ PFU VV-GP. In some experiments, naive mice were infected i.p. with 2 × 10⁷ PFU LCMV-Arm to quantify LCMV viral RNA during primary infections or with 2 × 10⁷ PFU LCMV-Arm to assess P14 proliferation.
LCMV real-time quantitative PCR
Spleens were isolated from mice during primary or secondary LCMV infection, and small samples (mean, ~9.5 mg) were stored in RNAlater (QIAGEN, Valencia, CA) at −80°C. RNA was isolated and DNA was digested using the RNasey mini kit with on-column DNase digestion, according to the manufacturer’s instructions (QIAGEN). cDNA was generated with 200 nM the following primer 5′-CAGGTGCAGGTGGTGTGTTTGCACC-3′ (Valuegene, San Diego, CA), specific for the genomic (negative) sense of the LCMV nucleoprotein RNA, with Multiscribe RT (Applied Biosystems, San Diego, CA). Reaction conditions were as follows: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Real-time PCR was performed as described (10) using TaqMan Universal PCR master mix (Applied Biosystems). Reaction conditions: 25°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Absolute copy numbers of LCMV RNA were determined by comparison with a series of standard dilutions of the plasmid pT7-5, which encodes the S segment of LCMV (11) (a kind gift of Prof. Juan Carlos de la Torre, The Scripps Research Institute). Data were acquired on a Bio-Rad iCycler and analyzed with iCycler iQ software (Bio-Rad, Hercules, CA).

Standard intracellular cytokine staining
A total of 2 × 10^6 isolated splenocytes was incubated for 6 h directly ex vivo with GolgiPlug (BD Biosciences) and 1 μM the synthetic peptides GP33-41, or GP276-286 (GenScript, Piscataway, NJ). To determine the functional avidity of memory cells, splenocytes were incubated with various concentrations of the above synthetic peptides, as previously described (2). Following peptide stimulation, the cells were Fc blocked and surface stained with CD8α and CD44. Cells were subsequently fixed and permeabilized with Cytofix/Cytoperm and stained for the cytokines IFN-γ (XMG1.2; BioLegend, San Diego, CA), TNF (MP6-XT22; BioLegend), and IL-2 (JES6-5H4; BD Biosciences).

Direct intracellular cytokine staining to identify T cells that are producing IFN-γ in vivo
As described previously (7, 12, 13), 250 μg brefeldin A (BFA; Sigma, St. Louis, MO) was injected i.v. into mice at various points during secondary LCMV infection. Mice were sacrificed 6 h after injection. Single-cell suspensions, isolated from the peritoneal cavity by lavage or from the spleen by disruption, were immediately processed on ice, Fc blocked, and stained for surface marker and intracellular cytokine expression as above. BFA (10 μg/ml) was included in all media and staining buffers until cells were fixed and permeabilized. T cells were not stimulated ex vivo with synthetic peptide.

Flow cytometry
Isolated lymphocytes, collected from homogenized spleens, peritoneal cavity, or blood, were Fc blocked with anti-CD16/32 1:200 (BD Biosciences, CA) and immunophenotyped with fluorescent Abs (eBioscience, San Diego, CA and BioLegend, CA) for the following cell surface markers: CD8α and CD44 (1M7), Thy1.1 (HIS51 or OX-7), CD45.1 (A20), PD-1 (J43), Tim-3 (RM3-23), Lag-3 (C97W8), and CXCR3 (CXCR3-173). D'GP33-41 MHC class I tetramers were provided by the National Institutes of Health Tetramer Core Facility (Emory University, Atlanta, GA). Annexin V and 7-aminocoumarin D staining was determined using an AnnexinV PE Apoptosis Detection kit (eBioscience) after surface staining, according to the manufacturer’s instructions. For staining of intracellular markers, surface-stained splenocytes were fixed and permeabilized with either Cytofix/ Cytoperm (BD Biosciences) or a Fix/Perm (QIAGEN) buffer. CFSE dilution was determined by flow cytometry. In other experiments, memory P14 cells were generated and assessed as follows. Splenocytes were isolated from congenic Thy1.1+ or Thy1.1+GFP+ mice, and the frequency of transgenic CD8+ T cells (Vα27/Vγ8.1/0.2+) was determined using flow cytometry. A total of 2 × 10^6 of these naive transgenic CD8+ T cells was injected i.v. into naive mice; assuming a “take” of 10%, recipient mice possess ~200 transgenic T cells. The mice were then infected with LCMV-Arm (2 × 10^9 PFU). Four to five weeks later, recipient mice were bled, and the frequency of transgenic P14 CD8+ T cells was determined. Based on these P14 cell frequencies, the mice were segregated into three equivalent groups.

In vivo cytotoxicity assay
As previously described (14), splenocytes from congenic Ly5a (CD45.1) mice were isolated and loaded with 1 μM the LCMV synthetic peptides GP33-41, or NP366-374, and with the irrelevant control influenza A peptide NP366-374 (Genscript) for 1 h at 37°C. After washing, peptide-loaded splenocytes were labeled for 7 min at room temperature with 5, 1.0, or 0.1 μM CFSE. Approximately 5 × 10^5 of each of the three populations of peptide-loaded and CFSE-labeled splenocytes (∼15 × 10^5 total cells) were injected i.v. into naive mice (negative controls) or into LCMV-immune mice either without, or at 6 or 24 h after, secondary infection. Two hours after the inoculation of the CFSE-labeled target cells, the recipient mice were sacrificed, and their spleens were harvested. Donor splenocytes were identified by staining for CD45.1, and the proportions of CFSElow, CFSEmiddle, and CFSEhigh cells were determined. The percentage of specific cytotoxicity was calculated as previously described (14): 1 − (% LCMV peptide labeled in infected mice/% control peptide labeled in infected mice/(% LCMV peptide labeled in naive mice/% control peptide labeled in naive mice)) × 100. E/T ratios were calculated by determining the number of GP33-41 or NP366-374 MHC class I tetramer–reactive CD8+ memory T cells in the spleens of individual mice and comparing them with the average number of GP33-41- or NP366-374-loaded target cells in naive, control mice. Nonlinear regression lines were calculated using GraphPad Prism 6.

Results
Immediately following secondary infection, CD8+ memory T cells produce IFN-γ in vivo and suppress virus replication, but they terminate IFN-γ production within hours
To better characterize the kinetics of the effector response of memory CD8+ T cells to secondary virus infection, LCMV-immune mice (5–8 wk postprimary infection) were rechallenged with LCMV, and the number of memory CD8+ T cells that actively produced IFN-γ in vivo was assessed at various times p.i., beginning at 3 h and extending to 48 h. Six hours prior to harvesting the spleens for analysis, the mice were inoculated with BFA, which enhances the ability to identify IFN-γ–producing T cells (12, 13). As shown in Fig. 1A–C, IFN-γ− cells were detectable within 3 h of secondary infection, and the number of responding cells peaked at ~6 h p.i. Similar to previous in vivo and ex vivo reports (6, 13, 15), IFN-γ production within total CD8+ T cells was transient, with ~80% of cells terminating IFN-γ synthesis by 24 h p.i. The number of IFN-γ–producing cells had decreased to background levels by 48 h p.i. (Fig. 1B, 1C), a situation that was maintained until ≥96 h p.i. (data not shown). We analyzed the kinetics of IFN-γ production in one epitope-specific CD8+ T cell population using D'GP33-41 MHC class I tetramers. GP33-41-specific cells rapidly responded to secondary infection, indicating that LCMV glycoprotein epitopes are presented more rapidly than previously proposed (16), and they reached their...
maximal IFN-γ production within 6–12 h (Fig. 1D, 1E). It is well-established that LCMV-specific memory T cells can reduce the titers of infectious virus measured at several days postchallenge, after memory T cell division has begun (6). However, one round of LCMV replication takes ∼8–12 h (17), so we investigated the possibility that memory T cells may exert antiviral effects before a single round of virus replication has occurred. To maximize our ability to detect antiviral effects that might occur prior to the production of infectious particles, we used the quantity of LCMV RNA as our yardstick, rather than virus titer. As shown in Fig. 1F, LCMV RNA rapidly accumulated within the spleens of naive mice (red), reaching levels of 10⁷ genome copies/mg within 12 h p.i. and the difference between LCMV-immune and naive mice was highly statistically significant at this very early time point (p < 0.0021). This containment of viral replication extended to ≥24 h p.i., although, as shown in Fig. 1A–E, the majority of CD8⁺ T cells were, by that time, not producing IFN-γ. However, the early control of infection, although very substantial, was not absolute, because a further ∼25-fold reduction in viral RNA occurred between 24 and 72 h p.i.

**IFN-γ production by peritoneal CD8⁺ memory T cells is also rapid but unsustained**

It was possible that the rapid in vivo on/off response observed in splenic memory T cells was organ specific. As we explained previously (12), the in vivo BFA approach has a technical limitation: it is best applied to tissues from which T cells can be rapidly and readily isolated; it cannot be used to assess T cell response in “solid” tissues whose disruption requires, for example, enzymatic incubation at 37°C. Hence, we chose to evaluate extrasplenic in vivo T cell responses in the peritoneal cavity, from which cells can be quickly obtained using peritoneal lavage. As shown in Fig. 2, CD8⁺ memory cells in this location (the site of viral inoculation) displayed an extremely rapid on/off response. By 6 h postsecondary infection, ∼30% of CD8⁺ T cells were actively producing IFN-γ, and this rapidly declined as early as 12 h p.i. (Fig. 2B). This proportional increase was paralleled by an increase in the absolute number of IFN-γ-producing cells (Fig. 2C). Next, tetramer staining was applied to determine the responses of epitope-specific cells. The proportion of endogenous GP₃₃–₄₁-specific cells that was producing IFN-γ also increased markedly at 6 h p.i. and declined thereafter (Fig. 2D). The reduction in the number of cells making IFN-γ in vivo at 12–24 h p.i. cannot be explained by egress from the peritoneal cavity, because the absolute number of GP₃₃–₄₁-specific cells in the peritoneal cavity remained relatively stable during that 24-h time period (Fig. 2E); the early, transient loss of detectable cells (t = 6 h) is explained by the temporary reduction in cell surface TCR that occurs on highly activated CD8⁺ T cells. In summary, LCMV-specific memory T cells in both the peritoneal cavity (Fig. 2) and the spleen (Fig. 1) respond to secondary challenge by quickly making IFN-γ and then quickly terminating production.

**FIGURE 1.** Immediately following secondary infection, CD8⁺ memory T cells produce IFN-γ in vivo and suppress virus replication, but they terminate IFN-γ production within hours. LCMV-immune mice were infected i.p. with 2 × 10⁶ PFU LCMV-Arm, and IFN-γ-producing CD8⁺ T cells in the spleen were enumerated by flow cytometry at the indicated time points (see Materials and Methods). CD8⁺ T cells were not stimulated ex vivo with exogenous peptide. (A) Representative dot plots of splenocytes harvested at 0, 6, and 24 h p.i. (gated on CD8⁺ T cells). The frequency (B) and absolute number (C) of total CD8α⁺CD44hiIFN-γ⁺ cells within the spleen were determined. The frequency (D) and absolute number (E) of IFN-γ-producing D⁹GP₃₃–₄₁-specific memory CD8⁺ T cells (CD8α⁺CD44hi+) also were assessed. Independent experiments were carried out nine times (A–C) or seven times (D, E). (F) To determine the protective efficacy to the response, naive mice and LCMV-immune mice were infected i.p. with 2 × 10⁶ PFU LCMV-Arm. At the indicated time points, LCMV genome copies in the spleen were enumerated using quantitative real-time PCR. Data for naive mice are from two independent experiments; data for LCMV-immune mice are from eight independent experiments. In (B–F), the numerals adjacent to each column/symbol indicate the number of individual mice at each time point. +p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Termination of IFN-γ synthesis occurs despite the continued presence of immunostimulatory viral Ag

One explanation for the loss of IFN-γ–producing CD8+ T cells is that viral Ag is quickly reduced to a level that is insufficient to stimulate memory CD8+ T cells. The presence of LCMV RNA in secondarily infected mice at 24 h p.i. (Fig. 1F, green) suggested that Ag probably also would be present. To directly determine whether immunostimulatory virus Ag was available, the experiment shown in Fig. 3A was carried out. At t = 0, naive mice or LCMV-immune mice were either sham infected or infected with LCMV. Twenty-four hours later, naive CFSE-labeled P14 cells were transferred into the mice, and P14 proliferation was assessed 48 and 72 h thereafter (72 and 96 h postsecondary infection/sham), the spleens were harvested, and CFSE dilution was determined using flow cytometry. Representative FACS plots (gated on transferred P14 CD8+ T cells) are shown. Data are representative of three experiments (LCMV immune) or one experiment (LCMV naive). As indicated in panel (A), panels (B) and (C) refer to sham-infected and LCMV-infected mice, respectively.

Cell transfer (Fig. 1F, green) and demonstrates that viral Ag was present at this time point, when endogenous memory cells have largely terminated in vivo IFN-γ synthesis. It is also noteworthy that there is sufficient immunostimulatory Ag to drive responses by naive sensor P14 cells, but memory cells, which are generally better able to respond to very low levels of Ag, are cytokine silent. Thus, the rapid termination of IFN-γ production by virus-specific CD8+ memory T cells and the maintenance of a nonresponsive (refractory) state appear to be active processes, rather than merely a passive consequence of Ag loss.

The loss of IFN-γ+ CD8+ T cells from the spleen at 24 h p.i. cannot be explained by cell death or egress

Because sufficient Ag was present, we sought an alternative explanation for the rapid disappearance from the spleen of IFN-γ–producing CD8+ T cells in vivo between 24 and 96 h following virus challenge. To assess the possibility that the loss of memory cells was due to their physical removal from the spleen, through
death or egress, we quantified the absolute numbers of endogenous GP33-specific virus-specific T cells within the spleen using MHC class I tetramers; cell numbers were reduced (~3-fold) within the first 12 h of secondary infection (Fig. 4A). This reduction in tetramer+ cells might have been caused by downregulation of TCR on responding cells, as previously observed in the peritoneal cavity (Fig. 2E). To control for this, we rechallenged LCMV-immune mice that contained near-endogenous levels of P14 memory cells, which can be enumerated in a TCR-independent manner using the congenic marker Thy1.1. Within 24 h of secondary LCMV infection, the absolute number of these transgenic CD8+ memory T cells also decreased by ~3-fold (Fig. 4B), confirming that the observed reduction in endogenous cells was not the consequence of TCR downregulation. Thus, during the recall response, the numbers of IFN-γ–producing T cells (Fig. 1) and total epitope-specific cells (Fig. 4A, 4B) both change during the first 24–48 h of secondary virus infection. To better understand the relationship between the kinetics of these two events, summaries of both datasets are presented in Fig. 4C. Both datasets are normalized to their value at t0; subsequent values are plotted as the fold change. The small reduction in total cell number begins almost immediately; however, during this time, the number of IFN-γ+ cells increases markedly. Most importantly, between 12 and 24 p.i., the total cell number (○) remains relatively stable or even increases, but the number of IFN-γ–producing cells (●) rapidly declines. Thus, the small reduction in T cell number does not temporally coincide with, and thus appears not to underpin, the loss of IFN-γ–producing cells. Furthermore, if the observed reductions in the number of IFN-γ–producing cells in the spleen (Fig. 1) and in the peritoneal cavity (Fig. 2C) were due to the selective egress of IFN-γ–synthesizing cells from those locations, one might expect to see a transient increase in IFN-γ–producing cells in the blood. However, there was no statistically significant increase in the frequency of GP33–41+–specific cells in the blood (Fig. 4D), and IFN-γ–producing cells were essentially undetectable (data not shown). Next, we assessed whether virus-specific cells underwent apoptosis following secondary LCMV infection. Previous work showed that virus-specific memory CD8+ T cells are subject to IFNα/β-mediated attrition and apoptosis during the early stages of LCMV infection (18, 19). Therefore, we measured the expression of annexin V upon memory P14 cells during secondary LCMV infection. The frequency of total annexin V+ P14s was significantly upregulated ($p < 0.001$) within 24 h of secondary LCMV infection (Fig. 4E). Resting LCMV-specific memory CD8+ T cells also express greater levels of the pro-survival protein Bcl-2 compared with activated effector cells (20, 21); Bcl-2 expression has been associated with enhanced survival and proliferation of memory T cells (21–23). Therefore, we assessed Bcl-2 expression on endogenous memory T cells to determine whether secondary LCMV influences Bcl-2 expression and may influence T cell survival. Interestingly, within 24 h of secondary LCMV infection, Bcl-2 levels were significantly upregulated ($p < 0.01$) on GP33–41–specific memory CD8+ T cells (Fig. 4F). Thus, the regulation of apoptosis following secondary infection is complex, but apoptosis alone cannot explain the cessation of IFN-γ production by virus-specific CD8+ T cells.

**FIGURE 4.** The loss of IFN-γ+ CD8+ T cells from the spleen at 24 h p.i. cannot be explained by cell death or egress. (A) LCMV-immune mice were infected i.p. with 2 × 10^6 PFU LCMV-Arm, and D^*GP33–41* tetramers were used to identify memory CD8+ T cells at the indicated time points. (B) A total of 2 × 10^6 naive transgenic P14 CD8+ T cells was adoptively transferred i.v. into naive recipients, which were subsequently infected i.p. with 2 × 10^6 PFU LCMV-Arm. 5–6 wk after the primary infection, at which time the recipient mice contained memory P14 cells (as shown in t = 0 group, which received a sham infection), mice were challenged i.p. with 2 × 10^6 PFU LCMV-Arm, and transgenic P14 cells were enumerated 6 or 24 h later. (C) Fold changes in the number of tetramer+ cells and IFN-γ–producing cells. (D) The frequency of circulating D^*GP33–41* CD8+ T cells within the blood during secondary LCMV infection was evaluated. (E) Annexin V and 7-aminoactinomycin D reactivity was assessed upon transgenic memory P14 cells during secondary LCMV infection. Bcl-2 expression was determined within D^*GP33–41* memory CD8+ T cells during secondary infection (F). Representative histograms (isotype control shown in gray) and normalized geometric MFI are shown. Data are the summation of 14 (A), 4 (B), or 2 (D–F) independent experiments. In (A), (B), and (D), the numerals within each bar indicate the number of individual mice at each time point. *0.05 ≤ $p < 0.1$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ****p ≤ 0.0001.
After responding to reinfection, LCMV-specific CD8+ memory T cells rapidly lose their capacity to make multiple cytokines ex vivo.

We next assessed whether, during the early hours of the recall response, memory CD8+ T cells showed any changes in their capacity to secrete IFN-γ, as well as TNF and IL-2, in response to ex vivo Ag stimulation. Splenocytes from three groups of LCMV-immune mice were compared: resting mice (i.e., not subjected to secondary infection), mice reinfected with LCMV and evaluated at the peak of in vivo IFN-γ production (6 h p.i.), and mice reinfected with LCMV and evaluated at 24 h p.i., when in vivo IFN-γ production had dramatically declined. Following incubation in vitro with two immunodominant LCMV epitopes, CD8+ memory T cells were stained for cytokine production (Fig. 5A).

As expected, resting cells (t = 0) of both epitope specificities were readily detected, and many of them were multifunctional (i.e., the cells synthesized TNF or TNF and IL-2, in addition to IFN-γ). Significantly fewer (p < 0.0002) peptide-responsive (IFN-γ+) CD8+ T cells were detected at 24 h p.i.; this is consistent with the small numerical reduction in memory cells that was detected using tetramers (Fig. 4A, 4B). More importantly, the profile of cytokines that was produced by responding cells in response to ex vivo peptide stimulation changed over time. As shown in Fig. 5B, as early as 6 h p.i., the ratio of multifunctional cells (gray + white) to monofunctional cells (IFN-γ-producing, black) was reduced in both epitope-specific populations, and this decline in multifunctionality (i.e., a relative increase in the black segments of the pie charts in Fig. 5B) was greater at the 24-h time point. The progressive and hierarchical decline in multifunctionality is a hallmark of T cell exhaustion, which was previously associated with chronic virus infections (24–26). Other investigators (27) reported that, perhaps surprisingly, LCMV-specific T cells harvested from chronically infected mice (i.e., “classically exhausted” T cells) have near-identical in vitro functional avidities compared with cells harvested from animals that have cleared an acute LCMV infection. Therefore, we next asked whether the functional avidity of epitope-specific memory cells was similarly maintained during the 24-h period in which they show features of “exhaustion.”

This proved to be the case: memory cells analyzed prior to infection (0 h) or at 6 or 24 h postsecondary challenge had very similar functional avidities (Fig. 5C, 5D).

Inhibitory receptors are upregulated upon responding CD8+ cells in vivo, and expression levels of T-bet, Eomes, and CXCR3 rapidly change

The above data caused us to ask whether responding memory cells might display additional characteristics of exhaustion. Exhausted T cells upregulate inhibitory receptors, such as PD-1, Tim-3, and Lag-3 (28–31). At the peak of IFN-γ production (between 6 and 12 h), approximately a third of responding CD8+ T cells were...
positive for PD-1 (Fig. 6A–D); thereafter, as in vivo IFN-γ production waned, PD-1 became significantly enriched upon the remaining responding CD8+ T cells and, within 24 h, ~60% of the IFN-γ+ CD8+ T cells expressed PD-1 (Fig. 6A–D). The increase in the frequency of PD-1 at 18 and 24 h p.i. expression coincided with a significant increase in the coexpression of the inhibitory receptors Tim-3 (Fig. 6A, 6C) and Lag-3 (Fig. 6B, 6D) upon responding CD8+ T cells. Next, we evaluated expression of the T-box transcription factors T-bet and eomesoderm (Eomes), which are known to play important roles in regulating T cell abundance and function (32–34). A recent report suggested that the relative levels of T-bet and Eomes expression in CD8+ T cells changed as the cells responded during a chronic virus infection, with T-bethigh cells declining in number and Eomeshigh cells becoming more frequent (35). In CD8+ memory T cells, T-bet was downregulated 6 h after secondary infection, and this downregulation was broadly maintained up to 24 h p.i. (Fig. 6E, 6F), whereas Eomes expression was relatively unchanged at 6 h postsecondary infection but increased significantly on responding cells at 24 h p.i. (Fig. 6G, 6H). The observed downregulation of T-bet (Fig. 6E, 6F) was temporally concurrent with upregulation of PD-1 (Fig. 6A–D); this is consistent with the recently demonstrated capacity of T-bet to inhibit PD-1 expression (36). We also assessed the expression, on LCMV-specific CD8+ memory T cells, of the chemokine receptor CXCR3, which is upregulated by T-bet (37). As T-bet expression diminished following secondary infection, CXCR3 expression also was similarly and significantly downregulated (Fig. 6I, 6J).

CD8+ memory T cells responding to poxvirus infection also express IFN-γ rapidly but transiently and then display phenotypic exhaustion

We considered the possibility that CD8+ memory T cells develop an exhausted phenotype only following infection by LCMV, a small RNA virus. To address this question, we evaluated their responses to an infection by VV, a large DNA virus whose biology differs substantially from that of LCMV. To this end, LCMV-immune mice were infected with a recombinant VV-GP. To allow the evaluation of in vivo cytokine production in the hours following VV-GP infection, some of the infected mice received BFA, as described in Materials and Methods. Representative dot plots are shown in Fig. 7A, and the frequency and number of responding cells during the 48 h p.i. are presented in Fig. 7B and 7C. IFN-γ-producing cells were absent at t₀, abundant within 12 h, and almost undetectable 24 h after VV-GP infection. Notably, IFN-γ production was not resumed, even at the 48-h time point. Near-identical kinetics were observed in epitope-specific (GP33–41) memory cells (Fig. 7D), which showed a 3-fold reduction in number (Fig. 7E), similar to that observed for LCMV (Fig. 4B). The rapid termination of IFN-γ production, as well as the failure to resume synthesis during the first 48 h p.i., occurred even though infectious VV-GP was present throughout this period (Fig. 7F). To assess the multifunctionality of CD8+ memory T cells in the hours following VV-GP infection, a similar approach was taken, but the mice did not receive BFA; splenocytes were harvested, and their ex vivo responsivenes to both GP33–41 (Fig. 7G) and GP276-286 (Fig. 7H) peptides were determined. As expected from Fig. 7A–E, a reduction in the total number of responding cells is evident. However, there also is a loss in multifunctionality, best observed in the related pie charts (Fig. 7I, 7J), which is particularly notable in the first 12 h p.i. Thus, regardless of the nature of the infecting virus, CD8+ memory T cells quickly make IFN-γ and rapidly terminate IFN-γ synthesis in vivo. Furthermore, the cells remain IFN-γ− until ≥48 h p.i., despite the presence of virus and Ag, and they very rapidly lose their ability to elaborate multiple cytokines following ex vivo Ag stimulation.

**FIGURE 6.** Inhibitory receptors are upregulated upon responding CD8+ cells in vivo, and expression levels of T-bet, Eomes, and CXCR3 change rapidly. LCMV-immune mice were infected i.p. with 2 × 10^6 PFU LCMV-Arm and BFA was administered, as described in Materials and Methods. At the indicated times p.i., splenocytes were harvested and gated on IFN-γ-producing CD8+ memory T cells (CD8α+IFN-γ+), and the frequencies of cells expressing PD-1 with or without Tim-3 (A, C) or PD-1 with or without Lag-3 (B, D) were determined. The expression of the transcription factors T-bet (E, F) and Eomes (G, H), as well as the chemokine receptor CXCR3 (I, J), were determined on DGP33–41-specific memory CD8+ (CD8α+CD44high) T cells in the spleen. Data in bar graphs are presented as mean ± SEM and are the summation of six (C, D) or two or three (F–J) independent experiments. Each time point represents 7–11 mice. *0.05 ≤ p > 0.01, **0.01 ≤ p > 0.001, ***0.001 ≤ p > 0.0001.
CD8+ memory cells remain cytolytic throughout the recall response

The above data show that, during the recall response to an arenavirus and a poxvirus, CD8+ memory T cells terminate IFN-γ synthesis within ∼12–24 h p.i. However, there is no resulting increase in LCMV RNA levels (Fig. 1F) or in VV load (Fig. 7E), suggesting that other effector functions might remain intact. T cell exhaustion is accompanied by an additional change in T cell effector function: the loss of cytolytic activity when measured in vitro. However, perhaps surprisingly, in vivo cytotoxicity often remains intact. For example, during chronic LCMV infections, although CD8+ T cell cytokine secretion is dysfunctional and ex vivo cytolytic activity is severely abrogated (25, 27, 38, 39), in vivo cytosisis and granzyme B upregulation remain relatively untouched (40–42). Oxe-nius and colleagues (43) replicated these findings in the LCMV model and also showed that it applies to HIV infection of humans.
Therefore, we determined whether there was any change in the in vivo cytolytic capacity of CD8\(^+\) memory T cells during their recall response to secondary virus infection. Consistent with previous observations (14), resting CD8\(^+\) memory T cells exhibited CTL activity, eliminating GP\(_{33-41}\) and NP\(_{396-404}\)-coated cells from the spleen (Fig. 8A, 0 h). Secondary LCMV infection caused only minor changes in overall in vivo cytotoxicity, with most target cells being cleared within 2 h of being inoculated into mice at both the 6- and 24-h p.i. time points (Fig. 8A). Total GP\(_{33-41}\)-specific cytotoxicity (Fig. 8B) declined only slightly over the 24-h p.i. time period and, when the estimated in vivo E:T ratio (see Materials and Methods) was taken into account, the per-cell cytotoxicity appeared to be very similar at each of the three postsecondary time points (Fig. 8C). Identical analyses were applied to evaluate NP\(_{396-404}\)-specific cytotoxicity (Fig. 8D, 8E) and this, too, was well maintained; indeed, at 24 h following secondary infection there was, if anything, a slight increase in per-cell cytotoxicity. Finally, we assessed the expression, within LCMV-specific T cells, of granzyme B, a key component of the cytolysis machinery (44, 45); as shown in Fig. 8F and 8G, granzyme B expression significantly increased as early as 6 h p.i. (∼2-fold, \(p < 0.001\)) and was further upregulated at 24 h p.i. (3-fold, \(p < 0.001\)). Therefore, in contrast to in vivo cytokine secretion, in vivo cytolyis is relatively unaffected following secondary LCMV infection; we speculate that this may explain why, despite the rapid termination of IFN-γ synthesis by ∼12 h, LCMV RNA and infectious VV do not subsequently increase and are ultimately cleared.

### Discussion

The protective capacity of CD8\(^+\) memory T cells is well recognized (2, 5, 6, 46–52), but the rapidity with which protection is conferred, as well as the subsequent in vivo status of the responding CD8\(^+\) T cells, remains unclear because most studies focused on relatively late time points (>24 h p.i.), and memory cell responses were examined ex vivo/in vitro as surrogates for in vivo effector activity. In this study, we examined and quantified the recall responses of memory CD8\(^+\) T cell activity in vivo and within hours of a secondary virus infection, long before the memory cells have begun to divide. Following secondary infection with LCMV-Arm, we observed rapid, but fleeting, in vivo production of IFN-γ by CD8\(^+\) T cells within the spleen (Fig. 1), confirming previous in vivo (7, 13) and ex vivo (6) observations. A similar on/off response to secondary LCMV infection was observed in CD8\(^+\) memory cells in the peritoneal cavity, indicating that this pattern of response is not restricted to secondary lymphoid tissues (Fig. 2). The expression of IFN-γ was accompanied by a remarkably rapid suppression of viral replication, beginning before a single round of virus replication has been completed, and leading to an ∼99.5% reduction in LCMV RNA within 12 h (Fig. 1F). We speculate that the evolutionary benefit of a rapid recall response is maximized because the pathogen may be at its most vulnerable immediately following infection, when a limited number of virus-infected cells is present. However, viral RNA levels decreased further between 24 and 72 h p.i., indicating that this early suppression of viral replication is incomplete.

Why is production of IFN-γ, a potent antiviral effector molecule, terminated before the virus has been eradicated? We suggest that this occurs to prevent immunopathology; if correct, the rapid cessation of IFN-γ synthesis by CD8\(^+\) T cells is likely to be an active regulatory event. IFN-γ production is dependent upon continued contact with cognate Ag, and IFN-γ production is quickly terminated when contact is broken (53, 54). Thus, one obvious reason for the rapid loss of IFN-γ-producing CD8\(^+\) T cells in vivo is a reduction in the abundance of immunostimulatory Ag. However, LCMV RNA (Fig. 1F), as well as immunostimulatory LCMV Ag, is present at 24 h after secondary infection (Fig. 3C), a time point at which most endogenous cells are cytokine silent. Although we did not experimentally demonstrate the presence of Ag following VV challenge, infectious virus was present for >48 h (Fig. 7D), indicating that the downregulation of the VV-driven recall response (Fig. 7A–D) also is likely to be an active process.

The observation that memory T cells quickly lost the capacity to synthesize IFN-γ in vivo led us to evaluate their responsiveness to ex vivo peptide stimulation. We confirmed that there was a reduction in the absolute number of cells that could respond; however, more importantly, using a standard criterion of “quality,” the ability to produce multiple cytokines, we also identified a reduc-

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**FIGURE 8.** CD8\(^+\) memory cells remain cytolytic throughout the recall response. LCMV-immune mice were infected i.p. with 2 × 10\(^6\) PFU LCMV-Arm. At 0, 6, and 24 h postsecondary infection, they received CFSE-labeled CD45.1\(^+\) (Ly5a) target cells coated with one of the three indicated peptides (LCMV GP\(_{33-41}\) or NP\(_{396-404}\) or the irrelevant control influenza A peptide NP\(_{366-374}\)). Naive mice acted as negative control. Naive mice acted as negative control. Naive mice acted as negative control. Naive mice acted as negative control. Naive mice acted as negative control. Naive mice acted as negative control. Naive mice acted as negative control. Naive mice acted as negative control. Naive mice acted as negative control.

A) Representative graphs gated on CD45.1\(^+\) CFSE\(^+\) cells. (B) Percentage of GP\(_{33-41}\)-specific cytotoxicity at each time point. (C) Correlation between cytotoxicity and estimated in vivo E:T ratios. (D and E) Equivalent cytotoxicity data for NP\(_{396-404}\). Granzyme B expression was determined within D\(\text{GFP}_{33-41}\)\(^+\) memory CD8\(^+\) T cells during secondary infection. Representative graphs (F; isotype control shown in gray) and normalized geometric MFI (G). Data in (B–E) are a summation of three independent experiments (n = 12). Data in (F) and (G) are a summation of six independent experiments (n = 23). *0.05 ≤ p > 0.01, **0.01 ≤ p > 0.001, ***0.001 ≤ p > 0.0001.
tions in T cell quality after the initial response. This was true following infection with both LCMV (Fig. 5) and VV (Fig. 7). A loss of multifunctionality has long been considered a hallmark of T cell exhaustion, a phenomenon that is associated with many cancers, as well as with chronic viral infections, such as those established by HIV, hepatitis B virus (HBV), hepatitis C virus, and some strains of LCMV (reviewed in Refs. 26, 55). Therefore, we were surprised to see this rapid qualitative decline occurring during an acute infection and within hours of Ag contact. For this reason, we next assessed another marker of exhaustion, the elevated expression of the inhibitory receptors PD-1, Tim-3, and Lag-3 (28–31, 56, 57). All three inhibitory molecules were quickly upregulated on responding CD8+ memory T cells (Fig. 6). Finally, we evaluated the cytotoxic activity of memory cells during the early part of the recall response and found that both GP33–41-specific and NP96–104-specific memory cells remain cytolytically active at 24 h p.i. (Fig. 8); indeed, NP96–104-specific effect may even have increased slightly on a per-cell basis (Fig. 8E). During chronic LCMV infection, and consistent with the term “exhaustion,” in vitro cytolytic activity is severely abrogated (25, 27, 38, 39). However, cytolytic activity may be near normal when measured in vivo, and granzyme B upregulation is retained in the T cells (43, 58), indicating that the term “exhaustion” may be too broad. Other investigators (43) noted this discrepancy during LCMV clone 13 infection of mice, as well as during HIV infection of humans, and termed the phenomenon “split exhaustion.” Divergence between cytokine and cytolytic activity also was demonstrated in a model in which HBV-specific CD8+ T cells were adoptively transferred into HBV-transgenic mice that constitutively express HBV Ag in hepatocytes (59, 60). In this model, which more closely resembles a chronic infection, the cytolytic and noncytolytic effector functions of the transferred cells were regulated asynchronously and in an oscillatory manner, suggesting that nonprofessional APCs (hepatocytes) were affecting the behavior of CD8+ T cells. As those investigators noted, evolution may have shaped the regulation of T cell effector functions to reduce the profound immunopathology that is associated with cytokine expression, while permitting the virus-specific T cells to retain some degree of antiviral effect. Thus, for example, the fact that LCMV clone 13 infection is ultimately cleared may reflect the ongoing cytolytic activity of the so-called “exhausted” T cells.

Therefore, we propose that the T cell exhaustion that is observed during chronic infections should not be viewed as the culmination of a series of slowly developing pathological changes, but instead as a prolongation of the natural physiological response of T cells. The data from this study suggest that the phenotypic changes of “exhaustion” are entirely normal, that they develop over a few hours, and that they result in the T cells’ entering a refractory state during which they no longer synthesize multiple cytokines in response to Ag contact. We showed previously that, if the secondary virus infection is cleared, the memory T cell population gradually regains full functionality over a period of 1–3 wk (61). In contrast, we propose that if the infection is chronic (e.g., LCMV e13), recurrent Ag contact prevents the cells from exiting the normal refractory phase, which eventually becomes locked in place by epigenetic changes; consistent with our proposition, a very recent article (62) demonstrated that, during prolonged HIV infection, epigenetic programming at the PD-1 locus gradually becomes irreversible. Strikingly, this rapid tripartite sequence of the normal CD8+ T cell recall response—cytokine production/antiviral effect/exhaustion—is completed before memory T cell division has even begun. These observations were possible only because memory cells respond in synchrony and are sufficiently abundant to allow immediate analysis. However, we speculate that the same rapid on/off regulation may occur during the primary response to acute infection and that it had not been identified previously because by the time the primary effector cells have expanded to a detectable number they are no longer in synchrony, so some cells will be multifunctional, and others not; standard flow cytometry provides a population average, but fails to reveal the phenotypic diversity of the individual responding cells.

Consistent with this notion, two recent publications (63, 64) explicitly showed that the cells that constitute the primary CD8+ T cell response do not differentiate in lockstep; rather, individually responding CD8+ T cells, even when sharing the same TCR specificity, exhibit striking variability in differentiation stage and functional status. We suggest that, as an acute infection wanes, Ag clearance allows individual responding cells to asynchronously exit their refractory period, leading to the gradual restoration of a multifunctional CD8+ T cell pool that, as we showed some time ago (61), is a characteristic that distinguishes primary from memory CD8+ T cell populations. However, if Ag contact is recurrent, as occurs during chronic infections and/or in the presence of suppressive cytokines (65, 66), we propose that the refractory phase is prolonged; these physiologically normal genetic changes become epigenetically imprinted on the cells and their progeny, consistent with the finding that repeated Ag stimulation in vivo causes a series of stepwise changes in gene regulation, with each successive stimulation causing an expansion of the number of genes being regulated (67). Viewed from this perspective, the exhaustion that is observed in chronic infections and cancer does not represent the end stage of a slowly developing and pathological T cell dysfunction; rather, it results from rapidly occurring and physiologically normal T cell changes that have been locked in place by an abnormal antigenic environment.

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Disclosures
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References


