

# Attrition of T Cell Memory: Selective Loss of LCMV Epitope-Specific Memory CD8 T Cells following Infections with Heterologous Viruses

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## Summary

Using a variety of techniques, including limiting dilution assays (LDA), intracellular IFN $\gamma$  assays, and D<sup>b</sup>-IgG1 MHC dimer staining to measure viral peptide-specific T cell number and function, we show here that heterologous virus infections quantitatively delete and qualitatively alter the memory pool of T cells specific to a previously encountered virus. We also show that a prior history of a virus infection can alter the hierarchy of the immunodominant peptide response to a second virus and that virus infections selectively reactivate memory T cells with distinct specificities to earlier viruses. These results are consistent with a model for the immune system that accommodates memory T cell populations for multiple pathogens over the course of a lifetime.

## Introduction

Antigen-specific CD8 T cells generated during acute viral infections enter the memory pool and provide host resistance on reexposure to the virus. The development of antigen-specific T cell staining using tetrameric (Altman et al., 1996) or dimeric (Greten et al., 1998) peptide/MHC complexes as well as intracellular cytokine staining has made it possible to visualize antigen-specific T cells. Studies indicate that a high portion of CD8 T cells can be virus specific during the acute and memory phase of the immune response (Butz and Bevan, 1998; Callan et al., 1998; Flynn et al., 1998; Murali-Krishna et al., 1998); 40%–60% of the CD8 T cells in LCMV-infected mice can be shown to be virus specific during the acute phase of the response, and over 10% of the CD8 T cells remain virus specific after the immune system returns to homeostasis (Murali-Krishna et al., 1998). Virus-specific CTL memory is extremely stable, as demonstrated originally by LDA, which showed that CTL precursor (pCTL) frequencies for several viruses and for each of three LCMV-encoded immunodominant epitopes (NP396, GP33, and GP276) can remain stable for up to 2 years (Oehen et al., 1992; Hou et al., 1994; Lau et al., 1994;

Mullbacher, 1994; Selin et al., 1996; Doherty et al., 1997; Lin and Welsh, 1998).

The profound seeding of the memory T cell pool with such a high proportion of virus-specific cells after a single systemic virus infection poses a problem for the accommodation of memory T cells specific for multiple pathogens over the course of a lifetime. The memory T cell pool would rapidly fill up unless (1) the pool of memory T cells continuously expands, (2) preexisting memory T cells are deleted with each successive infection, or (3) the pool fills with T cells cross-reactive between two or more pathogens. It is possible that all three mechanisms are at work, at least to some degree. The proportion of CD8 T cells expressing memory markers such as CD44 does increase with age, but homeostatic regulation controlling the size of the spleen and lymph nodes places an upper limit on the total number of memory cells, precluding numbers which could accommodate many infections producing memory T cells at the levels produced by LCMV. Thus, there must be a deletion of preexisting memory populations when the host responds to another pathogen.

Marked decreases in pCTL occur in mice sequentially infected with a series of viruses that elicit systemic infections and strong CD8 T cell responses (Selin et al., 1996). Mice infected with LCMV and then, at 2 or more month intervals, with Pichinde virus (PV), vaccinia virus (VV), and murine cytomegalovirus (MCMV) reduced pCTL frequencies specific to all previous viruses with each successive viral infection. On average, one systemic virus infection reduced pCTL specific to an earlier virus by 3-fold, and two additional infections reduced pCTL to an earlier virus by 8-fold. The degree of pCTL reduction was not, however, proportionally equal between different viruses, as, for example, an infection that reduced LCMV-specific pCTL by 2-fold in the same mice reduced PV-specific pCTL by 5-fold. This suggests that qualitative changes relating to the T cell specificity may be influencing the degree of memory T cell attrition. These data in support of the loss of memory CD8 T cells by heterologous virus infections were generated by LDA, which depended on outgrowth of T cells and their cytolytic function. They did not distinguish between deleted T cell populations versus present but functionally inactive T cell populations, now known to exist at high levels in persistent virus infections (Oxenius et al., 1998; Zajac et al., 1998).

An important factor that might influence the decline in CTL memory after heterologous viral infections is whether or not putatively unrelated pathogens can cross-reactively stimulate memory T cells specific to previously encountered viruses. Although the T cell response to an infection is predominantly specific for the virus, it is also degenerate in that many of the virus-specific T cells can recognize other antigens. It has been calculated that a single TCR may recognize up to a million different peptide sequences (Mason, 1998). Our studies have shown that viruses once thought not to be cross-reactive at the levels of T cells can elicit cross-reactive responses if an immune system biased by a

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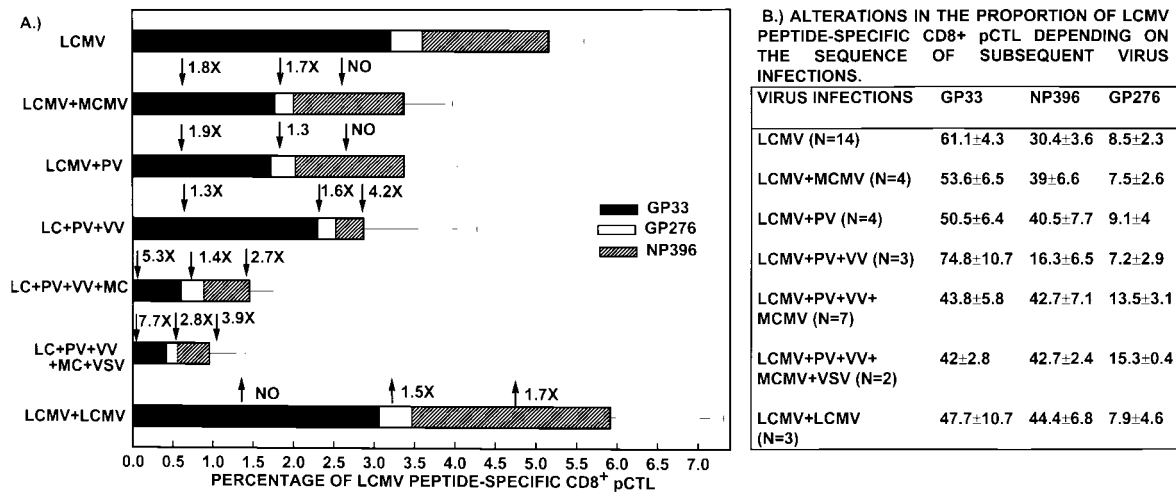


Figure 1. Selective Deletion of LCMV Epitope-Specific Memory pCTL by Subsequent Heterologous Virus Infections Resulting in Alterations in the Proportion of LCMV Peptide-Specific CD8 pCTL

C57BL/6 mice were infected with heterologous viruses sequentially, and splenocytes were harvested from mice in the resting immune state and set up in LDAs to determine the LCMV epitope-specific pCTL/f as described in Experimental Procedures. In (A) the data are listed as percent of CD8 to make it easier to compare to the intracellular IFN $\gamma$  and D<sup>b</sup>-IgG1 MHC dimer staining data. (B) is a LCMV epitope-specific proportional summary of the data from these same mice. The number of mice per group is recorded in (B). The numbers displayed in (A) represent the fold decrease of each epitope-specific population as compared to the LCMV-immune mice alone. "NO" means that there was no decrease in that epitope-specific population.

memory T cell pool specific for one virus is challenged with a heterologous virus. A low frequency of memory T cells in the starting population that can be stimulated cross-reactively with the second virus quickly expands and may enhance viral clearance of the second infection or contribute to immunopathogenesis (Selin and Welsh, 1994; Selin et al., 1994, 1998; Welsh et al., 1997). This type of cross-reactive expansion might enable unrelated viruses to qualitatively as well as quantitatively change the memory T cell pools specific for previously encountered infectious agents. We show here that heterologous viruses quantitatively delete and qualitatively alter the memory pool of T cells specific to a previously encountered virus.

## Results

### Selective Deletion of LCMV Epitope-Specific Memory pCTL by Subsequent Heterologous Virus Infections

Having previously demonstrated that pCTL frequencies analyzed on LCMV-infected targets decrease with each of several successive heterologous infections (Selin et al., 1996), we questioned whether there were equal reductions in the frequencies to each of three LCMV-encoded immunodominant peptides. The purpose of this experiment was to determine if there were qualitative changes in the memory CTL response to LCMV as a consequence of other viral infections. Figure 1B shows by LDA the proportion of the LCMV-specific memory T cell response accounted for by the 3 peptides in 14 mice analyzed over a period of 2 years. Note that the hierarchy is GP33>NP396>GP276 and that the standard deviations for these values are small. Thus, divergences in

these proportions and hierarchy as a consequence of heterologous infections can be shown to be highly statistically significant.

Figure 1A demonstrates by LDA that heterologous viral infections cause an overall reduction of the LCMV-specific pCTL frequency, as shown here by summing the frequencies of the three immunodominant peptides. As shown previously (Selin et al., 1996), homeostasis in lymphoid number occurred by 6 weeks after each infection. The numbers of splenic leukocytes after 2 ( $1.1 \times 10^8 \pm .1$  SEM,  $n = 12$ ), 3 ( $1.1 \times 10^8 \pm .1$ ;  $n = 7$ ), 4 ( $1.0 \times 10^8 \pm .1$ ;  $n = 8$ ), or 5 ( $0.9 \times 10^8 \pm .1$ ;  $n = 4$ ) infections were no greater than after the initial LCMV infection ( $0.9 \times 10^8 \pm .1$ ;  $n = 14$ ). Similarly, the percent of CD8 spleen cells did not significantly change over time: LCMV ( $14\% \pm 1.4\%$ ); LCMV+PV ( $13\% \pm 2.1\%$ ); LCMV+PV+VV ( $14.8\% \pm 2.3\%$ ); LCMV+PV+VV+MCMV ( $12\% \pm 1.1\%$ ); LCMV+PV+VV+MCMV+VSV ( $14.6\% \pm 1.7\%$ ). Since there were no significant changes in the total CD8 number per mouse, the data are presented as the percent of antigen-specific CD8 T cells.

The combined frequencies derived from targets pulsed with the three peptides are about double that of the frequencies we have reported with virus-infected targets, which may be less efficient at detecting low-affinity responses. One subsequent virus infection with either MCMV or PV resulted in an approximately 1.5-fold (about 33%) reduction in the combined LCMV peptide-specific pCTL, from 5.2% of the CD8 splenic T cells to 3.5%. Two subsequent viral infections (PV+VV) resulted in a 1.7-fold (44%) decrease in LCMV-specific pCTL to 2.9% of the CD8. Three subsequent virus infections (PV+VV+MCMV) resulted in a 3.5-fold (71%) reduction to 1.5% of the CD8 ( $p < .001$ ), while four virus infections

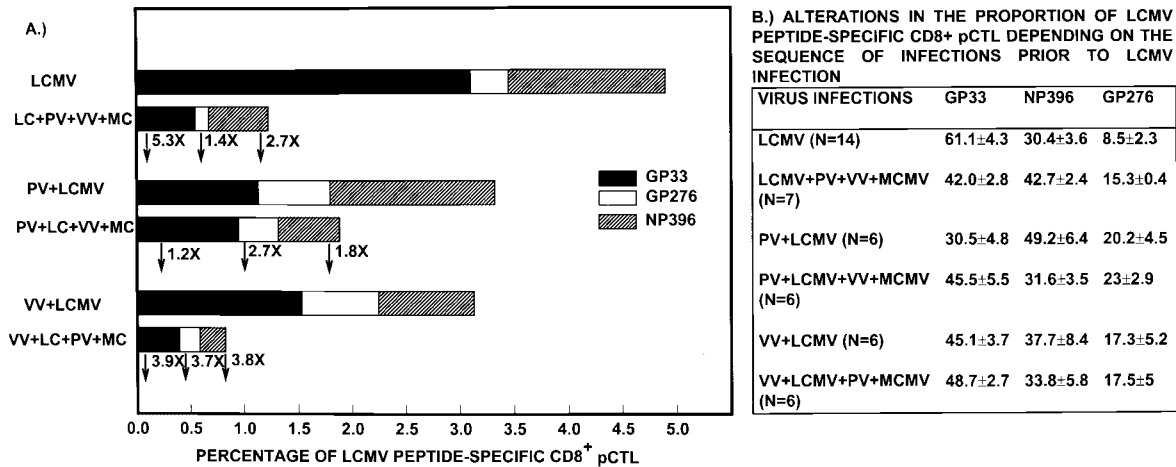


Figure 2. Prior Infection with a Heterologous Virus before Exposure to LCMV Alters LCMV Epitope-Specific Responses and Their Subsequent Deletion Resulting in Alterations in the Proportion of LCMV Peptide-Specific CD8 pCTL

(B) is an LCMV epitope-specific proportional summary of the data from these same mice. The number of mice per group is recorded in (B). The numbers displayed in (A) represent the fold decrease of each epitope-specific population as compared to the LCMV-immune mice alone.

(PV+VV+MCMV+VSV) resulted in a 5.4-fold (82%) decrease to 0.96% of the CD8 ( $p < .04$ ). In contrast, challenge of LCMV-immune mice with LCMV caused a slight elevation in the LCMV-specific pCTL frequency, though this increase was not statistically significant.

Figures 1A and 1B demonstrate that this total combined loss of LCMV epitope-specific memory pCTL after heterologous viral infections is selective and dependent on the virus sequence. Figure 1A shows that PV infection after LCMV resulted in a predominantly GP33-specific reduction that reversed if the mice were then infected with VV. In the LCMV-immune mouse, 61.1% of the pCTL were GP33-specific, but after PV infection this decreased to 50.5%, and the NP396-specific frequency increased from 30.4% to 40.5% of the memory pool. Subsequent VV infection of LCMV+PV-immune mice dramatically altered the peptide-specific response. There appeared to be a preservation of GP33-specific pCTL by VV but a loss in NP396-specific memory pCTL ( $p < .03$ ), as at this point the GP33-specific response represented 74.8% of the LCMV-specific memory pool. When MCMV followed VV infection, there was a further reduction in the total LCMV-specific pCTL, leaving the host with 5.3-fold decrease in GP33-specific pCTL ( $p < .005$ ), but only a 1.4-fold decrease in GP276-specific and a 2.7-fold decrease in NP396 responses compared to the LCMV-immune control. At this point GP33 (43.8%) and NP-396 (42.7%) specific pCTL represented about equal portions of the memory pool, and the proportion of GP276-specific pCTL had increased to 13.5%. VSV infection further decreased the GP33-specific pool so that it was 7.7-fold less than the original memory pool in LCMV-only immune mice ( $p < .04$ ), and the proportion of the response that was GP276-positive further increased to 15.3%. Rechallenge of LCMV-immune mice with LCMV resulted in proportional increases in the NP396-specific pCTL. Collectively, these results suggest that there is a mechanism that results in the attrition of memory T cells specific to viruses to which the host

has been previously exposed and that this mechanism may involve a selective process, as not all epitope-specific populations are reduced in equal proportions.

#### History of a Prior Infection with a Heterologous Virus before Exposure to LCMV Alters the LCMV Epitope-Specific Responses and Their Subsequent Deletion Pattern

A prior exposure to heterologous viruses before LCMV infection also affects the LCMV-specific T cell pool (Figures 2A and 2B). Prior immunity to PV resulted in 1.6-fold lower total LCMV-specific pCTL (5.2% vs 3.3%), and the epitope-specific pCTL repertoire was qualitatively different. NP396 represented 49.2% of the repertoire as compared to only 30.4% in the control LCMV-immune repertoire. There was also a greater portion of pCTL specific to GP276 (20.2%) in comparison to the control LCMV-immune mice (8.5%). When the PV+LCMV-immune mice were subsequently exposed to VV and then to MCMV, there was a greater (2.7-fold) deletion of the NP396-specific memory pool than the GP33-specific pool, whose proportion rose from 30.5% to 45.5%.

Mice previously immune to VV before infection with LCMV also had a 1.7-fold lower total LCMV-specific pCTL pool. Their T cell repertoire to LCMV was also altered in comparison to the LCMV-only pool, with a more equal proportion of GP33 (45.1%) and NP396 (37.7%) specific pCTL. The GP276-specific response was also increased proportionally and represented 17.3% versus 8.5% of the LCMV-only pool. The VV+LCMV-immune mice, when subsequently exposed to PV and then MCMV, demonstrated an equal proportional loss against each of the three peptides in comparison to the VV+LCMV-immune mice. The total number of LCMV-specific pCTL was quite reduced in the VV+LCMV+PV+MCMV-immune mice. These experiments indicate that when the memory T cell pool contains T cells specific to a previously encountered virus,

the nature of the T cell response to a subsequent viral infection is altered.

### Selective Reduction of Epitope-Specific Memory CD8 T Cells as Assayed by Intracellular IFN $\gamma$ Staining

Because LDAs require first the outgrowth of T cells in culture and then a functional cytotoxicity assay, we deemed it important to analyze T cell attrition by additional mechanisms. We therefore used the technique of intracellular IFN $\gamma$  staining.

Figure 3i shows in age-matched mice that subsequent heterologous viral infections elicited an overall decrease in the percentage of LCMV-specific IFN $\gamma$ -producing CD8 memory T cells. Totalling the peptides NP396-404, GP33-43, and GP276-286, PV infection of LCMV-immune mice resulted in an approximately 1.6-fold decrease (total %=7.3) in LCMV-specific IFN $\gamma$ -producing CD8 T cells; two subsequent viruses (PV+VV) resulted in a 1.8-fold (total percent = 6.3) decrease in LCMV-specific IFN $\gamma$ -producing CD8 T cells; three subsequent viruses (PV+VV+MCMV) resulted in a 3.9-fold decrease (total percent = 3.7) in LCMV-specific IFN $\gamma$ -producing CD8 T cells.

We have up to this time used the GP33-43 peptide in our LDA CTL analyses based on the assumption that in the 18 hr incubation on target cells, it will be processed by cellular proteases and will be presented both as an H-2D<sup>b</sup> (GP33-41) as well as an H-2K<sup>b</sup> (GP33-43) epitope. However, in the much shorter intracellular IFN $\gamma$  assay, it may not be processed as efficiently. Also, the D<sup>b</sup>-IgG1 MHC dimer, which will be described in the next section, will only load with the GP33-41 peptide. Therefore, in the IFN $\gamma$  assay presented in Figure 3, the T cells were also stimulated with GP33-41. The GP33-41 peptide gave very similar results to GP33-43, except that it defined a higher frequency of the LCMV-specific memory CD8 T cells (Figure 3i). Using the GP33-41 epitope to detect frequencies, 14.6% of the CD8 cells were scored as virus specific; this was reduced to 3.6% after three additional virus infections.

The intracellular IFN $\gamma$  assay results also demonstrated a selective reduction in LCMV epitope-specific CD8 memory T cells similar to that observed in LDAs. For example, after three subsequent viruses there was a 5.5-, 3.9-, and 2.4-fold decrease in GP33-43-, NP396-404-, and GP276-286-specific CD8 memory T cells, respectively. There was also a very reproducible selective loss of memory T cells producing the highest amount of IFN $\gamma$ , as those cells staining most brightly were greatly reduced in frequency after subsequent infections (Figure 3). The NP396-specific IFN $\gamma$  staining mean fluorescence intensity decreased 81% from 1241 to 237 after three subsequent infections, while GP33-41 dropped 46% from 695 to 377 and GP276 dropped 23% from 489 to 377. This might indicate that higher affinity cells specific for LCMV are preferentially lost on subsequent heterologous viral infections.

Figure 3ii provides additional studies showing that the LDA and IFN $\gamma$  techniques give similar results in paired assays, although the IFN $\gamma$  assay was about 2-fold more efficient. The IFN $\gamma$  assay demonstrated proportionally higher GP276 responses than the LDA technique, but

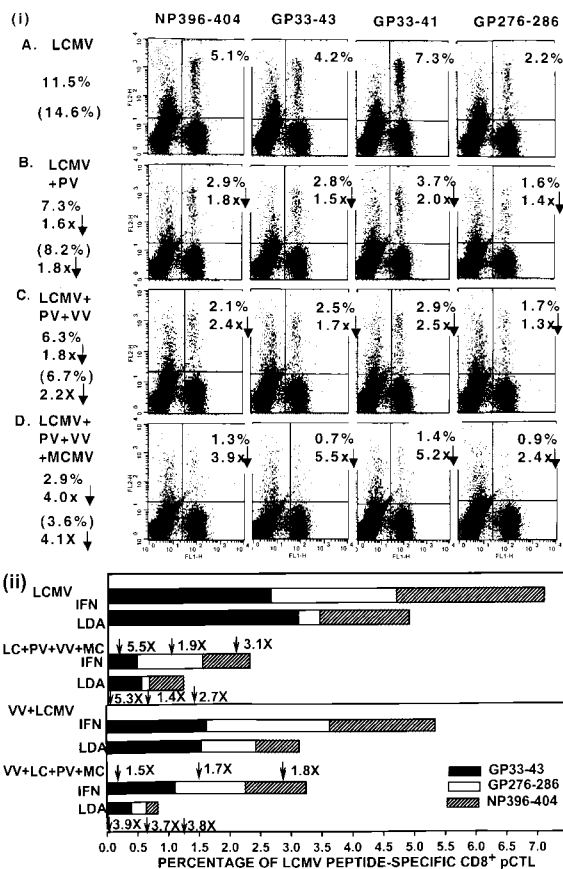


Figure 3. Selective Reduction of LCMV-Specific Memory CD8 T Cells as Assessed by Intracellular IFN $\gamma$  Staining

(i) Selective reduction of LCMV epitope-specific memory CD8 T cells by subsequent heterologous virus infections as assessed by intracellular IFN $\gamma$  staining. Each panel represents a pool of splenocytes from three similarly infected mice. These are representative data from one of three similar experiments. The x axis represents CD8 FITC staining and the y axis represents IFN $\gamma$  PE staining. The numbers recorded in the right upper quadrants represent the percent of IFN $\gamma$ -staining CD8 cells specific for that epitope. The number below this represents the fold decrease of each epitope-specific population from the LCMV-only immune mice. The two different forms of GP33, GP33-43 and GP33-41, were used in these experiments. The percentage to the left of each group is the total LCMV-specific CD8 frequency (NP396-404, GP33-43, and GP276-286) and below that is the fold decrease from the LCMV-only immune mice. The percentage to the left of each group presented in brackets is the total LCMV-specific CD8 frequency summing NP396-404, GP33-41, and GP276-286 responses and below that is the fold decrease from the LCMV-only immune mice.

(ii) Comparison of selective deletion of LCMV epitope-specific memory CD8 T cells by heterologous virus infections by LDA and intracellular IFN $\gamma$  staining. The pCTL/f in LCMV-immune mice and in LCMV+PV+VV+MCMV-immune mice is the mean of 14 and 7 different mice, respectively. The LCMV-immune and LCMV+PV+VV+MCMV-immune mice IFN $\gamma$  results represent the mean of two experiments in which three mice were pooled. The VV+LCMV-immune and the VV+LCMV+PV+MCMV-immune mice data for both techniques are based on the means of the same six mice. The numbers displayed in the figure represent the fold decrease of each epitope-specific population as compared to the LCMV-immune or VV+LCMV-immune mice.

both assays showed a very similar proportional decrease in each epitope-specific response in LCMV-immune mice challenged with three subsequent viruses. The greatest proportional decrease in both assays was that of the GP33 response (5.3- or 5.5-fold). The observation that the GP276-specific CD8 population may represent a greater proportion of the IFN $\gamma$ -producing cell response may indicate functional differences in the LCMV epitope-specific memory populations or the possibility that GP276-specific pCTL do not grow out well in LDAs.

#### Selective Reduction of Epitope-Specific Memory Cells as Assessed by D<sup>b</sup>-IgG1 MHC Dimer Staining

To directly enumerate antigen-specific memory T cells, we generated D<sup>b</sup>-IgG1, a soluble divalent D<sup>b</sup> complex, and used it to visualize antigen-specific CD8 T cells. To confirm the specificity of the reagent, D<sup>b</sup>-IgG1 complexes were loaded with peptides of interest and used to stain spleen cells from mice infected with LCMV or PV (Figure 4i). In a pool of splenocytes from two day 8 LCMV-infected mice, a total of 27.4% of the CD8 cells scored as LCMV specific. In contrast, very little staining was seen on T cell populations from control mice (data not shown) or from PV-infected mice (Figure 4i), which have a vigorous expansion of activated T cells, much like that of LCMV.

The levels of LCMV-specific memory CD8 cells detectable by the D<sup>b</sup>-IgG1 MHC dimer (8.8%) were comparable to though slightly lower than that detected by intracellular IFN $\gamma$  staining (Figure 4ii), mainly due to the lower efficiency of the GP33-41 dimer. After subsequent infections (PV+VV+MCMV) there was a 1.8-fold decrease (to 4.9%), and after four viruses there is a 2.7-fold decrease (to 3.3%) in LCMV-specific CD8 T cells. There was also evidence of a selective loss of epitope-specific memory CD8 T cells, as after four subsequent virus infections there was a 4.3-fold loss in GP33-specific CD8 memory T cell loss but only a 2.9-fold loss of NP396-specific memory cells. The deletion of antigen-specific memory T cells seen using D<sup>b</sup>-IgG1 were a little less dramatic than for LDA or the intracellular IFN $\gamma$  technique. This could imply that there are some functionally inactive T cells still present in the memory population, but this could be a technical issue of resolution of distinct populations over background binding, which was usually about 1%.

#### Reactivation of LCMV Epitope-Specific Memory T Cells by Acute Infection with VV or PV

LCMV-specific memory CTL can be reactivated from LCMV-immune mice by acute infections with heterologous viruses such as VV or PV (Yang et al., 1989; Selin et al., 1994). We have demonstrated cross-reactive T cell clones specific between LCMV and PV and between LCMV and VV, but the relative importance of cross-reactivity versus nonspecific bystander activation in the reactivation of LCMV-specific memory cells has not been clarified. The following experiments were designed to determine whether this reactivation in bulk is a non-specific bystander activation or whether there is selectivity in this reactivation, which would be suggestive of a role for cross-reactivity. Mice were immunized to the

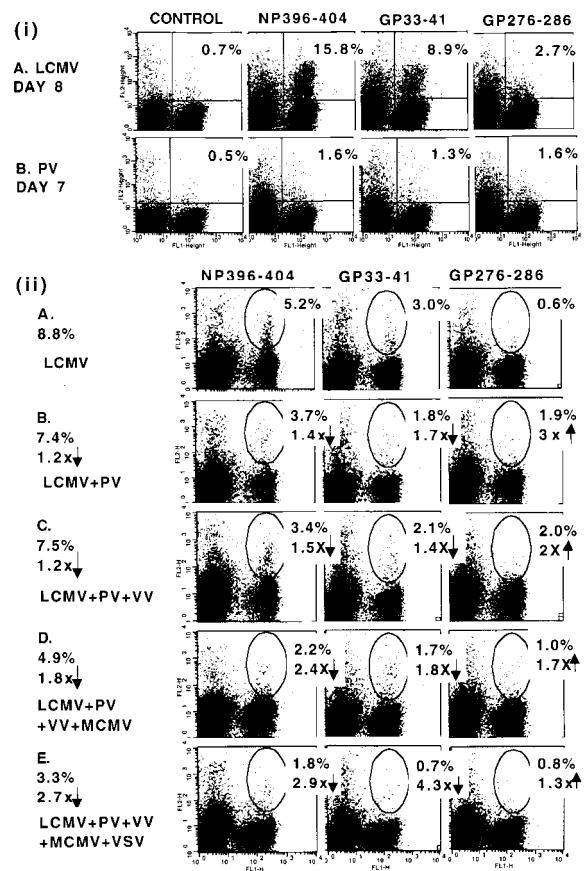


Figure 4. Selective Reduction of LCMV-Specific Memory CD8 T Cells as Assessed by D<sup>b</sup>-IgG1 MHC Dimer Staining

(i) D<sup>b</sup>-IgG1 MHC dimer staining of LCMV day 8 infected and PV day 7 infected splenocytes. The x axis represents CD8 FITC staining, and the y axis represents the peptide-D<sup>b</sup>-IgG1 MHC dimer staining. The y axis in the control FACS represents the secondary anti-IgG1 mAb without D<sup>b</sup>-IgG1 MHC dimer. The numbers recorded in the right upper quadrant of each FACS plot represent the percentage of CD8 T cells specific for that epitope.

(ii) Selective reduction of LCMV epitope-specific memory CD8 T cells by heterologous viruses as assessed by D<sup>b</sup>-IgG1 MHC dimer staining. Each data point represents a pool of splenocytes from three similarly infected mice. These data are representative of three similar experiments. The x axis represents CD8 FITC staining and the y axis represents D<sup>b</sup>-IgG1 MHC dimer staining. The numbers recorded in the right upper quadrant of each FACS plot are the percentage of CD8 T cells specific for that epitope. The number below this represents the fold change of each epitope-specific population from the LCMV-immune mice. The percentage to the left of each group is the total LCMV-specific CD8 frequency and below that is the fold decrease from the LCMV-immune mice.

LCMV parent strain or to different LCMV mutant viruses that had partial deletions of their responses to the NP epitope (NPV), GP33 epitope (GP1V), or all three immunodominant epitopes (GPNPV) (Figure 5A). These viruses contain single amino acid alterations in the specific epitopes and were generated by Oldstone et al. (1995) by culturing the virus in the presence of a T cell clone specific to the epitope that was to be deleted. Figure 5A shows bulk CTL assays of splenocytes from acutely infected mice 8 days postinfection. The parent strain generated a response with the normal hierarchy

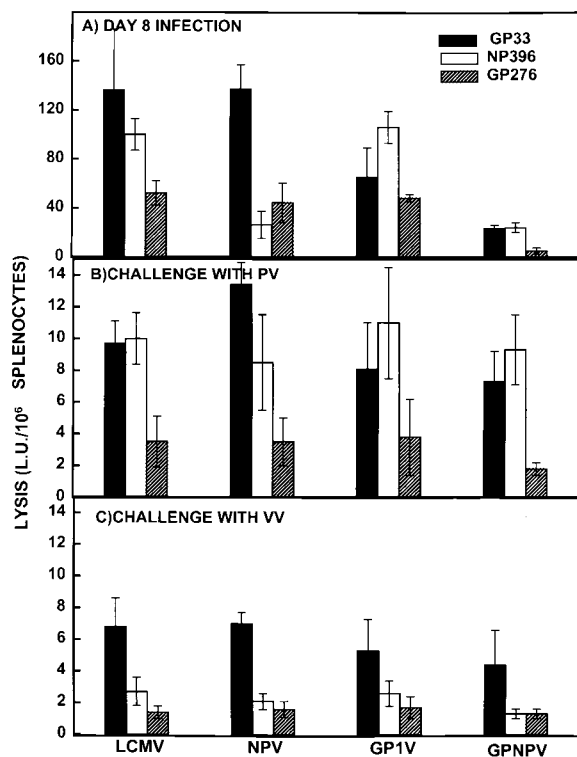


Figure 5. Heterologous Viral Challenge of Mice Immune to LCMV Mutant Viruses

(A) LCMV epitope-specific bulk CTL responses in mice acutely infected (Day 8) with LCMV or the three LCMV mutant viruses: NPV (deletion of NP epitope), GP1V (deletion of GP33 epitope), and GPNPV (deletion of all three LCMV epitopes). Reactivation of different LCMV epitope-specific memory T cells in LCMV mutant virus immune mice by acute infection with PV (B) or VV (C). The target cells used are peptide-coated RMA-S cells. Each data point is the mean of three different experiments.

of GP33>NP396>GP276, much like that observed with LDA for pCTL in immune mice. The NP396 mutant (NPV) generated a much reduced response to NP396, the GP33 mutant (GP1V) had a reduced response to GP33, and the mutant in all three epitopes (GPNPV) generated a weak response to all three epitopes, much as would be expected. There was in each case, however, still some level of recognition against the putatively deleted epitopes, presumably by T cells that do not require the particular amino acids that were deleted to interact with this peptide. These viruses nevertheless gave us the opportunity to generate LCMV-specific memory T cell populations containing different proportions of memory T cells specific to the three immunodominant epitopes.

When the LCMV mutant immune mice were challenged with VV, there was a preferential reactivation of GP33-specific CTL, regardless of which LCMV variant was used to immunize the mice. VV does not induce an LCMV GP33-specific CTL response in naive mice not immunized against LCMV (data not shown). These results indicate that the VV-induced reactivation of LCMV-specific CTL is a selective process more consistent with a cross-reactivity than a bystander reactivation mechanism. When LCMV mutant-immune mice were challenged with PV, an arenavirus with more (but still distant)

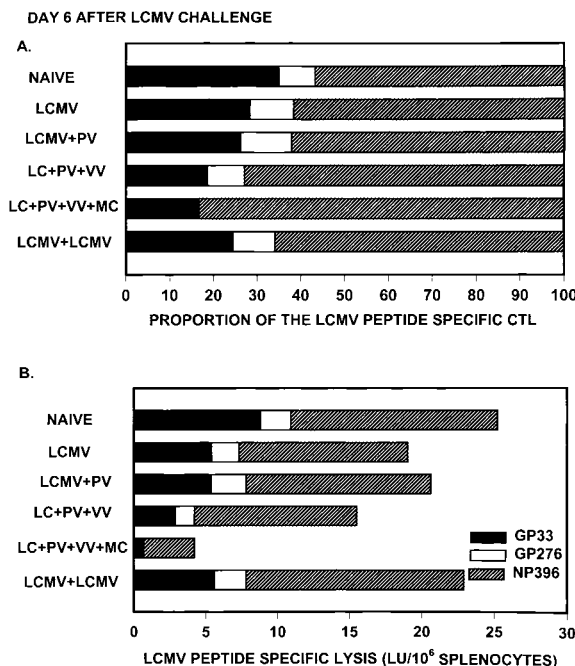


Figure 6. Alterations in the LCMV Epitope-Specific Usage upon Rechallenge with a Secondary LCMV Infection after Subsequent Heterologous Virus Infections

(A) demonstrates the proportion of the LCMV peptide-specific CTL and (B) demonstrates the LCMV epitope-specific lysis (LU/10<sup>6</sup>). The target cells were RMA-S cells incubated overnight with a 25 μM concentration of peptide.

homology to LCMV, both NP396 and GP33 epitope-specific memory CTL were reactivated, suggesting that both of these epitopes may be cross-reactive between LCMV- and PV-specific CD8 T cells; no such activity was found in naive mice infected with PV (Selin and Welsh, 1997). Nevertheless, there were proportional changes in epitope-specific responses that did not reflect the hierarchy established by the original LCMV infection. For instance, the NPV acutely infected mouse had a 7-fold greater GP33-response compared to NP396, whereas the NPV-immune mice challenged with PV demonstrated a significant reactivation of the NP396-specific response, which was only 1.7-fold less than the GP33-specific CTL response. It is also noteworthy that acute PV infection proportionally increased the NP396 response in mice immune to wild-type LCMV, and this is reflected in the data on pCTL frequencies in LCMV+PV-immune mice, which showed proportionally higher NP396 responses than LCMV-only immune mice (Figure 1B).

#### Alterations in the Acute LCMV Epitope-Specific Response upon Rechallenge with LCMV after Subsequent Heterologous Virus Infections

If subsequent heterologous virus infections were altering the LCMV-specific memory CTL repertoire, mice re-challenged with LCMV should have altered specificities of their secondary CTL response, as shown in Figures 6A and 6B. Here either naive mice, LCMV-immune, LCMV+PV-immune, LCMV+PV+VV-immune, LCMV+

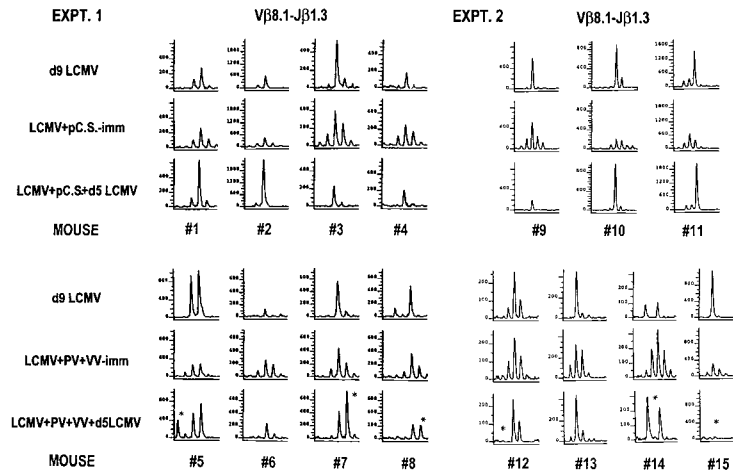


Figure 7. Modulations in the LCMV-Specific TcR Usage as Assessed by Vβ8.1-Jβ1.3 Spectratype Analysis upon Rechallenge with LCMV after Subsequent Heterologous Virus Infections

Experiment 1: peripheral blood (PB) samples from four control mice were obtained at 9 days after primary LCMV infection (d9 LCMV), 6 weeks after injection of purified culture supernatant (LCMV+pC. S.-imm), and 5 days after secondary LCMV infection (LCMV+pC. S.+d5 LCMV). PB from another four mice that had received four virus infections were obtained at 9 days after primary LCMV infection, 6 weeks after VV infection (LCMV+PV+VV-imm), and 5 days after secondary LCMV infection (LCMV+PV+VV+d5 LCMV). Experiment 2: PB from three control mice and four multiple virus-infected mice were obtained as described in Experiment 1. Changes in the spectratypes are marked with asterisks.

PV+VV+MCMV-immune, or mice infected with LCMV twice previously were challenged with LCMV. At 6 days postinfection, we quantified which LCMV immunodominant peptide-coated targets were lysed in bulk CTL assays. Figure 6B shows that the total magnitude of the secondary CTL response decreased after several viral infections, a result consistent with our LDA data showing reductions in the total CTL memory pool (Figure 1). There also were qualitative differences in the CTL response, as it would appear that as each virus was added, the host decreased its reactivation of GP33-specific CTL, favored reactivation of NP396-specific CTL, and maintained fairly consistent proportions of reactivated GP276-specific CTL. This, with some exceptions, parallels the selective alterations observed in the LCMV memory CD8 pool by LDA and intracellular IFN $\gamma$  staining, but it should be noted that a secondary infection will cause a further selection of the T cell repertoire, and a secondary LCMV infection of mice immune only to LCMV drives a selective proportional expansion of NP396-specific CTL and decline of GP33-specific CTL. Note that LCMV+PV+VV-immune mice have proportionally high levels of GP33-specific pCTL as shown by LDA. However, this does not occur when the LCMV+PV+VV-immune mouse is rechallenged in vivo with LCMV. Upon secondary challenge, the host consistently uses the NP396-specific repertoire preferentially. Therefore, there appears to be a selection for the NP396-specific response, which happens to be the highest affinity response (Hudrisier et al., 1996). The GP33 memory pool that is documented to proportionally increase after PV and VV challenge in LDA may be of low affinity for LCMV and therefore not highly reactivated by LCMV in vivo. The results from this experiment are consistent with the concept that heterologous viral infections alter preexisting memory T cell pools and show that the secondary bulk CTL response to a virus is quantitatively and qualitatively altered by intervening viral infections.

#### Modulations in the TcR Usage as Assessed by Spectratype Analysis

The acute LCMV infection in C57BL/6 mice induces many dominant clones of T cells within the Vβ8.1 population. Many of these clones can be revealed by CDR3

spectratype analyses, which have shown that some clones are so dominant that their spectratypes can be observed for many months after infection (Lin and Welsh, 1998). Other less dominant clones appearing during the acute infection become unresolvable by spectratype analysis in the memory state, probably due to dilution with naive T cells, but they will then reemerge on secondary challenge with LCMV. We hypothesized, based on the data presented above in this report, that intervening viral infections might sufficiently alter the T cell repertoire such that a secondary virus challenge would elicit a considerably different T cell repertoire than that observed during the original acute infection. For this experiment to be properly interpreted, we needed to examine windows in the T cell repertoire where LCMV induced a distinct spectratype during the acute infection, where the spectratype reverted to a Gaussian distribution in the memory state, where intervening viral infections did not substantially alter this Gaussian distribution, and where LCMV would reactivate the original dominant clones. We would then ask whether the intervening infections changed the repertoire induced by LCMV.

Figure 7 shows that the conditions for proper interpretation of this experiment were met by examining the Vβ8.1 CDR3 spectratypes emanating from Jβ1.3 primer extension reactions of individual mice whose blood was sequentially sampled as a source of T cells. Here, we are examining LCMV-induced T cell responses rather than responses clearly defined as LCMV specific, as it was not feasible to purify LCMV peptide-specific T cells from peripheral blood by D<sup>b</sup>-IgG1 MHC dimer sorting. LCMV-immune mice received subsequent infections with PV and VV and then a secondary 5 day acute infection with LCMV. Control LCMV-immune mice were inoculated with culture fluid from the cells in which PV and VV were grown and then challenged with LCMV. All of the control mice showed a skewed response in the acute infection, a Gaussian distribution in the memory state after subsequent culture fluid injections and before secondary LCMV challenge, and a pronounced dominant peak on secondary challenge, thus fulfilling the conditions for an interpretable analysis (Figure 7). LCMV-immune mice subsequently inoculated with PV and VV

all demonstrated Gaussian spectratypes prior to the secondary LCMV challenge. After this challenge, however, three out of four mice in each of the two experiments demonstrated new spectratype peaks on secondary LCMV infection. This is consistent with the concept that these mice modulated their LCMV-induced TcR repertoire as some new memory T cell clones expanded and other normally anticipated LCMV-induced T clones disappeared.

## Discussion

We have shown here that heterologous virus infections can cause significant decreases in otherwise stable virus-specific CD8 T cell memory. This virus-induced decrease in T cell memory had previously been documented using LDA against virus-infected targets (Selin et al., 1996). In the present study, similar total decreases were observed by summation of LCMV epitope-specific CD8 cells by LDAs, by flow cytometry for intracellular IFN $\gamma$ -producing cells, and by frequency analysis of CD8 T cells binding D<sup>p</sup>-IgG1 MHC dimers. Decreases in the secondary bulk CTL response to LCMV-encoded peptides were also shown to occur after several intervening viral infections. It is noteworthy that the tetramer, D<sup>p</sup>-IgG1 MHC dimer, and the IFN $\gamma$ -producing cell assay techniques are all excellent techniques to quantitate antigen-specific cells, but they are all limited by their ability to resolve a signal over background staining by FACS analysis. The LDA is orders of magnitude more sensitive than these procedures in that it can accurately detect much lower frequencies of antigen-specific T cells. More than 10% of the CD8 T cell pool in LCMV-immune mice was shown to be LCMV-specific by the intracellular IFN $\gamma$  assay, as shown previously (Murali-Krishna et al., 1998), and comparable numbers on LCMV-specific memory CD8 T cells were shown here for the first time with D<sup>p</sup>-IgG1 MHC dimer staining. Given this high frequency of antigen-specific memory cells, the reduction in memory T cells on subsequent infection is not surprising, as the memory pool would fill to capacity very quickly if the host were to maintain antigen-specific memory pools of this size for all the pathogens it would encounter in a lifetime. In order to accommodate an increasing memory pool in a limited shelf space, the simplest method would be to delete some of the preexisting memory cells as new infections occur.

This deletion of the preexisting memory pool may be a consequence of an antigen-specific expansion of CD8 T cells in response to a subsequent infection, followed by a competition for protective niches in the spleen and lymphoid organs as the immune system downregulates at the end of infection. It is noteworthy that CD8 T cell responses to PV and VV in LCMV-immune mice occur more rapidly and limit spread of the virus, but they reach substantial levels and subsequently impact the LCMV-specific memory pool (Selin et al., 1994, 1996, 1998; Welsh et al., 1997). The CD4 T cell pool does not increase in size as dramatically during these viral infections, either alone or during sequential infections, and there is less of a loss in the stable LCMV-specific CD4 memory T cells after heterologous viral infections (Varga and

Welsh, 1998a); (S. M. V., L. K. S., and R. M. W., unpublished data). Alternatively, there may be direct mechanisms that selectively eliminate memory cells in order to make room for a newly responding population. During the acute response to LCMV infection, there is in fact an attrition of T cells not specific to the virus. For example, transgenic or else tolerized T cell populations known to be non-cross-reactive with LCMV decline in number during a vigorous T cell response to LCMV (Zarozinski and Welsh, 1997). These non-LCMV-specific T cells display enhanced reactivity with Annexin V, which detects cells in the early stages of apoptosis (Welsh and McNally, 1999). Other cytokines or even virus infection of lymphocytes might also contribute to the loss of memory T cells, but such nonspecific mechanisms are difficult to reconcile with selectivity.

In this study we show that there is not only a quantitative reduction in CD8 T cell memory after heterologous infections but also a qualitative alteration. The relative proportions of memory T cells specific to each of the three LCMV-encoded immunodominant peptides changed in relationship to each other after heterologous infections, and IFN $\gamma$ -producing cell assays suggested that the cells most vigorously synthesizing IFN $\gamma$  may be selectively lost. Additionally, we noted that a prior history of a virus infection can influence the hierarchy of the T cell response to immunodominant peptides of a second unrelated virus on challenge. For example, the hierarchy of the memory pool laid down by an LCMV infection of naive mice differed from that laid down by LCMV infection of PV- or VV-immune mice. These qualitative changes in memory occurring as a consequence of prior or subsequent infections with heterologous viruses may well reflect the proclivity of memory T cells to cross-react with other antigens. We have previously defined cross-reactive memory T cell responses between heterologous viruses, leading to the postulation that any deletion in T cell memory would not affect all memory cells equally.

Indeed, the results presented here are consistent with the concept that CD8 memory T cells cross-reactive with the challenge virus are maintained while the non-cross-reactive memory T cells are deleted. When LCMV immune mice were acutely infected with VV, the LCMV GP33-specific CTL were preferentially reactivated. In LCMV+PV+VV-immune mice there has been a greater loss of NP396-specific pCTL and retention of GP33-specific pCTL, suggesting that the potentially cross-reactive T cells were maintained. It would thus appear that the highly efficient immune system is able to maintain homeostasis by saving space with two mechanisms, either the deletion of a portion of the memory CD8 T cell pool or the preservation of cross-reactive CD8 memory T cells.

## Experimental Procedures

### Virus Infection of Mice

LCMV (strain Armstrong), VV (strain WR), PV (strain AN3739), and VSV (strain Indiana) were propagated in L929 or BHK21 cells (Welsh et al., 1976; Yang et al., 1989). MCMV (strain Smith) was obtained from salivary glands of infected BALB/c mice (Bukowski et al., 1983). Mutant strains of LCMV with partial deletions of their responses to the NP epitope (NPV), GP33 epitope (GP1V), or all three (NP396,

GP33, GP276) immunodominant epitopes (GPNPV) were kindly donated to us by Dr. M. B. A. Oldstone (Scripps Institute, San Diego, CA) (Oldstone et al., 1995). C57BL/6 (H-2<sup>b</sup>) male mice were purchased from The Jackson Laboratories and used at 2–26 months of age. For acute virus infections, mice were injected i.p. with  $4 \times 10^4$  PFU of LCMV,  $1-2 \times 10^6$  PFU of VV,  $10^6-10^7$  PFU of PV,  $10^4$  PFU of MCMV, or  $10^8$  PFU of VSV.

#### Cell Lines

KO (H-2<sup>b</sup>), an SV40-transformed kidney cell line derived from a C57BL/6 mouse (Tanaka and Tevethia, 1988) was propagated in DMEM (GIBCO Laboratories) supplemented with  $5 \times 10^{-5}$  M 2-ME. MC57G (H-2<sup>b</sup>), a fibroblast cell line from C57BL/6 mice was propagated in Eagle's MEM (GIBCO Laboratories). KO and MC57G cells were infected with LCMV at a multiplicity of infection (MOI) of 0.01–0.02 PFU/cell and incubated for 2 days at 37°C. The RMA-S cell line (H-2<sup>b</sup>) is a T cell lymphoma that has been mutated, resulting in loss of the *TAP2* gene (Attaya et al., 1992). These cells were propagated in RPMI-1640 medium (Sigma Chemical) as described (Selin and Welsh, 1997).

#### Infection Protocol with Heterologous Viruses

After the rise and fall of the acute T cell response to one virus and when the immune system had returned to homeostasis (usually 6 weeks or longer), the mouse was challenged with another virus. These viruses were used either in crude tissue culture supernatants or were purified over sucrose gradients and diluted in PBS. Control naive mice were either left uninoculated or else were inoculated with tissue culture media or with culture media sedimented like virus over a sucrose gradient. The control mice were always age matched to the experimental group and housed under exactly the same pathogen-free conditions as the experimental group for the identical time period. All mice used were healthy with no evidence of any underlying disease.

#### LDA for Peptide-Specific pCTL

Adult C57BL/6 mice were inoculated i.p. with the sequence of viruses indicated, and at the indicated times postinfection spleens were harvested. The LCMV epitope-specific p/CTL frequency per CD8<sup>+</sup> T cell was quantified by LDAs of unsorted cells performed as previously described (Selin et al., 1994).

#### Cytotoxicity Assays

Cell-mediated cytotoxicity was determined using a standard microcytotoxicity assay (Selin et al., 1994). Varying numbers of effector leukocytes were plated in triplicate to achieve the desired E/T ratio. <sup>51</sup>Cr-labeled MC57G target cells ( $5 \times 10^5$ ), either uninfected or infected with virus, or RMA-S cells, either untreated or pulsed with peptides, were added to all wells, and after a 6 hr incubation at 37°C the supernatant was harvested and counted. Data are expressed as percent specific <sup>51</sup>Cr-release =  $100 \times [(\text{experimental cpm} - \text{spontaneous cpm}) / \text{maximum release cpm} - \text{spontaneous release cpm}]$ . Lytic units were calculated using the exponential fit method (Pross et al., 1981) provided by software from Proteins International. One lytic unit was defined as the number of effector cells required to lyse 5% of a population of  $5 \times 10^3$  targets in a 6 hr CTL assay. The spontaneous release for each target used in these assays was less than 20%.

#### Peptide Pulsing of Targets

RMA-S or KO cells were pulsed as previously described (Selin and Welsh, 1997) overnight at  $2-3 \times 10^6$  cells in 3 ml of complete media in  $60 \times 15$  mm tissue culture plates (Becton Dickinson) with various peptides at a 200  $\mu$ M concentration unless otherwise indicated. The LCMV H-2D<sup>b</sup>-restricted immunodominant peptides (Hudrisier et al., 1996; Varga and Welsh, 1998b) NP396–404 (FQPQNGQFI), GP33–41 (KAVYNFATC), and GP276–286 (SGVENPGGYCL) and the H-2K<sup>b</sup> epitope GP33–43 (KAVYNFATCGI) were synthesized by Dr. Robert Carraway (University of Massachusetts Medical Center). These peptides were purified to >95% homogeneity by reverse-phase HPLC.

#### Intracellular IFN $\gamma$ Staining

Splenocytes were stimulated with peptide (5  $\mu$ g/ml) in the presence of 10 U/ml human recombinant IL-2 and 10  $\mu$ g/ml Brefeldin A (Sigma) for 5 hr at 37°C. The cells were stained with tricolor-conjugated anti-mouse CD8 (clone CT-CD8, Caltag) and fixed with 2% paraformaldehyde as described previously (Varga and Welsh, 1998b). They were then permeabilized with 0.5% saponin before adding PE-conjugated rat anti-mouse anti-IFN $\gamma$  mAb (PharMingen) or control PE-conjugated rat IgG1 isotype. They were analyzed on either a FACS 440 (Becton Dickinson) or FACStar Plus (Becton Dickinson).

#### D<sup>b</sup>-IgG1 MHC Dimer Staining

T cells were stained with a mouse IgG1-H-2D<sup>b</sup> fusion protein in which the antibody heavy chain was fused to the MHC class I  $\alpha$  chain such that MHC class I was expressed on the antigen-binding domain of the antibody molecule (Dal Porto et al., 1993; Greten et al., 1998). When these molecules are incubated with  $\beta$ 2-microglobulin and the appropriate peptide, a complete peptide-loaded MHC class I molecule is formed, and this can be used for the enumeration of antigen-binding T cells. In our hands, this has shown similar percentages of LCMV-specific CD8 T cells as that reported with MHC tetramers (Murali-Krishna et al., 1998). For these studies, the D<sup>b</sup>-IgG1 MHC dimer (2  $\mu$ M) was prepared as previously described for other class I molecules (Dal Porto et al., 1993), incubated with  $\beta$ 2-microglobulin (4  $\mu$ M) and appropriate peptide (80  $\mu$ M) for 10 days. For staining, 16  $\mu$ l of the peptide-D<sup>b</sup>-IgG1 MHC dimer mixture was incubated on splenocytes for 3–4 hr at 4°C. After washing, the cells were incubated for 20 min with biotinylated anti-mouse IgG1 (PharMingen) at 4°C. The cells were washed and then incubated with streptavidin PE and CD8 FITC for 20 min at 4°C. They were then washed and fixed with 2% paraformaldehyde before analysis by FACS 440 or FACStar Plus.

#### CDR3 Length Spectratyping

The V $\beta$ 8.1, C $\beta$ , and J $\beta$  primer sequences were used as previously described (Pannetier et al., 1993; Lin and Welsh, 1998). V $\beta$ 8.1 and C $\beta$  primers used for RT-PCR reactions were synthesized by BioSynthesis. Fluorescent J $\beta$  primers were synthesized and labeled at the 5' end with fluorophores by PE Applied Biosystems. Total RNA was extracted from peripheral blood by the acid-guanidinium thiocyanate-phenol-chloroform method (Lin and Welsh, 1998). RNA samples, equivalent to 0.12 ml of blood, were amplified by using a GeneAmp RNA PCR kit (Perkin-Elmer) with V $\beta$ 8.1 and C $\beta$  primers, according to the manufacturer's instructions.

Two microliters of the amplified PCR products were subjected to a cycle of run-off with radiolabeled J $\beta$  primers or five cycles of run-off with fluorophore-labeled J $\beta$  primers. One microliter of the fluorescent products was mixed with an equal volume of gel-loading buffer and loaded onto a 4.75% acrylamide sequencing gel. The results were analyzed on an automated DNA sequencer using GeneScan software (PE Applied Biosystems).

#### Statistical Analyses

The ANOVA test was used for data analysis where appropriate.

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