Independent Regulation of Lymphocytic Choriomeningitis Virus-Specific T Cell Memory Pools: Relative Stability of CD4 Memory Under Conditions of CD8 Memory T Cell Loss

Steven M. Varga, Liisa K. Selin and Raymond M. Welsh

*J Immunol* 2001; 166:1554-1561; 
doi: 10.4049/jimmunol.166.3.1554

http://www.jimmunol.org/content/166/3/1554

Why *The JI*?
- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**
This article cites 51 articles, 29 of which you can access for free at:
http://www.jimmunol.org/content/166/3/1554.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Independent Regulation of Lymphocytic Choriomeningitis Virus-Specific T Cell Memory Pools: Relative Stability of CD4 Memory Under Conditions of CD8 Memory T Cell Loss

Steven M. Varga, Liisa K. Selin, and Raymond M. Welsh

Infection of mice with a series of heterologous viruses causes a reduction of memory CD8+ T cells specific to viruses from earlier infections, but the fate of the virus-specific memory CD4+ T cell pool following multiple virus infections has been unknown. We have previously reported that the virus-specific CD4+ Th precursor (Thp) frequency remains stable into long-term immunity following lymphocytic choriomeningitis virus (LCMV) infection. In this study, we questioned whether heterologous virus infections or injection with soluble protein CD4 Ags would impact this stable LCMV-specific CD4+ Thp memory pool. Limiting dilution analyses for IL-2-producing cells and intracellular cytokine staining for IFN-γ revealed that the LCMV-specific CD4+ Thp frequency remains relatively stable following multiple heterologous virus infections or protein Ag immunizations, even under conditions that dramatically reduce the LCMV-specific CD8+ CTL precursor frequency. These data indicate that the CD4+ and CD8+ memory T cell pools are regulated independently and that the loss in CD8+ T cell memory following heterologous virus infections is not a consequence of a parallel loss in the memory CD4+ T cell population. The Journal of Immunology, 2001, 166: 1554–1561.

Received for publication October 27, 2000. Accepted for publication November 1, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

© 2001 by The American Association of Immunologists 0022-1767/01/$02.00

---

Abbreviations used in this paper: LDA, limiting dilution assays; LCMV, lymphocytic choriomeningitis virus; Thp, Th precursor; PV, Pichinde virus; VV, vaccinia virus; MCMV, murine CMV; KLH, keyhole limpet hemocyanin; CD62L, CD62 ligand; CTLp, CTL precursor.
independently? We questioned whether heterologous virus infections and soluble protein Ags would perturb the stable LCMV-specific memory CD4+ Thp and demonstrate in this study that memory CD4+ T cells can resist deletion after a series of heterologous viral infections, even under conditions resulting in considerable memory CD8+ T cell loss (3, 12, 14). This argues that the size and specificity of the CD4+ and CD8+ memory T cell pools are regulated independently and that the loss in virus-specific CD8+ T cell memory is not simply a consequence of a concurrent attrition of the memory CD4+ T cell population.

Materials and Methods

Mice

C57BL/6 (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Male mice were used at 2–10 mo of age for most experiments.

Viruses, protein Ags, and inoculations of mice

The LCMV Armstrong strain and the AN3739 strain of Pichinde virus (PV) were propagated in baby hamster kidney cells (BHK21), as described previously (21). The WR strain of vaccinia virus (VV) was propagated in L929 cells (22). Murine CMV (MCMV), strain Smith, was obtained from the salivary glands of infected BALB/c mice (23). For acute virus infections, mice were inoculated i.p. with 5 × 106 PFU LCMV, 4 × 106 PFU PV, or 1 × 106 PFU VV, or 1 × 106 PFU MCMV. To prevent CD4+ T cell activation against cell debris or FBS Ags present in the virus stock, the virus preparations were either directly diluted in PBS (LCMV and MCMV) or purified over sucrose gradients and diluted in PBS (PV and VV), as described previously (12, 15). Mice receiving multiple virus infections were immunized with a sublethal dose of one virus, rested for at least 8 wk to allow the immune system to return to homeostasis, and then challenged with the next virus. Immune mice used in all memory experiments were infected previously (12, 15). Mice receiving multiple virus infections were immunized over sucrose gradients and diluted in PBS (PV and VV), as described previously (12, 15). Mice receiving multiple virus infections were immunized with a sublethal dose of one virus, rested for at least 8 wk to allow the immune system to return to homeostasis, and then challenged with the next virus. Immune mice used in all memory experiments were infected previously (12, 15). Mice receiving multiple virus infections were immunized over sucrose gradients and diluted in PBS (PV and VV), as described previously (12, 15). Mice receiving multiple virus infections were immunized with a sublethal dose of one virus, rested for at least 8 wk to allow the immune system to return to homeostasis, and then challenged with the next virus. Immune mice used in all memory experiments were infected previously (12, 15). Mice receiving multiple virus infections were immunized over sucrose gradients and diluted in PBS (PV and VV), as described previously (12, 15). Mice receiving multiple virus infections were immunized with a sublethal dose of one virus, rested for at least 8 wk to allow the immune system to return to homeostasis, and then challenged with the next virus. Immune mice used in all memory experiments were infected previously (12, 15). Mice receiving multiple virus infections were immunized over sucrose gradients and diluted in PBS (PV and VV), as described previously (12, 15). Mice receiving multiple virus infections were immunized with a sublethal dose of one virus, rested for at least 8 wk to allow the immune system to return to homeostasis, and then challenged with the next virus. Immune mice used in all memory experiments were infected previously (12, 15). Mice receiving multiple virus infections were immunized over sucrose gradients and diluted in PBS (PV and VV), as described previously (12, 15). Mice receiving multiple virus infections were immunized with a sublethal dose of one virus, rested for at least 8 wk to allow the immune system to return to homeostasis, and then challenged with the next virus. Immune mice used in all memory experiments were infected previously (12, 15). Mice receiving multiple virus infections were immunized over sucrose gradients and diluted in PBS (PV and VV), as described previously (12, 15).

Cell preparations and flow cytometry

Mice were sacrificed by cervical dislocation, and spleens were removed aseptically. Splenic leukocytes were obtained by preparing single-cell suspensions from spleens and treating them with 0.84% NH4 Cl to lyse the erythrocytes, as described previously (24). For multicolor FACS analysis, 1–106 cells were stained with PE-conjugated anti-CD4 (clone H129.19) and either anti-CD44 (clone IM7) or anti-CD62 ligand (CD62L; clone R3-34; BD PharMingen). At least 60,000 events were acquired and analyzed in two-color mode using a Becton Dickinson (San Jose, CA) FACS cytometer, and between 10,000 and 20,000 events were acquired from each sample. The data were analyzed using CellQuest software (Becton Dickinson). Intracellular IFN-γ staining

Intracellular cytokine staining was performed as described in detail previously (5). Briefly, −2 × 106 cells were stimulated for 5 h in the presence of 10 U/ml of recombinant human IL-2 (BD Pharmingen) and 10 μg/ml of brefeldin A (Sigma) in the presence (5 μg/ml) or absence of one of the two LCMV MHC class II-restricted peptides, GP53–43 (KAVYNFATCGI) or NP396–404 (FQPQNGQFI) peptides.

LDA of virus-specific precursors

LDA to detect LCMV-specific CD4+ Thp were performed as described previously (4). To determine the frequency of peptide-specific CD4+ Thp, peritoneal exudate cells were pulsed with 5 μg/ml of one of the two known LCMV MHC class II-restricted peptides, GP61–80 and NP309–328. For determinations of KLH or OVA-specific CD4+ Thp, peritoneal exudate cells were pulsed with 100 μg/ml of the appropriate protein Ag. The frequencies were corrected for the purity of the sorted CD4+ populations. LDA to detect LCMV-specific CD8+ CTL precursor (CTLp) were performed as described previously (3, 13).

Statistical analysis

CD4+ Thp and CD8+ CTLp frequencies were calculated using χ2 analysis according to the method of Taswell (26) on a computer program kindly provided by Dr. Richard Miller (University of Michigan, Ann Arbor, MI).

Results

T cell distribution and activation phenotype of CD4+ T cells following acute viral infections

Fig. 1 shows the percentage of CD4+ and CD8+ T cells in the spleen of C57BL/6 mice acutely infected with either LCMV, PV, or MCMV. Also shown is the increase in the total leukocyte number in the spleen that occurs during each of these virus infections (3). In agreement with previous work (4, 24), LCMV infection results in a 2-fold increase in the total leukocyte number in the spleen and a conversion of the CD4 to CD8 ratio from 2.1 to 1.2–3. Each of the other viruses also induced an increase in the total leukocyte number in the spleen as well as a conversion of the CD4 to CD8 ratio (Fig. 1), but none of the other viruses induced these changes to the extent LCMV infection does. Correlating with the inversion of the CD4 to CD8 ratio, each of the viruses also induced a marked increase in the total number of CD8+ T cells. Fold-increases in the total number of CD8+ T cells per spleen at day 9 p.i. were as follows: LCMV, 5.6; PV, 1.7; and MCMV, 2.2. In contrast, LCMV, PV, and VL infection induced no increase in the total number of CD4+ T cells in the spleen, whereas MCMV infection induced only a slight 1.3-fold (i.e., 30%) increase. It has been reported that there is an increase in the proportion of CD4+ T cells expressing increased amounts of CD44 and lower amounts of CD62L following an acute LCMV infection (4, 6, 27, 28). Fig. 2 shows that each of these virus infections similarly induces the modulation of the activation markers CD44 and CD62L on gated CD8+ T cells, consistent with an activated cell phenotype (29, 30). To further examine the extent to which each of these viruses activate CD4+ T cells, we performed intracellular IFN-γ staining on PMA- and ionomycin-stimulated CD4+ T cells isolated from naive mice or mice acutely infected with either LCMV, PV, or MCMV. As can be seen in Fig. 3, each of the viruses stimulates increased frequencies of IFN-γ-producing CD4+ T cells. This, combined with the up-regulation of activation markers discussed above, suggests that all of these viruses are capable of inducing an activated CD4+ T cell response, even though the total number of CD4+ T cells in the spleen remains relatively constant.

LCMV-specific CD4+ Thp frequency does not decline following multiple heterologous virus infections

LCMV-specific CD4+ Thp and CD8+ CTLp frequencies remain quite stable in long-term immunity (1–6). However, heterologous virus infections have a profound impact on the memory CD8+ T cell pool by causing reductions in the CD8+ CTLp frequency to earlier virus infections (3, 12, 14). In this study, we examined the stability of the LCMV-specific CD4+ Thp frequency following

Downloaded from http://www.jimmunol.org/ by guest on October 29, 2017
multiple heterologous virus infections to determine whether these heterologous viruses could also induce a similar perturbation in the memory CD4\(^+\) T cell compartment. We focused on experiments examining LCMV-immune mice that had been challenged with 1–3 heterologous viruses. Fig. 4 shows that the LCMV-specific CD4\(^+\) Tp does not decline (within the 95% confidence limits) following three heterologous virus infections (PV, VV, and MCMV) in three individual experiments using a pool of three mice per group. A CD8 LDA was performed in one of these assays (Fig. 4, bottom group) to show that within the same mice, the LCMV-specific CD8\(^+\) CTLp frequency does decline, as we have previously reported (3). The CD8\(^+\) CTLp frequency declined 6-fold in LCMV- and PV-immune mice and 18-fold in LCMV-, PV-, VV-, and MCMV-immune mice as compared with LCMV-immune mice. This decline in CTL memory is more dramatic than the usual 4-fold reduction we usually see with this virus combination, but we presented this in this study to show that in some experiments major declines in CD4\(^+\) T cell memory can occur under conditions of CD4\(^+\) T cell stability. Experiments on the stability of LCMV-specific T cell memory were also done in mice infected first with PV and then sequentially with LCMV, VV, and MCMV. Fig. 5 shows three experiments indicating that the LCMV-specific CD4\(^+\) Tp frequency did not decline after two additional infections. In contrast, CD8\(^+\) Tp to LCMV declined by more than 2-fold (Fig. 5, bottom), a result consistent with our previous studies (12).

Our most recent work examining the fate of the virus-specific CD8\(^+\) CTLp following multiple heterologous virus infections has revealed that the ratio of the LCMV peptide-specific CD8\(^+\) T cells is altered following each virus infection (12). This work shows that although CTLp specific to each of the three immunodominant MHC class I-restricted peptides are reduced, the CTLp of some specificities are affected more than others, indicating that the reduction of the memory CD8\(^+\) T cell pool that occurs following heterologous virus infections is a partly selective event. To address this issue with CD4\(^+\) T cell memory, we performed additional experiments using the two known LCMV-encoded MHC class II-restricted CD4 peptides to follow the fate of the virus peptide-specific memory CD4\(^+\) Tp under these same conditions. Table I shows two independent LDA experiments, using a pool of three mice per group, in which there is no significant decline in the LCMV-specific CD4\(^+\) Tp frequency to whole virus or to either of the two LCMV MHC class II-restricted CD4 peptides in LCMV-, PV-, VV-, and MCMV-immune mice. Thus, in a total of five independent LDA experiments (using a pool of three mice per group) shown in Table I and Fig. 4, there was <2-fold decrease in the LCMV-specific CD4\(^+\) Tp frequency to whole virus and in two experiments (using a pool of three mice per group) there was <2-fold decrease in the LCMV-specific CD4\(^+\) Tp frequency to the GP61–80 epitope. The NP309–328 epitope represents a much smaller proportion of the IL-2-producing LCMV-specific CD4\(^+\) T

![FIGURE 1.](https://www.jimmunol.org/figure1.png)
Intracellular staining of peptide-specific IFN-γ in peptide-treated cells from uninfected mice, and background NP396–404 peptide. Of note is that virtually no signal was found in peptide-treated cells from three mice per group. These experiments, each group represents a pool of splenic lymphocytes from mice either uninfected (naive) or infected with either LCMV, VV, PV, or MCMV for 9 days. Splenocytes were stained with anti-CD4 and either anti-CD44 or anti-CD62L mAb. A CD4 gate was applied, and the histograms represent the percentage of gated CD4+ T cells that fall within that region is displayed. Data shown are representative of three separate experiments with two individual mice per experiment.

Intracellular staining of peptide-specific IFN-γ-producing CD4+ T cells following multiple heterologous virus infections

LCMV-specific memory CD4+ and CD8+ T cells can be detected following stimulation with peptide and IL-2 in the presence of brefeldin A and staining for intracellular IFN-γ. In this study we questioned whether the frequency of LCMV-specific memory CD4+ T cells remained stable by this assay, as it did using the IL-2-based assay, and whether the results were truly independent of the type of virus used. In this study, we used four different viruses: LCMV, VV, PV, and MCMV. Mice were infected with each virus individually, and the frequency of peptide-specific CD4+ T cells was measured 9 days after infection. The results were compared with those from mice that were infected with a combination of the four viruses. The data showed that the frequency of peptide-specific CD4+ T cells was not significantly different from the frequency in mice that were infected with a single virus. This was true regardless of whether the virus was LCMV, VV, PV, or MCMV. The data also showed that the frequency of peptide-specific CD4+ T cells was not significantly different from the frequency in naive mice.

The same mice, substantial losses in NP396–404 peptide-specific CD8+ T cell memory occurred after the additional virus infections. Fig. 6B plots the levels of Ag-specific T cells as a function of each virus infection. There is some experimental variation, and in this experiment NP396–404 peptide-specific CD8+ T cell frequencies declined with each successive infection; GP33–43 peptide-specific CD8+ T cell frequencies showed an overall decline, though in this case there was a modest rebound after the fourth infection. In contrast, the GP61–80 peptide-specific CD4+ T cell frequencies remained stable after four viruses. There was a small dip in the GP61–80 peptide-specific CD4+ T cells after the third virus, VV, in this and two other experiments, but it is noteworthy that after the fourth infection the GP61–80 frequencies returned to normal. It is clear that the dynamics in shifting frequencies of the CD4+ and CD8+ T cell populations remain independent of each other.

Table II shows additional data with both GP61–80 and NP396–328 peptide-specific CD4+ T cells in a group of mice immunized 2 years previously to the group presented in Fig. 6. In this study, CD4+ T cell frequencies were analyzed from individual mice and are presented as the means ± SDs. In the experiment shown in Table II, upper panel, the mice had a relatively high NP396–328 response, but, like the GP61–80 response, it did not decline after three additional virus infections. Compared with this are CD8+ T cell frequencies to the MHC class I GP33–43 peptide from this earlier group of mice. These data, which represent the mean of two separate experiments, each with a pool of three mice per group, show a 5-fold decline in the GP33–43 peptide-specific CD8+ T cell frequencies.
cell memory, as we have previously published (12). In a separate series of experiments displayed in Table II, lower panel, LCMV-specific memory CD4\(^+\) T cell responses were quantified in mice that first received a PV infection and then were infected with LCMV, VV, and MCMV. Again, in two separate experiments with a total of six individual mice per group, both GP61–80 and NP309–328 peptide-specific memory CD4\(^+\) T cell responses remained relatively stable. These experiments collectively show, at the single-cell level, that the CD4\(^+\) and CD8\(^+\) memory T cell pools are independently regulated after subsequent infections and that the CD4\(^+\) T cell pool appears to be more stable under these conditions of infection.

**LCMV-specific CD4\(^+\) Thp frequency remains stable following protein Ag immunization**

One reason why there is no dramatic decline in the CD4\(^+\) Thp frequency following multiple heterologous virus infections may be that all of these virus infections induce stronger CD8\(^+\) than CD4\(^+\) T cell responses (Fig. 1). In an attempt to induce a CD4\(^+\) T cell response under conditions that do not preferentially expand CD8\(^+\) T cells, we examined the LCMV-specific CD4\(^+\) Thp frequency in LCMV-immune mice challenged with the complex protein Ag KLH in CFA that there was <2-fold decrease in the LCMV-specific CD4\(^+\) Thp frequency to whole virus and there was <2-fold decrease in the LCMV-specific CD4\(^+\) Thp frequency to the NP309–328 epitope. The LCMV-specific CD4\(^+\) Thp frequency to the GP61–80 epitope declined 3.3-fold in one of three experiments, but this drop was not consistent, as the other two experiments demonstrated no such significant decline (within the 95% confidence limits, and average decline in the three experiments was 1.2-fold). Likewise, challenge of LCMV-immune mice with CFA alone resulted in <2-fold reduction in the LCMV-specific CD4\(^+\) Thp frequency to whole virus and the NP309–328 epitope, whereas there was a 2.2-fold decrease to the GP61–80 epitope in only one of the three experiments. Finally, two additional experiments were performed in which LCMV-immune mice were challenged with KLH in CFA followed by OVA in a different adjuvant (TiterMax Gold). In each of these experiments there was <2-fold decrease in the LCMV-specific CD4\(^+\) Thp frequency to whole virus or to either of the two MHC class II-restricted peptides (Table III). A measurable protein Ag-specific CD4\(^+\) Thp frequency was detectable in all of these experiments, demonstrating that each of these immunizations efficiently stimulated a CD4\(^+\) T cell response (see Table III). These results demonstrate that regardless of the CD4\(^+\) T cell stimuli, the LCMV-specific CD4\(^+\) Thp frequency remains relatively stable.

### Table I. LCMV-specific CD4\(^+\) T cell precursor frequencies in multiply immune mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Uninfected APC</th>
<th>LCMV-infected APC</th>
<th>GP-pulsed APC</th>
<th>NP-pulsed APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCMV</td>
<td>6.067 (5,998–7,490)(^a)</td>
<td>1,751 (1,356–2,471)</td>
<td>1,842 (1,370–2,809)</td>
<td>5.106 (3,896–7,409)(^b)</td>
</tr>
<tr>
<td>L+P+V+M(^c)</td>
<td>6,118 (4,818–8,380)</td>
<td>1,736 (1,306–2,585)</td>
<td>1,275 (973–1,849)</td>
<td>4,260 (3,318–5,948)(^b)</td>
</tr>
<tr>
<td>LCMV</td>
<td>9,475 (7,485–12,904)</td>
<td>816 (678–1,024)</td>
<td>894 (736–1,136)</td>
<td>3,445 (2,843–4,370)</td>
</tr>
<tr>
<td>L+P+V+M</td>
<td>11,135 (8,242–17,153)</td>
<td>1,342 (1,102–1,714)</td>
<td>970 (807–1,214)</td>
<td>3,991 (3,143–5,463)</td>
</tr>
</tbody>
</table>

\(^a\) 95% confidence limits.

\(^b\) Not significantly different (within the 95% confidence limits) from uninfected APC controls.

\(^c\) Mice were infected with LCMV (L), PV (P), VV (V), and MCMV (M) at 2-mo intervals and were sampled at least 2 mo after the last infection.
even though modest declines are sometimes observed. However, none of the Ags tested could stimulate CD4⁺ T cell responses to the magnitude of virus-induced CD8⁺ T cell responses.

Discussion

We show in this study that heterologous viral infections or soluble protein CD4⁺ T cell Ags inoculated in the presence of adjuvants either only moderately reduce or, in most cases, have no impact on the preservation of the LCMV-specific CD4⁺ T cell memory, monitored either by LDA for IL-2-producing cells or by flow cytometry for IFN-γ-producing cells. In contrast, heterosexual viral infections reproducibly cause significant decreases in otherwise stable virus-specific CD8⁺ T cell memory (3, 12, 14). These virus-induced decreases in CD8⁺ T cell memory have now been shown by LDA for CTLp (3), by flow cytometry for IFN-γ-producing cells in response to MHC class I peptides (Ref. 12, Table II, and Fig. 6), and by frequency analyses of CD8⁺ T cells binding chimeric IgG-MHC molecules loaded with MHC class I viral peptides (12). This dichotomy, which is illustrated well in Table II, Fig. 6, and by the third experiment in Fig. 4, indicates that CD4⁺ and CD8⁺ memory T cell pools are regulated independently of each other. This means that the loss in CD8⁺ T cell memory is not secondary to a loss in CD4⁺ T cells, and it also indicates that whatever factors drive the loss of memory CD8⁺ T cells do not cause attrition of the CD4⁺ T cell memory population.

The differences in the stabilities of the CD4⁺ and CD8⁺ memory T cell pools may not be due to any inherent differences in their regulation but instead may be a consequence of the apparently higher frequency of virus-specific CD8⁺ than CD4⁺ T cells within the memory pool (1– 6, 8, 11). Work in the LCMV system has revealed that >10% of the CD8⁺ T cells in LCMV-immune mice are LCMV-specific (8, 11, 12). If memory CD8⁺ T cells for each pathogen a host experiences were preserved at a similar frequency, the CD8⁺ T cell memory pool would quickly fill to capacity. These memory T cells could be accommodated by a continual expansion of the pool, but, even though the memory pool does moderately increase in size with age, homeostatic regulation limits this expansion and the size of lymphoid organs. Therefore, accommodation of new memory cells should require a deletion of some of the memory cells pre-existing in the pool. Assessments of the frequency of LCMV-specific CD4⁺ memory T cells suggest that they may be substantially lower than CD8⁺ memory T cells (Refs. 3–5, Table II, and Fig. 6).

Table II. Frequency of IFN-γ-producing peptide-specific CD4⁺ and CD8⁺ T cells in multiply immune mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Percentage of IFN-γ⁺ CD4⁺ T Cells</th>
<th>Percentage of IFN-γ⁺ CD8⁺ T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GP61–80</td>
<td>NP309–328</td>
</tr>
<tr>
<td>LCMV immune</td>
<td>0.76 ± 0.43 (3)</td>
<td>1.30 ± 0.83 (3)</td>
</tr>
<tr>
<td>LCMV + PV + VV + MCMV</td>
<td>0.83 ± 0.68 (3)</td>
<td>1.51 ± 0.91 (3)</td>
</tr>
<tr>
<td>PV + LCMV</td>
<td>1.07 ± 0.38 (6)</td>
<td>0.45 ± 0.12 (6)</td>
</tr>
<tr>
<td>PV + LCMV + VV + MCMV</td>
<td>1.20 ± 0.40 (6)</td>
<td>0.47 ± 0.11 (6)</td>
</tr>
</tbody>
</table>

a Splenocytes from immune C57BL/6 mice were stimulated with either one of the two LCMV MHC class II-restricted peptides, GP61–80 and NP309–328, or a MHC class I-restricted peptide, GP33–43, in the presence of IL-2 and brefeldin A for 5 h as described in Materials and Methods. The numbers shown represent the percentage of CD4⁺ IFN-γ⁺ or CD8⁺ IFN-γ⁺ T cells in the spleen. The first two rows display CD4 data from a single experiment with three individual mice per group, whereas the bottom two rows represent CD4 data from two separate experiments with a total of six individual mice per group. The CD8 data shown in the first two rows represent the mean of two separate experiments, each with a pool of three mice per group. All data are shown as the mean ± SD. The number in parentheses refer to the total number of mice per group.

b Significantly different as compared to LCMV-immune mice; p < 0.05.
Another factor relevant to memory pool “shelf-space” issues relates to the different dynamics of the CD4+ and CD8+ T cell responses during acute viral infections, as illustrated in Fig. 1. Viruses induce major expansions in the number of CD8+ T cells but not CD4+ T cells (3, 4). During that expansion period CD8+ T cells not specific for the virus do not increase in number and, therefore, are diluted out (31). After clearance of virus, the cells in the immune system undergo apoptosis and return to preinfection levels (32). The contraction of the CD8+ T cell response is much more dramatic than that of the CD4+ T cell response, and competition for protective niches in the homeostatic spleen would be much more vigorous for the CD8+ than for the CD4+ T cells. Such a competition might be disadvantageous for the survival of pre-existing memory CD8+ T cells. Should the CD4+ T cell number expand as dramatically as the CD8+ T cells do during viral infection, there might be similar attrition of the CD4+ T cells. However, few pathogens or protein Ags stimulate CD4+ T cell responses to the levels that CD8+ T cells get stimulated by viral infections.

A third possible reason for more selective deletion of CD8+ than CD4+ T cell memory may rest on differential sensitivity of these memory populations to apoptotic events. We have recently found that pre-existing memory T cells not specific for an infecting virus undergo apoptosis and decline in number and that such apoptosis is more dramatic in the CD8+ than in the CD4+ T cell compartment (33). In this model there would be an active depletion of memory cells exposed to cytokines in the absence of Ag stimulation, in contrast to a more passive dilution and competition model as described above.

A fourth factor that may be of significance in the preservation of memory T cell populations is whether or not they display cross-reactivity to subsequently encountered Ags. It is well established that any given TCR can see more than one ligand (34–38), and in some cases these cross-reactive interactions can lead to altered T cell responses and effector functions (39–41). We have observed in our experiments on the decline of CD8+ T cell memory after heterologous viral infections that cross-reactive memory T cells may be preserved at the expense of the non-cross-reactive ones, which are deleted (12). It is thus possible that CD4+ T cell memory is more stable because there is a higher level of CD4+ T cell cross-reactivity between heterologous Ags or even endogenous self-Ags. In LCMV-immune mice challenged with VV there is a very rapid and profound CD4+ and CD8+ memory-like T cell response that includes CD8+ CTLp cross-reactive between LCMV and VV (15). If cross-reactivity at the CD4+ T cell level exists, it may be more difficult to reduce the CD4+ T cell memory by heterologous infections. However, if cross-reactivity were to explain memory CD4+ T cell stability, it would have to be dramatic and very wide-spread, as the stability was observed in epitopes expressed on different proteins, and even diverse Ags such as KLH and OVA, in their respective adjuvants, failed to reproducibly induce a decline in CD4+ T cell memory.

Persistence of the original Ag could also potentially influence the relative stabilities of CD4+ vs CD8+ T cell memory. Soluble protein Ags have been shown to persist in vivo in the form of immune complexes associated with follicular dendritic cells (42, 43). This might serve as a reservoir for CD4+ T cell Ags to exogenously access the class II Ag-presenting pathway. The preservation of CD8+ T cell Ags would most likely require a continued live virus infection of cells to engage the endogenous MHC class I pathway.

The resistance of the LCMV-specific memory CD4+ T cell pool to deletion following heterologous virus infections may play a role in preserving the remaining virus-specific CD8+ CTLp. In support of this, recent work in the influenza virus model has demonstrated that depletion of CD4+ T cells from Igg-/- mice results in diminished influenza virus-specific memory CD8+ T cell responses (44). Immunity against each of the viruses used in this study is primarily
dependent on MHC class I-restricted CD8\(^+\) CTL (3), which can often function independently of CD4\(^+\) T cells to mount a protective response (45–49). However, recent work has suggested that CD4\(^+\) T cells may play a role in the long-term maintenance of memory CD8\(^+\) CTL to viruses such as LCMV (20). There is a continually cycling population of LCMV-specific CD8\(^+\) CTLp that are capable of mediating cytolysis ex vivo against highly sensitive target cells (3, 4, 50, 51), and the continued presence of CD4\(^+\) T cells may help maintain the continued cycling of a small frequency of virus-specific memory CD8\(^+\) CTLp, possibly through the production of cytokines such as IL-2. Thus, the maintenance of the LCMV-specific CD4\(^+\) T cell frequency may help to maintain the remaining virus-specific CD8\(^+\) CTLp to ensure long-term protective immunity.

**Acknowledgments**

We thank Keith Daniels for his technical assistance and Tammy Krumpoch and Barbara Fournier for their help with the FACS analysis.

**References**