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Counting Antigen-Specific CD8 T Cells: A Reevaluation of Bystander Activation during Viral Infection

Kaja Murali-Krishna,*‡ John D. Altman,*‡ M. Suresh,* David J. D. Sourdive,* Allan J. Zajac,* Joseph D. Miller,* Jill Slansky,† and Rafi Ahmed*§
*Emory Vaccine Center and
Department of Microbiology and Immunology
Emory University School of Medicine
Atlanta, Georgia 30322
†Department of Oncology
Johns Hopkins University School of Medicine
Baltimore, Maryland 21205-2196

Summary

Viral infections induce extensive T cell proliferation in vivo, but the specificity of the majority of the responding T cells has not been defined. To address this issue we used tetramers of MHC class I molecules containing viral peptides to directly visualize antigen-specific CD8 T cells during acute LCMV infection of mice. Based on tetramer binding and two sensitive assays measuring interferon-γ production at the single-cell level, we found that 50%–70% of the activated CD8 T cells were LCMV specific (2×10^7 virus-specific cells/spleen). Following viral clearance, antigen-specific CD8 T cell numbers dropped to 10^6 per spleen and were maintained at this level for the life of the mouse. Upon rechallenge with LCMV, there was rapid expansion of memory T cells, but after infection with the heterologous vaccinia virus there was no detectable change in the numbers of LCMV-specific memory CTL. Therefore, much of the CD8 T cell expansion seen during viral infection represents antigen-specific cells and warrants a revision of our current thinking on the size of the antiviral response.

Introduction

Viral infections are often characterized by substantial activation and expansion of CD8 T cells. This is seen in humans during the acute phase of infection with viruses that replicate systemically, such as human immunodeficiency virus (HIV), Epstein-Barr virus (EBV), measles and cytomegalovirus (Koup et al., 1994; Pantaleo et al., 1994, 1997; Griffin, 1995; Callan et al., 1996; Oldstone, 1996; Wills et al., 1996; Borrow et al., 1997; Steven et al., 1997). Expansions of CD8 T cells are also seen in experimental models such as infection of mice with vaccinia virus (VV), vesicular stomatitis virus, murine γ-herpesvirus, and lymphocytic choriomeningitis virus (LCMV) (Lau et al., 1994; Sunil-Chandra et al., 1994; Bi et al., 1995; Cousens et al., 1995; Murata et al., 1996; Selin et al., 1996; Doherty et al., 1997; Slifka et al., 1997). Because of the potential for bystander activation to initiate autoimmune reactions or to shape the memory T cell repertoire, there has been considerable interest in determining how much of this expansion represents virus-specific CD8 T cells and how much of it is due to activation of T cells not specific to the immunizing antigen (Nahill and Welsh, 1993; Razvi et al., 1995; Selin et al., 1996; Tough and Sprent, 1996; Tough et al., 1996; Ehl et al., 1997; Zarozinski and Welsh, 1997).

The T cell proliferation seen during viral infections in vivo could in theory result from a number of mechanisms: antigen-driven expansion of specific T cells, stimulation of cell division by cross-reactive antigens, or cytokine-mediated bystander activation (Oldstone, 1987; Beverley, 1990; Matzinger, 1994; Unutmaz et al., 1994; Cao et al., 1995; Tough et al., 1996; Zal et al., 1996). Limiting dilution analysis (LDA) to quantitate virus-specific cytotoxic T lymphocytes (CTL) has shown that only a small fraction (1%–5% at most) of the activated CD8 T cells are antigen specific at the peak of the primary response (Doherty et al., 1992; Ahmed and Gray, 1996; Zinkernagel et al., 1996). This has led to the hypothesis that most of the CD8 T cell expansion is not antigen specific and represents bystander activation and/or cross-reactive stimulation of nonspecific cells. Experimental evidence for cytokine-mediated bystander T cell proliferation during viral infection has come from studies by Tough et al. (1996), who showed that type 1 interferons can induce proliferation of activated ($CD44^{hi}$) CD8 T cells and that this proliferation occurs independently of signaling through the T cell receptor (TCR). Two recent studies (Ehl et al., 1997; Zarozinski and Welsh, 1997) have questioned the contribution of bystander activation in viral infection, but the issue remains unresolved because the specificity of the majority of the responding T cells has not been defined.

In this study we have reexamined the question of specificity. This is a critical issue because the current models of T cell activation during viral infection are based on the notion that most (>95%) of the proliferating cells are nonspecific (Tough and Sprent, 1996; Tough et al., 1996). We used tetramers of major histocompatibility (MHC) class I molecules containing viral peptides to directly visualize antigen-specific CD8 T cells during acute LCMV infection. Based on tetramer binding and two sensitive functional assays to measure interferon-γ (IFNγ) production at the single-cell level, we found that at the peak of the primary and secondary responses, 50%–70% of the activated CD8 T cells were LCMV specific. Thus, the size of the antiviral response is considerably larger than our current estimates, and much of the expansion seen during viral infection represents expansion of antigen-specific T cells.

Results

Size of the Primary Virus-Specific CD8 T Cell Response

Adult mice infected with LCMV (Armstrong strain) generate a potent immune response and clear the virus within 8–10 days. It is well established that clearance of this

‡These two authors contributed equally to this work.
§To whom correspondence should be addressed (email: ra@microbio.emory.edu).

acute LCMV infection is mediated by CD8 CTL (Ahmed et al., 1984; Byrne et al., 1984; Moskophidis et al. 1987; Fung-Leung et al., 1991; Kagi et al., 1994; Walsh et al., 1994). The kinetics of the virus-specific CTL response and the expansion of CD8 T cells that occurs during the course of an acute LCMV infection have been extensively analyzed (Lau et al., 1994; Ahmed and Gray, 1996; Asano and Ahmed, 1996; Tough et al., 1996; Zimmerman et al., 1996). At the peak of the primary response, there is 10-fold or greater increase in the number of activated CD8 T cells in the spleen and blood, and bromodeoxyuridine (BrdU) labeling experiments have confirmed that all of the activated ($CD44^{hi}$) CD8 T cells undergo proliferation. However, quantitation of CTL precursor (CTL_p) frequencies by LDA has shown that only 1%–5% of the proliferating CD8 T cells are virus specific at the peak of the primary response (day 8 postinfection) (Moskophidis et al., 1993; Nahill and Welsh, 1993; Lau et al., 1994; Andersson et al., 1995; Razvi et al., 1995; Borrow et al., 1996; Selin et al., 1996; Zinkernagel et al., 1996). We have reexamined this issue using two sensitive functional assays: intracellular staining for IFNγ and single-cell IFNγ enzyme-linked immunospot (ELISPOT) assay. In addition, we have used tetramers of MHC class I molecules bound to viral peptides to directly visualize the antigen-specific CD8 T cells.

BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) mice were infected with LCMV, and at the peak of the primary response (day 8) the frequency of CD8 T cells specific for the different LCMV CTL epitopes was determined by functional assays. Spleen cells freshly explanted from infected mice were cultured in vitro (5 hr for intracellular cytokine stain and 36 hr for ELISPOT) either without stimulation (i.e., no peptide) or with peptides representing CTL epitopes defined in BALB/c and C57BL/6 mice (van der Most et al., 1996, 1998). We found that the size of the virus-specific response was remarkably high. As shown in Figure 1A, 70% of the CD8 T cells in C57BL/6 mice were LCMV specific; the strongest response was to peptide NP396-404 (33% ± 7.3% of CD8 T cells), followed by the responses to GP33-41 (25% ± 4.1% of CD8 T cells), GP276-286 (6% ± 2.8% CD8 T cells), NP205–212 (5.6% ± 3% of CD8 T cells), and GP92-101 (0.8% ± 0.6% of CD8 T cells). In BALB/c mice, the single immunodominant epitope NP118–126 constituted up to 50% (43% ± 7.4%) of the CD8 T cells. A representative set of the actual data along with specificity controls is shown in Figure 1B. Spleen cells from LCMV-infected mice made no IFNγ in the absence of stimulation, and peptide-stimulated spleen cells did not show any intracellular staining above background with the isotype control antibody, confirming the specificity of the intracellular IFNγ stain. An NP118-stimulated sample is shown in Figure 1B; there were 0.4% positive cells with the isotype control antibody versus 52% positive cells with the IFNγ-specific antibody. In addition, spleen cells from uninfected BALB/c mice did not produce any IFNγ after stimulation with any of the peptides (data not shown). The data in Figure 1C show that all of the NP396-404–specific CD8 T cells were $CD44^{hi}$. Similar results were seen with CD8 T cells responding to the other epitopes (data not shown).

Tetramers of MHC class I molecules containing the nominal antigenic peptide provide powerful reagents for quantitating antigen-specific CD8 T cells (Altman et al., 1996). This approach not only allows direct visualization of antigen-specific CD8 T cells, but also the analysis can be done on freshly explanted cells without any in vitro manipulations. Tetramers of L^d containing NP118–126 and D^b containing NP396-404 or GP33-41 were used to quantitate CD8 T cells specific for these epitopes. Frequencies obtained by tetramer analysis were in the same range as those determined by the IFNγ functional assays. A representative result is shown in Figure 2A: 23% of CD8 T cells in LCMV-infected C57BL/6 mice (day 8) bound the MHC D^b NP396-404 tetramer, and 56% of CD8 T cells in infected BALB/c mice bound the L^d NP118–126 tetramer. There was minimal (<0.1%) binding of these tetramers to CD8 T cells from naive (uninfected) mice. Similar specific staining results were obtained with an L^d–immunoglobulin fusion protein loaded with the NP118-126 peptide that was produced as described (Dal Porto et al., 1993) (data not shown).

By staining with tetramers in combination with BrdU labeling, it should be possible to determine the fraction of antigen-specific CD8 T cells that have undergone proliferation during the 8-day period of infection. As shown in Figure 2B, all of the tetramer-positive cells (both D^b NP396-404 specific and D^b GP33-41 specific) proliferated during LCMV infection. Although this is the expected result, this analysis provides direct proof of turnover of antigen-specific CD8 T cells in vivo. BrdU labeling experiments have been done previously with transgenic T cells (Bruno et al., 1996; Pihlgren et al., 1996; Zimmerman et al., 1996), but our studies provide documentation of in vivo proliferation of antigen-specific T cells in nontransgenic mice.

To further confirm the specificity of the MHC class I tetramers, D^b NP396-404 tetramer–positive and –negative CD8 T cells from C57BL/6 mice (day 8 postinfection) were sorted, and the two populations were tested for peptide-specific lytic activity and for secretion of IFNγ after stimulation with NP396-404 peptide. Based on the IFNγ ELISPOT assay, almost 100% of the tetramer-positive cells were NP396-404 specific (Figure 3). This result shows that all of the tetramer-binding cells could score in a functional assay and cross-validates both the physical (tetramer binding) and functional (IFNγ production) techniques we have used to quantitate antigen-specific CD8 T cells.

The ability to kill peptide-coated targets was also greatly enriched (>20 fold) in the sorted tetramer-positive CD8 T cell population (Figure 3). Even at the low effector:target ratio of 0.1:1, there was clearly observable killing of peptide NP396-404–coated targets. In contrast, CD8 T cells that did not bind the D^b NP396-404 tetramer (i.e., tetramer-negative cells) showed minimal to no killing of NP 396-404–coated targets but were able to kill GP33-41–coated targets. This further confirmed the specificity of the D^b NP396-404 tetramer by revealing that tetramer-binding cells were functionally enriched for killing of NP396-404–coated targets and that the tetramer-negative population contained few, if any, cells that scored as NP396-404 specific by functional assays. Finally, the data in Figure 3 show that

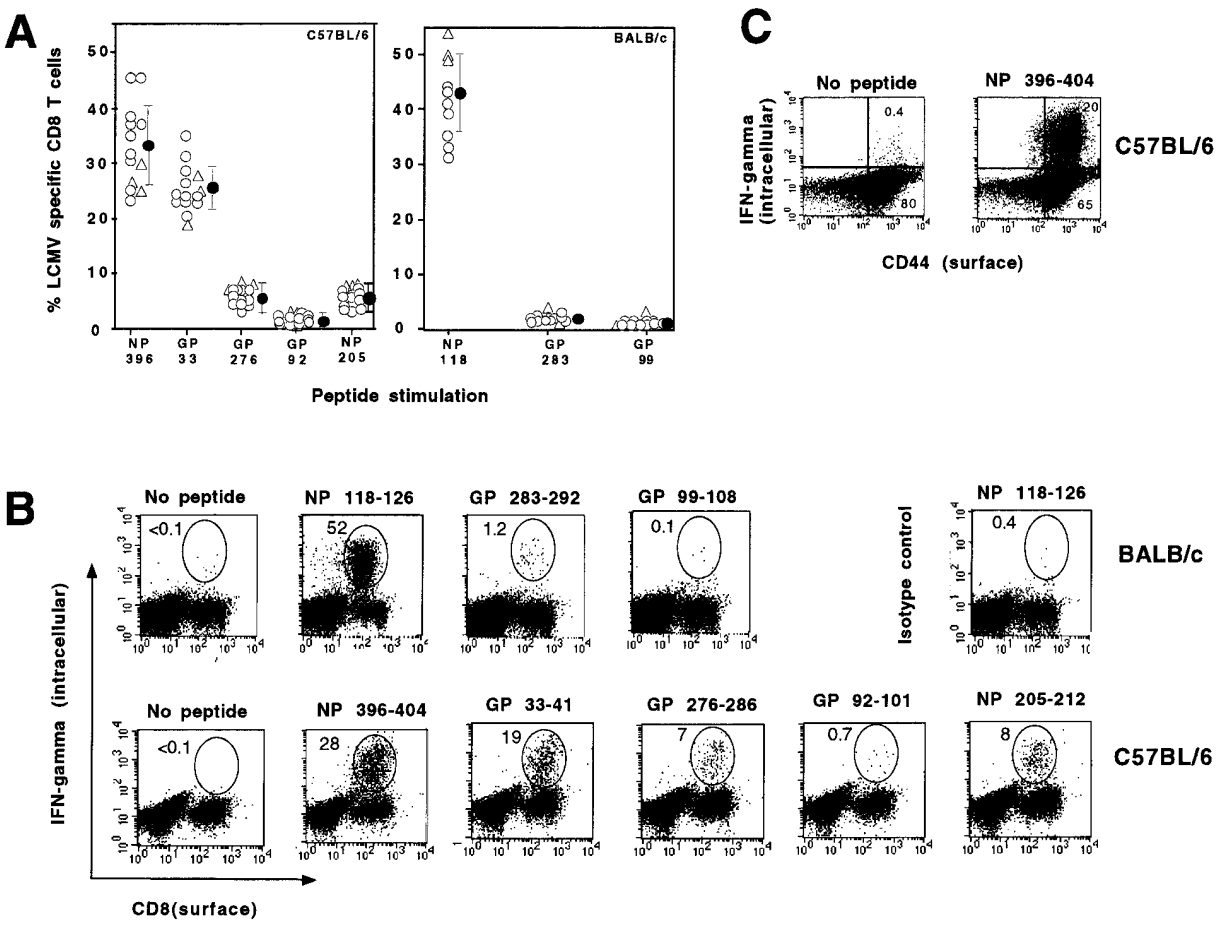

Figure 1. High Frequency of Virus-Specific CD8 T Cells during Primary LCMV Infection

(A) C57BL/6 and BALB/c mice were infected with LCMV, and 8 days later the frequency of specific CD8 T cells in the spleen was measured by single-cell IFNγ ELISPOT assay (open circles) or by intracellular IFNγ staining (open triangles). Data are presented as the percentage of CD8 T cells that make IFNγ upon stimulation with each of the indicated CD8 epitopes. Each point represents an individual mouse, and the filled circles represent the average values ± SD for each epitope.

(B) Spleen cells from LCMV-infected (day 8) BALB/c and C57BL/6 mice were cultured in vitro for 5 hr either with or without the indicated peptides and stained for intracellular IFNγ. The numbers shown indicate the percentage of CD8 T cells that are positive for the intracellular IFNγ stain. Isotype control antibody for anti-IFNγ was used routinely for all of the samples, and a representative analysis after NP118 stimulation is shown in the upper right corner. Data are representative of six experiments.

(C) Spleen cells from LCMV-infected (day 8) C57BL/6 mice were cultured in vitro for 5 hr either with or without peptide NP396-404. Cells were stained for surface CD8 and CD44 followed by intracellular IFNγ. Cells were gated on a $CD8^+$ population and analyzed for surface CD44 versus intracellular IFNγ stain. Numbers shown are the percentage of CD8 T cells that fall into the indicated quadrant.

MHC class I tetramers can be used to isolate a pure population of antigen-specific CD8 T cells.

Together, the results presented in Figures 1–3 show that the majority (50%–70%) of activated CD8 T cells are virus specific at the peak of the primary response to LCMV. This finding greatly revises our previous estimates of the size of the antiviral T cell response.

Kinetics of the Primary Response and Quantitation of Memory CD8 T Cells

The CD8 T cell response to acute LCMV infection consists of three distinct phases: (1) activation and expansion (days 1–8 postinfection); (2) death (days 8–30), during which more than 95% of the activated CD8 T cells die by apoptosis; and (3) memory (after day 30), when a stable pool of memory T cells persists for the life of the mouse. The frequencies of memory CD8 T cells to the various CTL epitopes in BALB/c and C57BL/6 mice were quantitated for more than 1 yr after infection by means of the two IFNγ assays and MHC class I tetramer binding. The results of these analyses are summarized in Figure 4.

In BALB/c mice, memory CD8 T cells specific for the immunodominant NP118–126 epitope could be readily detected at a remarkably high frequency of 1 in 10 CD8 T cells. In the sample shown in Figure 4C, 14% of the CD8 T cells were NP118–126 specific based on the intracellular IFNγ stain, and 13% were specific according to L^d NP118–126 tetramer binding. Similar frequencies were obtained by the ELISPOT assay (data not shown). Memory CD8 T cells specific for NP118–126 were maintained at these high frequencies for more than 1 yr. In fact, there was no change in either the total number or the frequencies of NP118-126–specific CD8 T cells

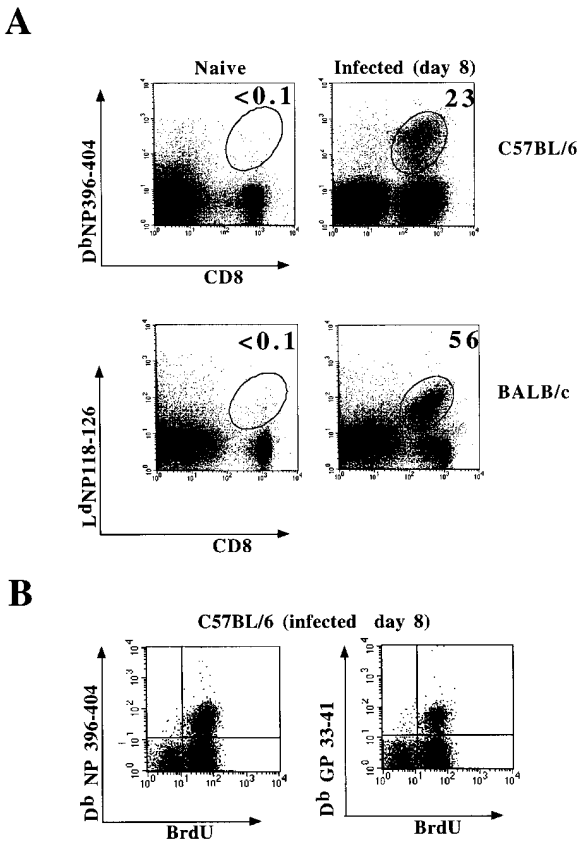

Figure 2. Visualization of Virus-Specific CD8 T Cells by MHC Class I Tetramers Containing Viral Peptide

(A) Spleen cells from naive or LCMV-infected (day 8) C57BL/6 and BALB/c mice were double-stained for surface CD8 and the indicated MHC class I tetramer–peptide complex. Numbers show the percentage of CD8 T cells that are tetramer positive. Data shown are representative of five such analyses.

(B) C57BL/6 mice were infected with LCMV and fed continuously with BrdU-containing water for 8 days. On day 8 postinfection, freshly explanted spleen cells were double-stained for surface CD8 and the indicated tetramer–peptide complex followed by intracellular staining for BrdU. Cells were gated on a $CD8^+$ population and analyzed for tetramer and BrdU double-positive cells. Data are representative of three similar experiments.

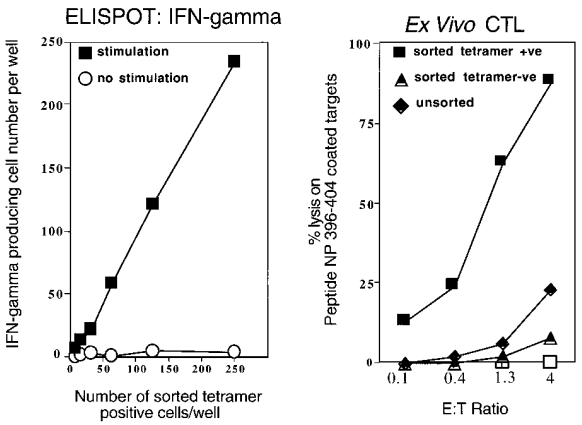

Figure 3. Isolation of a Pure Population of Antigen-Specific CD8 T Cells by Sorting MHC Tetramer–Positive Cells

Spleen cells from LCMV-infected (day 8) C57BL/6 mice were double-stained with anti-CD8 and tetramer D^b NP396-404. Tetramer and CD8 double-positive cells were sorted and assayed for single-cell IFNγ ELISPOT and ex vivo CTL activity. In the ELISPOT assay, sorted tetramer-positive cells were plated at a graded density starting from 250 cells/well and cultured with or without peptide stimulation. The data represent average values at each point and variation among duplicates was less than 7%. The sorted tetramer positive cells did not show any IFNγ production upon stimulation with another D^b-restricted peptide, GP33-41. In the ex vivo CTL assay, sorted tetramer-positive and tetramer-negative and unsorted total spleen cells were assayed on ^{51}Cr-labeled H-2^b targets that were coated with peptide NP396-404 (filled symbols) in a 5 hr ^{51}Cr-release assay. No lysis of uncoated targets was seen with any of the three effectors (open squares). The purity of the sorted population was 99% for tetramer-positive CD8 and 97% for tetramer-negative CD8. E:T, effector:target.

between day 30 and day 400 postinfection (Figure 4A). A similar pattern was seen with the subdominant K^d-restricted CTL epitope GP283-291, showing that long-term memory was equally well maintained for weak as well as strong epitopes. It is worth emphasizing that the number of NP 118-126– and GP283-291–specific CD8 T cells present in the memory pool was determined almost exclusively by the original burst size. There were approximately 20 x 10^6 NP118–126-specific CD8 T cells on day 8, and about 5% (1×10^6) of these cells survived to go into the memory pool. The same trend was seen with the GP283-291 response: at the peak there were approximately 1×10^6 specific CD8 T cells, and immune mice contained 5×10^4 GP283-specific memory CD8 T cells (i.e., 5% of the primary response). In C57BL/6 mice, frequencies of memory CD8 T cells for the two dominant epitopes (NP396-404 and GP33-41) were approximately 1 in 30 CD8 T cells and for the weaker GP276-286 epitope 1 in 70 (Figure 4B). As in BALB/c mice, frequencies for the three CTL epitopes were maintained over the long term and equally well, with minimal fluctuation. In addition, once again the size of the memory pool was a function of the burst size during the primary response. After adding responses to all of the CTL epitopes, the total number of LCMV-specific memory CD8 T cells in both BALB/c and C57BL/6 mice was in the range of 1×10^6/spleen (i.e., 10% of the total CD8 T cells).

The data in Figures 4C and 4D also demonstrate that memory T cells can respond very rapidly to restimulation with peptide. In these assays, freshly explanted memory CD8 T cells were stimulated for only 5 hr with peptide prior to staining for IFNγ. As is evident from the fluorescence-activated cell sorter (FACS) analysis, memory T cells were capable of making considerable amounts of cytokine in this short period. No IFNγ was made in the absence of peptide (data not shown). It is also worth noting that this state of quick responsiveness was maintained for extended periods; the samples shown in Figure 4C and 4D were taken from immune mice 240 and 270 days after infection.

The frequencies of virus-specific CD8 T cells obtained by the two IFNγ assays (intracellular stain and ELISPOT) and the MHC class I tetramer reagents were, for the most part, in general agreement with each other (Figures 1–4). However, these values were substantially higher

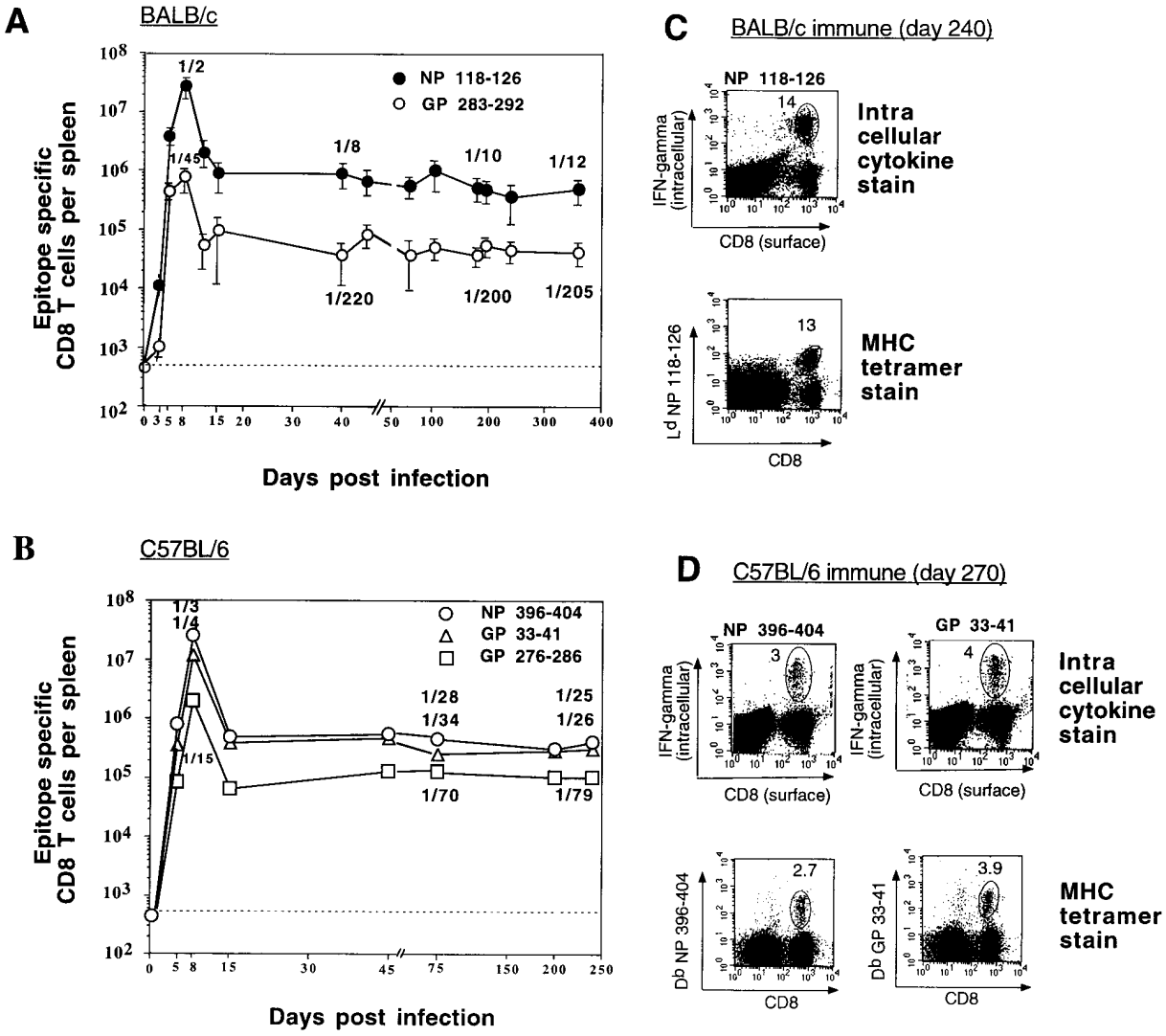

Figure 4. Quantitation and Visualization of Antigen-Specific Memory CD8 T Cells during LCMV Infection

BALB/c (A) and C57BL/6 (B) mice were checked at the indicated days postinfection for the number of virus-specific CD8 T cells in the spleen by IFNγ single-cell assays and MHC class I tetramer staining. Data represent average values obtained from three to five mice at each time point. The frequencies of peptide-specific cells/total CD8 T cells are indicated for each of the epitopes at selected time points. On day 3, the frequencies for NP118 were 1 in 1000 and for GP283, 1 in 10,000; on day 5, the frequencies were 1 in 4 for NP118 and 1 in 30 for GP283. In C57BL/6 mice the day 5 frequencies were 1 in 12 for NP396, 1 in 27 for GP33, and 1 in 180 for GP276. On day 0 postinfection the number of peptide-specific CD8 T cells was below detection (dotted line).

(C) Spleen cells from LCMV-infected BALB/c mice 240 days postinfection were incubated with peptide NP118–126 for 5 hr, followed by staining for surface CD8 and intracellular IFNγ. Antigen-specific cells from the same mouse were visualized by staining freshly explanted spleen cells with L^dNP118–126 tetramer.

(D) C57BL/6 mice 270 days postinfection were incubated in the presence of the indicated peptides followed by intracellular IFNγ staining, or freshly explanted spleen cells were stained with the indicated MHC tetramer–peptide complexes. Numbers represent the percentage of CD8 T cells that are antigen specific.

than published data on LCMV-specific CTL_p frequencies based on LDA. To compare these techniques directly, we quantitated CD8 T cells specific for different LCMV epitopes by LDA and IFNγ ELISPOT assay in acutely infected (day 8) and immune (day 120) C57BL/6 mice (Table 1). On day 8 postinfection, frequencies obtained by ELISPOT were 20- to 100-fold higher than precursor frequencies from LDA. In immune mice the differences were less, but ELISPOT numbers were still 10- to 20-fold higher than LDA numbers. It is noteworthy that among the three epitopes analyzed, the weaker epitope (GP276-286) showed the largest disparity in frequencies obtained by the two techniques.

Doubling Time of Antigen-Specific CD8 T Cells

The doubling time of virus-specific CD8 T cells during the expansion phase (days 1–8 postinfection) can be calculated from the data in Figure 4. The frequencies of CD8 T cells specific for a given epitope in naive mice are probably in the range of 10^{-4}– 10^{-5} per CD8 T cells,

Table 1. Quantitation of LCMV-Specific CD8 T Cells by LDA for CTL_p and IFNγ ELISPOT

LCMV Infection	Peptide	Mouse	Antigen-Specific CD8 T Cell Frequency		
			LDA for CTL_p	IFNγ ELISPOT	ELISPOT/LDA Ratio
8 days (acute)	NP396-404	1	1/122	1/4.9	25
		2	1/76	1/4.8	16
		3	1/162	1/4.0	40
	GP33-41	1	1/81	1/3.5	23
		2	1/152	1/7.1	21
		3	1/270	1/4.1	65
	GP276-286	1	1/818	1/22	37
		2	1/1900	1/19	100
		3	1/2700	1/14.9	180
120 days (memory)	NP396-404	1	1/420	1/35	12
		2	1/350	1/27.5	13
		3	1/400	1/34	12
	GP33-41	1	1/700	1/50	14
		2	1/500	1/31	16
		3	1/420	1/33	13
	GP276-286	1	1/2800	1/130	21
		2	1/1800	1/115	16
		3	1/2700	1/112	24

C57BL/6 mice were infected with LCMV (Armstrong) and the frequency of epitope-specific CD8 T cells in the spleen quantitated by LDA and IFNγ ELISPOT assay.

giving a baseline value of 10^2–10^3 epitope-specific CD8 T cells/spleen. Since this is a "soft" number, we have not used it in calculating the doubling time of antigen-specific CD8 T cells. However, the values obtained on days 3, 5, and 8 postinfection are based on highly specific and sensitive assays and should allow us to determine the number of divisions virus-specific CD8 T cells have undergone during this 5-day period of antigen-driven expansion. It should be pointed out that the increase in LCMV-specific CD8 T cells in the spleen truly represents cell proliferation and is not due simply to redistribution of T cells (i.e., migration of CD8 T cells to spleen). As shown in Figure 2B, all of the antigen-specific CD8 T cells in the spleen were BrdU positive. Moreover, during the acute phase of LCMV infection, not only was there an increase in the number of virus-specific CD8 T cells in the spleen, but similar expansions were seen in the blood and lymph nodes (data not shown). Thus, the increase in antigen-specific CD8 T cells in the spleen should reflect the division of these cells. As shown in Figure 4A, on day 3 after LCMV infection there were $1.2 \pm 0.3 \times 10^4$ NP118-126–specific CD8 T cells/spleen; by day 5 the number had increased to $4 \pm 1.0 \times 10^6$; and by day 8 the number had reached a peak of $2.8 \pm 1.0 \times 10^7$. This represented a 2300-fold increase (~11 divisions) in 5 days, with the fastest growth occurring between days 3 and 5, during which period NP118–126-specific CD8 T cells increased about 500-fold (9 divisions) with an estimated doubling time of 6–8 hr. Although the number of antigen-specific CD8 T cells continued to increase between days 5 and 8, there was a considerable slowing of the growth rate (2 or 3 divisions with an estimated doubling time of 24–30 hours), and this slow growth coincided with clearance of the virus (Lau et al., 1994; Asano and Ahmed, 1996).

It is useful to compare the expansion kinetics of the dominant NP 118 response with the weaker GP283 response (Figure 4A). On day 3, there were 10-fold fewer GP283-specific CD8 T cells, suggesting that either these cells were present at a lower frequency in naive mice and/or that the concentration of GP283 peptide was limiting during the early stages of infection. However, between days 3 and 5, CD8 T cells responding to NP118 or GP283 expanded at similar rates (doubling time 6–8 hr), implying that neither epitope was limiting during this period. After day 5, there was only minimal expansion of the subdominant response, suggesting that stimulus from the weaker epitope was considerably less than the dominant NP118 epitope. This simple model of viral peptide concentration driving T cell expansion is consistent with the kinetics of LCMV infection and the much higher binding affinity of the dominant NP 118 epitope to L^d compared with the binding of GP283 to its presenting molecule K^d (IC_{50} [concentration of peptide inhibiting 50% of the binding of the radiolabeled probe]: 1.3 nM for NP118 vs. 73 nM for GP283) (van der Most et al., 1996).

Effect of Homologous and Heterologous Virus Infection on LCMV-Specific Memory CD8 T Cells

We next examined the effect of homologous and heterologous virus infections on the frequency and effector function (killing) of LCMV-specific memory CD8 T cells. LCMV immune mice were divided into three groups: one group was left untreated; the second group was infected with VV; and the third group was rechallenged with LCMV. Seven days after infection, the frequency of LCMV-specific CD8 T cells and the presence of LCMV-specific and VV-specific CTL activity were determined. The results of a representative experiment (one of three) are shown in Figure 5. Rechallenge with homologous virus stimulated proliferation of LCMV-specific CD8 T cells and the development of virus-specific killer cells. In contrast, immune mice infected with VV did not show

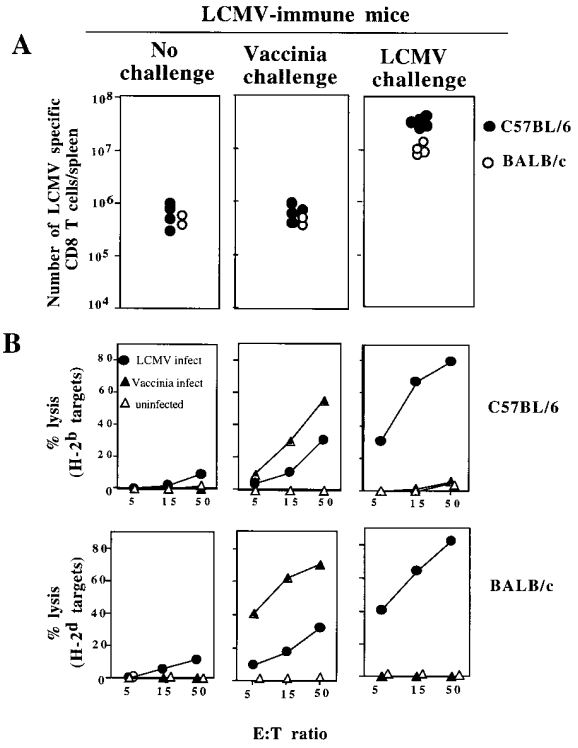

Figure 5. Expansion of Memory CD8 T Cells by Homologous but Not by Heterologous Virus Challenge

(A) LCMV-immune C57BL/6 or BALB/c mice 6 months postinfection were either untreated (no challenge), infected with VV, or infected with LCMV clone 13. On day 7 postinfection, spleen cells from individual mice were assayed for the number of antigen-specific CD8 T cells. The antigen-specific cell number was quantitated by tetramer staining and ELISPOT assay. Each point represents an individual mouse.

(B) Spleen cells from LCMV-immune C57BL/6 or BALB/c mice that were mock infected, VV infected, or LCMV clone 13 infected as described in (A) were tested for their ex vivo CTL activity on indicated targets. The targets used were H-2^b targets (in the case of C57BL/6 mice) or H-2^d targets (in the case of BALB/c mice) that were either uninfected, VV infected, or LCMV infected.

any detectable increase in the numbers of LCMV-specific memory CD8 T cells (Figure 5A). The VV-infected mice had enlarged spleens and lymph nodes and contained 20–40 $\times$ 10^6 CD8 CD44^{hi} cells/spleen compared to 5 $\times$ 10^6 in untreated immune mice. Thus, despite a substantial increase in the total number of activated CD8 T cells, there was minimal to no expansion of LCMV-specific CD8 T cells. However, spleen cells from VV-challenged LCMV immune mice exhibited a higher level of direct ex vivo LCMV-specific CTL activity compared to untreated immune mice (Figure 5B). The level of killing was about 3- to 5-fold higher based on lytic units. Taken together, these results show that infection with a heterologous virus does not increase the total number of LCMV-specific memory T cells but does induce killing function in this population of memory T cells.

The in vivo recall response after challenge with homologous virus was examined in more detail. LCMV-immune C57BL/6 mice were rechallenged with LCMV clone 13 (a variant of Armstrong), and the expansion of NP396-, GP33-, and GP276-specific CD8 T cells was monitored by tetramer staining and functional assays on days 3 and 5 post infection. Identification of NP396-specific CD8 T cells by D^b NP396 tetramers is shown in Figure 6A; Figure 6B presents a summary of data on all three epitopes. As shown in Figure 6A, there was rapid expansion of NP396-specific CD8 T cells after LCMV infection. Within 3 days, 38% of CD8 T cells were NP396 specific, and by day 5 this population had increased to 46%. By gating on D^b NP396 tetramer–positive cells and looking at the forward scatter (size), we found that more than 60% of the NP396-specific CD8 T cells were blasting on day 3 after infection, documenting the rapid recall response of memory T cells. This blasting population had decreased by day 5, consistent with data showing that immune mice resolve LCMV infection within 5 days (Lau et al., 1994). During the secondary in vivo response, the largest expansion was seen in NP396-specific CD8 T cells, followed by GP33 and GP276. When responses to all the epitopes were added together, more than 70% of the total CD8 T cells were LCMV specific on day 5 after rechallenge. Similar results were obtained when immune mice were rechallenged with LCMV Armstrong (data not shown). Thus, as in the primary infection, most of the CD8 T cell expansion during the recall response represents antigen-specific CD8 T cells.

Discussion

The main point of this study is that the size of the antiviral response is much greater than our previous estimates and that as many as 70% of the responding CD8 T cells during both primary and secondary infection can be virus specific. This finding, along with the results showing that a heterologous virus infection does not increase the number of memory T cells to an unrelated virus, provide compelling evidence that most of the CD8 T cell expansion seen during viral infection represents antigen-specific T cells. This should lead to a revision of our current models of viral-induced T cell proliferation that are based on the notion that most of the responding T cells are not specific to the infecting virus.

Why have previous studies underestimated the size of the virus-specific T cell response? In almost all viral systems examined to date, the LDA has been used to quantitate virus-specific CTL_p (Doherty et al., 1992; Ahmed and Gray., 1996; Zinkernagel et al., 1996). This technique involves plating graded numbers of cells in 96-well plates and stimulating them for 1–2 weeks in the presence of antigen plus interleukin-2 (IL-2). At the end of the culture period the contents of each well are tested for the presence of antigen-specific killer cells. An important caveat of this widely used assay is that only cells that are capable of dividing and surviving during the 1- to 2-week in vitro culture period will score as positive. Several recent studies have shown that activated T cells are prone to apoptosis upon restimulation with antigen—a phenomenon termed activation-induced cell death (AICD) (Nagata and Golstein, 1995). Thus, it is likely that many antigen-specific T cells either die or do not divide to a sufficient extent to score as positive in the LDA assay. In contrast, the techniques we have

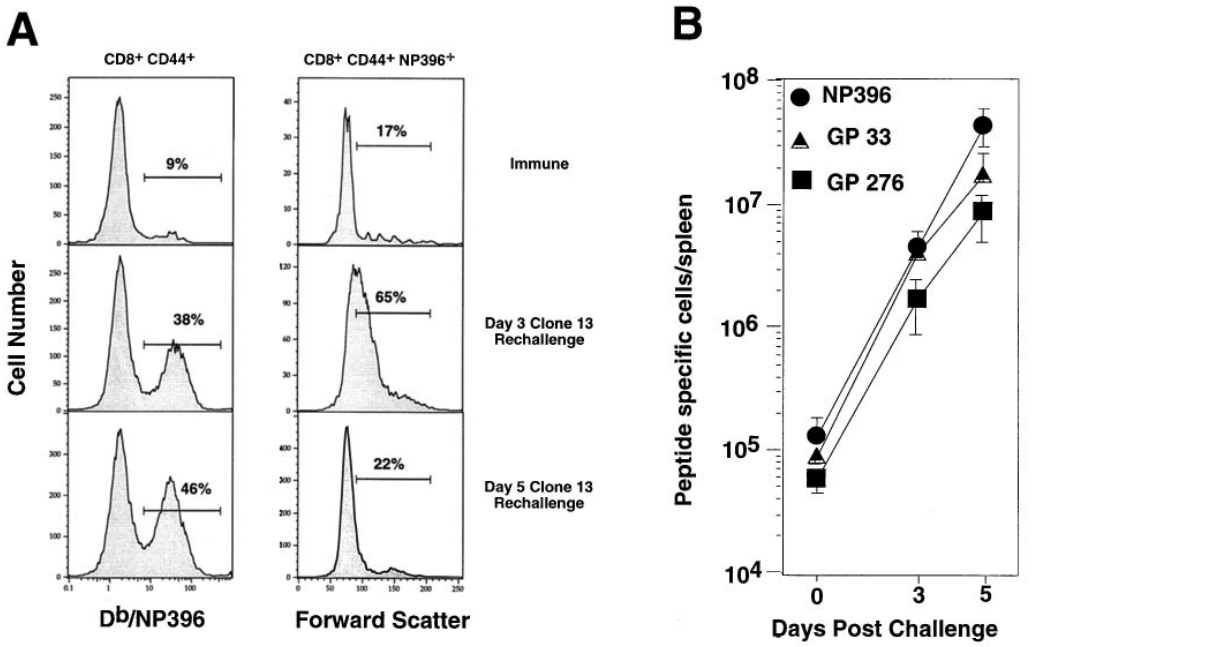

Figure 6. The Kinetics of LCMV-Specific CD8 T Cell Recall Responses

(A) C57BL/6 mice were immunized intraperitoneally with 2×10^5 pfu LCMV-Armstrong and rested 6 months prior to intravenous rechallenge with 2×10^6 pfu of LCMV clone 13. Splenocytes from rechallenged mice were analyzed 3 or 5 days later as indicated. (Left) Frequencies of NP396-404–specific cells per $CD8^+CD44^{hi}$ cells as determined by staining with D^b NP396-404 tetramers. (Right) The forward scatter of antigen-specific CD8 T cells (D^b NP396 tetramer–positive) cells. Gates for the blasting population were based on the forward scatter profile of the immune sample.

(B) Exponential division of memory CD8 T cells upon secondary rechallenge. LCMV immune C57BL/6 mice were rechallenged with LCMV clone 13 as described in (A). Antigen-specific CD8 T cell numbers specific for each of the indicated immunodominant epitope were quantitated using a single-cell IFNγ ELISPOT assay.

used do not have this limitation. This is especially true of staining with MHC class I tetramers, a method that involves no in vitro manipulations and allows direct visualization of the antigen-specific T cells. The two IFNγ functional assays require only a short period of in vitro stimulation (5 hr for intracellular stain and 24–36 hr for ELISPOT), and neither technique is dependent on long-term cell survival or proliferation.

Analysis of LCMV-specific CD8 T cells in acutely infected mice (day 8) showed that frequencies obtained by ELISPOT assay were 20- to 100-fold higher than numbers based on LDA (Table 1). At this time (i.e., day 8 postinfection) there is a large number of effector CD8 CTL, and it is likely that a substantial fraction of these highly activated cells are sensitive to AICD. Thus, it is not surprising that LDA analysis underestimated the true frequency of antigen-specific effector CD8 T cells. Although fewer differences were seen with memory CD8 T cells, it is notable that even in immune mice, which have very low if any effector CD8 T cells, only 10% of the LCMV-specific cells scored as positive by the LDA technique. Similar results have recently been reported for influenza virus (Lalvani et al., 1997). Thus, the LDA appears to underestimate the true frequency of both effector and memory T cells, with a greater underestimate for effector T cells. In addition to differences due to AICD, it is also possible that all virus-specific CD8 T cells can secrete IFNγ but only a subset of these cells can differentiate into killer cells.

Can our findings with the LCMV model be generalized to other viral infections, including those by human pathogens? It has been known for many years that acute EBV infection (infectious mononucleosis) is characterized by a massive increase in the number of CD8 T cells (Callan et al., 1996), and more recently it has also been shown that substantial CD8 T cell expansion occurs during primary HIV infection (Koup et al., 1994; Pantaleo et al., 1994, 1997). Increased numbers of activated CD8 T cells in the blood and cerebrospinal fluid of patients infected with human T cell leukemia virus type 1 (HTLV-1) have also been reported (Utz et al., 1996). It is likely that, similar to our findings with LCMV, the majority of the expanded CD8 T cells are EBV, HTLV-1, and HIV specific. In fact, recent studies analyzing TCR usage by CD8 T cells during acute EBV and HIV infections have shown expansions of a few dominant clones (Pantaleo et al., 1994, 1997; Callan et al., 1996). Such oligoclonal populations are most consistent with antigen-driven expansion of T cells. It will be interesting to see what frequencies are obtained in these human viral infections using the techniques we have described in this study. Even with viral infections that provide a much weaker stimulus to CD8 T cells, such as herpes simplex virus (HSV), it is possible that most of the responding cells are virus specific. A recent study (Cose et al., 1997) analyzing CD8 T cell responses in lymph nodes draining the site of HSV infection has documented expansion of CD8 T cells expressing a restricted TCR, suggesting

that a large proportion of the blasting cells may be HSV specific. Thus, it is likely that our study in the LCMV system, showing that more than 70% of the responding CD8 T cells are virus specific, will become a paradigm for viral infections in general.

It is well established that following viral clearance there is a precipitous drop in the number of antigen-specific CD8 T cells (Ahmed and Gray, 1996). During this period of death, more than 90% of the activated T cells undergo apoptosis. This down-regulation of the immune response is necessary to maintain homeostasis. In this study we have extended these findings to CD8 T cells responding to individual epitopes and have asked whether the contraction of the response is similar for dominant and subdominant epitopes. We found that the proportion of activated CD8 T cells that died was almost identical for dominant (NP118 in BALB/c; NP396 and GP33 in C57BL/6 mice) and subdominant (GP283 in BALB/c, GP276 in C57BL/6) responses. For each of the peptide-specific responses, approximately 5% of the activated CD8 T cells survived and went into the pool of memory T cells, and then long-term memory was equally well maintained for both strong and weak epitopes. Together these results show that the difference between a strong response and a weak response was not in the death phase nor in the maintenance of memory but was determined by the original burst size. These findings have clear implications for developing strategies to improve vaccines. They show that the size of the memory T cell pool is determined by the original burst size and emphasize the importance of initial priming in inducing long-term immunity.

We found that infection of LCMV immune mice with VV did not result in any detectable changes in the number of LCMV-specific memory T cells despite a substantial increase in the total number of activated CD8 T cells. In fact, as a result of this VV-driven expansion of non–LCMV-specific CD8 T cells, the frequency of LCMV-specific CD8 T cells was 2- to 3-fold lower at the peak of the VV response (day 7 postinfection). This shows that bystander activation plays a minimal role in the CD8 T cell expansion observed during viral infections. However, LCMV immune mice showed increased levels of LCMV-specific CTL activity after VV infection. Similar findings have been reported by others (Selin et al., 1996; Ehl et al., 1997). It is unlikely that this increased killing is due to VV-specific CTL that are cross-reactive to LCMV because naive mice infected with VV do not exhibit any detectable killing of LCMV-infected targets. So it appears that infection with a heterologous virus does not increase overall numbers of LCMV-specific CD8 T cells but increases the level of cytotoxicity. This effect could be mediated by cytokines (IL-12, for example) and/or could be due to a low-affinity TCR interaction that does not induce proliferation but causes differentiation of a subset of memory cells into killer cells.

In conclusion, this study provides definitive evidence that the majority of the CD8 T cells responding to a viral infection are antigen specific. This greatly revises our current thinking on the dynamics of T cell activation in vivo and warrants a reexamination of the prevailing models of viral-induced T cell proliferation.

Experimental Procedures

Virus Infection and Mice

Six- to 8-week-old male or female BALB/c and C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were infected with 2×10^5 pfu of LCMV Armstrong intraperitoneally and used at the indicated time points. For secondary rechallenge experiments, immune mice were injected with 2×10^6 pfu of LCMV clone 13 intravenously or with 2×10^6 pfu of VV intraperitoneally. Virus stocks were grown and quantitated as described previously (Ahmed et al., 1984).

Single-Cell ELISPOT Assay for IFNγ-Secreting Cells

The ELISPOT assay described by Taguchi et al. (1990) was modified to detect virus-specific CD8 T cells. First, 96-well filtration plates (Millipore, Bedford, MA) were coated with rat anti-mouse IFNγ antibody (clone R4–6A2, Pharmingen, San Diego, CA). Three- or 2-fold dilutions of responder cells in RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 50 mM 2-mercaptoethanol, and antibiotics (complete medium) were added into the wells along with 5×10^5 γ-irradiated (1200 rad) syngenic feeder cells and 10 units/well of recombinant human IL-2 (Pharmingen). Cells were incubated for 36 hr either with or without peptide stimulation (0.1 μg peptide/ml). The amino acid sequences of peptides used have been described (van der Most et al., 1996, 1998). After culture, the plates were washed followed by incubation with biotinylated anti-mouse IFNγ antibody (clone XMG 1.2, Pharmingen). Spots were developed using freshly prepared substrate buffer (0.3 mg/ml of 3-amino-9-ethyl-carbazole and 0.015% H_20_2 in 0.1 M sodium acetate [pH 5]). The frequency of peptide-specific CD8 T cells was calculated based on the percentage of CD8 T cells present in the responding population. Using this assay we could accurately measure a minimum of ten spots in 10^6 responder cells.

Flow Cytometry and FACS Analysis

Single-cell suspensions of spleen were prepared, and 10^6 cells were stained in phosphate-buffered saline containing 1% bovine serum albumin and 0.02% sodium azide (FACS buffer) for 30 minutes at 4°C followed by three washes in FACS buffer. Samples were acquired on either a FACScan flow cytometer or FACSCalibur instrument (Becton Dickinson, San Jose, CA). The data were analyzed using CELLQuest software (Becton Dickinson Immunocytometry Systems).

Intracellular IFNγ Staining

Spleen cells were cultured for 5 hr in 96-well flat-bottomed plates (Costar, Cambridge, MA) at a concentration of 1×10^6 cells/well in a volume of 0.2 ml complete medium supplemented with 10 units/well human recombinant IL-2 and 1 μl/ml Brefeldin A (Golgistop, Pharmingen) either with or without CTL epitope peptides. The peptides were used at a concentration of 0.1 μg/ml. After 5 hr of culture, the cells were harvested, washed once in FACS buffer, and surface stained in FACS buffer with phycoerythrin (PE)-conjugated monoclonal rat anti mouse CD8a (clone 53–6.7) antibody. After washing the unbound antibody, cells were subjected to intracellular cytokine stain using the Cytofix/Cytoperm kit according to the manufacturer's instructions (Pharmingen). For intracellular IFNγ staining we used FITC-conjugated monoclonal rat anti-mouse IFNγ antibody (clone XMG 1.2) and its isotype control antibody (rat IgG1) (Pharmingen).

Preparation of H-2D^b and H-2L^d Tetramers

The expression cassette for fusions to a BirA substrate peptide (BSP) (Schatz, 1993) was moved from pHN1-A2-BSP (Altman et al., 1996) on a SacI–Hind III fragment (containing the α3 domain of A2 plus the BSP) that was subcloned into the T7 polymerase vector pET-23a(+) (Novagen) cut with the same enzymes to create pET-23a-BSP. DNA coding for residues 1–280 of the soluble domain of D^b was amplified (from a D^b plasmid clone [a generous gift from S. Nathenson]) by the PCR with the 5′ primer GGGAATTCATATGGGCC CACACTCGATGCGG and the 3′ primer CGGGATCCGGACGGAGGA GGCTCCCA. This fragment was cut with NdeI and BamHI (sites underlined) and subcloned into pET-23a-BSP cut with the same enzymes (removing the α3 domain of A2) to create the plasmid pET23-D^b-BSP. Similarly, DNA coding for residues 1–280 of the L^d

was amplified (from a plasmid clone [a generous gift from Y. H. Chien]) with the 5′ primer GGAATTCCATATGGGCCCACACTCGATG CGG and the 3′ primer as above and identically subcloned to create pET23-L^d-BSP. The sequences of the MHC genes for both plasmids were verified by standard dideoxy sequencing. Each clone was used to transform the *Escherichia coli* strain BL21(DE3) and expression was induced with isopropyl-β-D-thiogalactopyranoside as described (Altman et al., 1993). Human β_2-microglobulin (β_2m) was expressed from the plasmid pHN1-β_2m as described (Garboczi et al., 1992). Folding, purification, biotinylation, and tetramer production of L^d and D^b peptide complexes was performed as described (Altman et al., 1996).

BrdU Staining

For BrdU labeling, LCMV (Armstrong)–infected mice were fed continuously with drinking water containing BrdU (0.8 mg/ml) for 8 days. On day 8 postinfection, freshly explanted spleen cells were surface stained with PE-conjugated anti-mouse CD8, Cychrome-conjugated anti-mouse CD44 (Pharmingen), and allophycocyanin-conjugated MHC tetramer of indicated specificity followed by intracellular BrdU stain as described (Tough and Sprent, 1994). For BrdU staining, FITC-conjugated mouse anti-BrdU antibody (Clone B44, Becton Dickinson) or its isotype control (mouse IgG1 antibody) were used.

Sorting of MHC Tetramer–Positive Cells

Freshly explanted spleen cells from LCMV-infected mice (day 8 postinfection) were surface stained with PE-conjugated anti-CD8 antibody and allophycocyanin-conjugated D^b NP396-404 tetramer in RPMI 1640 medium containing 1% fetal calf serum. CD8 T cells were sorted into tetramer-positive and -negative poulations in a Becton Dickinson FACS sorter.

Ex Vivo CTL Assay and LDA

Cytotoxic activity was tested in a standard 6 hr ^{51}Cr release assay as described previously. For VV infection, targets were infected at a multiplicity of infection of 10. Targets were coated with peptide NP396-404 or GP33-41 at a concentration of 0.1 μg/ml. LDA was performed as described previously (Lau et al., 1994; van der Most et al., 1996).

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