

T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens

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We show here that T cell cross-reactivity between heterologous viruses influences the immunodominance of virus-specific CD8⁺ T cells by two mechanisms. First, T cells specific for cross-reactive epitopes dominate acute responses to viral infections; second, within the memory pool, T cells specific for cross-reactive epitopes are maintained while those specific for non-cross-reactive epitopes are selectively lost. These findings suggest an immunological paradigm in which viral infections shape the available T cell repertoire, causing alterations in the hierarchies of both the primary and memory CD8⁺ T cell responses elicited by subsequent viral infections. Thus, immunodominance is a function of the host's previous exposure to unrelated pathogens, and this may have an impact on protective immunity and immunopathology.

Antigen recognition by CD8⁺ T cells has a degenerate nature¹, but the ramifications for T cell cross-reactivity *in vivo* are still not completely understood. Promiscuous antigen recognition has been observed for virus-specific CD8⁺ T cells, with cross-reactive responses between viruses as diverse as influenza and rotavirus² or influenza and hepatitis C virus³. Cross-reactive cytotoxic T lymphocytes (CTLs) against unrelated viruses such as lymphocytic choriomeningitis virus (LCMV), Pichinde virus (PV) and vaccinia virus were easily observed when hosts that were immune to one virus were challenged with a second virus, which promoted the outgrowth of cross-reactive clones⁴. We postulated that cross-reactivity between heterologous viruses is a relatively common event and can have marked consequences, including enhancement of protection against subsequent virus challenge⁵, induction of immunopathology⁶ and shaping of the T cell memory pool⁷. The recent identification of cross-reactive epitopes—which share seven of nine amino acids (aa)—between the common, but heterologous, human pathogens influenza and hepatitis C virus³, highlights the need for us to fully understand the repercussions of previous infections on an individual's ability to respond to subsequent challenges with pathogens. We define here distinct cross-reactive epitopes and show that virus cross-reactivity influences T cell immunodominance, changing hierarchies of the virus-specific CD8⁺ T cells during acute responses and in the memory pool.

Virus-specific CD8⁺ T lymphocytes recognize short, 8–11 aa-long, peptides that are derived from viral proteins and presented in the context of major histocompatibility complex (MHC) class I molecules on the surface of infected cells⁸. Of the many virus-derived peptides that

are processed and presented, relatively few stimulate strong CD8⁺ T cell responses. The peptides that are targets for virus-specific T cells are generally recognized in a distinct hierarchy of dominance with some being strong (immunodominant), others being weak to barely detectable (subdominant) and still others being detectable only in the absence of dominant epitopes (cryptic)⁹. The hierarchies of CD8⁺ T cell responses in mice of various haplotypes have been well defined for numerous viruses, including herpes simplex virus (HSV)¹⁰, influenza virus^{11,12}, LCMV^{13–16}, murine gammaherpesvirus 68^{17,18}, Sendai virus¹⁹, simian virus 40 (SV40)²⁰ and vesicular stomatitis virus (VSV)²¹. The determinants recognized in various virus models range from systems in which there is a single immunodominant epitope—such as HSV¹⁰, Sendai virus²² and VSV²¹—to more complex models—such as influenza^{12,23}, LCMV²⁴ and SV40²⁵—that have multiple dominant epitopes. The hierarchies of T cell responses elicited by viral infections of immunologically naïve animals are generally very stable.

The factors that influence immunodominance are not well understood, and the epitope hierarchy for T cell responses against any given virus is probably shaped by a combination of factors that operate concurrently. Elements that influence immunodominance hierarchies include the efficiency of peptide processing and presentation, the affinity of the peptide for the presenting MHC molecule, the overall number of peptide-MHC complexes, the available T cell receptor (TCR) repertoire and the phenomenon of immunodomination in which T cells specific for immunodominant peptides suppress responses to other viral epitopes⁹. Examination of human HIV-specific CD8⁺ T cell responses showed that immunodominance hierarchies may not be as stable in

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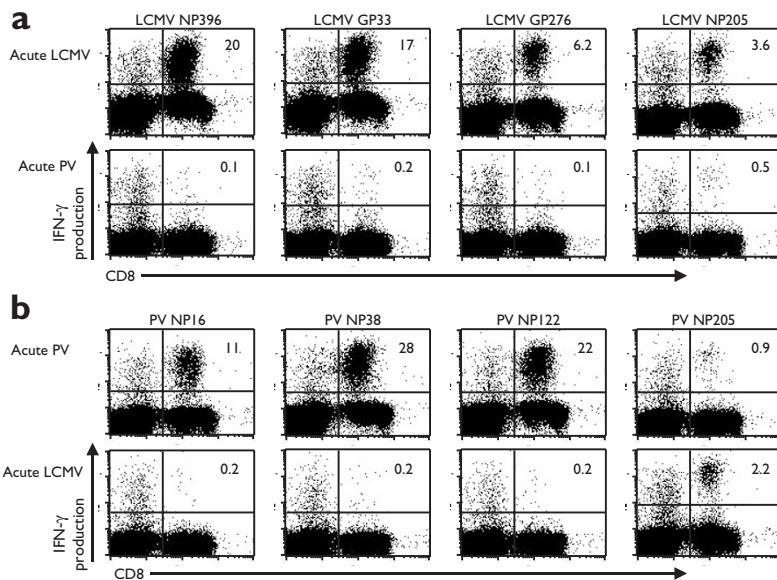


Figure 1. Stable immunodominance hierarchy of the CD8⁺ T cell responses elicited by LCMV or PV infection. Splenocytes from B6 mice acutely infected with either LCMV (8 days after challenge) or PV (7 days after challenge) were examined by intracellular IFN- γ staining after stimulation with either (a) LCMV-derived peptides or (b) PV-derived peptides. The numbers represent the percentage of IFN- γ -producing CD8⁺ T cells. Data are from an individual mouse and are representative of three separate experiments. LCMV NP396, LCMV NP(396–404); LCMV NP205, LCMV NP(205–212); LCMV GP33, LCMV GP(33–41); LCMV GP276, LCMV GP(276–286); PV NP16, PV NP(16–25); PV NP38, PV NP(38–45); PV NP122, PV NP(122–132); PV NP205, PV NP(205–212).

humans as in murine models. Virus-specific CD8⁺ T cells generated in individual HLA-A2⁺HIV-1⁺ patients showed extreme variability in hierarchies for known HLA-A2-restricted epitopes derived from HIV proteins²⁶. A plausible explanation for this variability is that the non-matched HLA molecules expressed between the patients alter the immunodominance hierarchies. For instance, the immunodominance hierarchy of H-2^b-restricted, influenza-specific T cell responses is changed in (H-2^b–H-2^k)F₁ mice²³. However, HIV-1⁺ patients that coexpress the same alleles, HLA-A2, HLA-A3 and HLA-B7, maintain marked diversity in the hierarchies of the HIV-specific T cell responses, suggesting that immunodominance may be influenced by factors that have yet to be determined²⁷.

We identify here four CD8⁺ T cell epitopes for PV and show that T cells specific for one PV-encoded subdominant epitope cross-react with a subdominant LCMV epitope. Our data indicate that previous infection with either LCMV or PV markedly amplifies the T cell response against the cross-reactive peptide after challenge with heterologous virus, but not with the homologous virus. In addition, previous infection with either LCMV or PV disrupted the overall hierarchy of epitope-specific T cells elicited after challenge with the heterologous virus. The alteration of immunodominance was evident in virus-specific T cell memory, with cross-reactivity affecting both the maintenance and the initial establishment of CD8⁺ memory as T cells specific for cross-reactive epitopes were maintained while non-cross-reactive T cells were lost.

Results

Hierarchy of virus-specific CD8⁺ T cell responses

Six virus-derived peptides have been identified as H-2^b-restricted epitopes for LCMV-specific CD8⁺ T cells^{13–16}; we evaluated four of these peptides (Fig. 1a). Two of the LCMV peptides are derived from the glycoprotein, GP(33–41) (KAVYNFATC) and GP(276–286) (SGVENPGGYCL), and two are derived from the nucleoprotein, NP(205–212) (YTVKYPNL) and NP(396–404) (FQPQNGQFI). The NP(396–404), GP(33–41) and GP(276–286) peptides are restricted by H-2D^b and NP(205–212) is restricted by H-2K^b. The hierarchy of the epitope-specific CD8⁺ T cell responses induced by LCMV infection was determined by intracellular interferon- γ (IFN- γ) staining (Fig. 1a). GP(33–41) and NP(396–404)

elicited strong responses, whereas GP(276–286) elicited a moderate response; all three peptides are considered immunodominant epitopes. NP(205–212) elicited weak responses and is considered a subdominant epitope¹⁵. This hierarchy of immunodominance is very reproducible between experiments and persists into memory²⁸. Additionally, we have defined four epitopes encoded for by PV, three immunodominant epitopes—NP(16–25) (RGLSNWTHPV), NP(38–45) (SALDFHKV) and NP(122–132) (VYEGNLTNTQL)—were identified by screening overlapping 20 aa that spanned the PV nucleoprotein and one very weak subdominant epitope, NP(205–212) (YTVKFPNM) (Fig. 1b). The PV peptides NP(16–25) and NP(122–132) are restricted by H-2D^b; and NP(38–45) and NP(205–212) are restricted by H-2K^b (data not shown). T cell responses elicited by either virus did not recognize the immunodominant peptides derived from the heterologous virus.

H-2K^b-restricted PV and LCMV epitope similarity

We tested the hypothesis that T cell cross-reactivity between heterologous viruses is a key factor in the development of immunodominance hierarchies for T cells. However, examination of the role cross-reactivity plays in shaping T cell hierarchies was complicated by the lack of defined cross-reactive peptides between viruses. LCMV and PV showed a very low amount of T cell cross-reactivity, which was detectable only after challenge of LCMV-immune mice with PV⁴. A comparison between the amino acid sequences of the LCMV-derived T cell epitopes and the corresponding PV sequences revealed a high degree of sequence similarity with the subdominant NP(205–212) epitope but limited similarity with the immunodominant peptides. The sequence of LCMV NP(205–212) contains the predicted K^b-binding motif²⁹, with a tyrosine at position 5 and a hydrophobic leucine at the COOH terminus. PV NP(205–212) varied from its LCMV counterpart at these two positions, with a phenylalanine at position 5 and a methionine at position 8; both amino acids can function as anchoring residues for MHC binding. The relative H-2K^b-binding affinities for LCMV NP(205–212) and PV NP(205–212) were compared with an MHC stabilization assay; they were identical, with peptide concentrations ranging from 100 μ M to 0.02 μ M (data not shown). Although these two epitopes share six of eight amino acid residues, their antigenic properties did not appear to be identical: infection with PV consistently

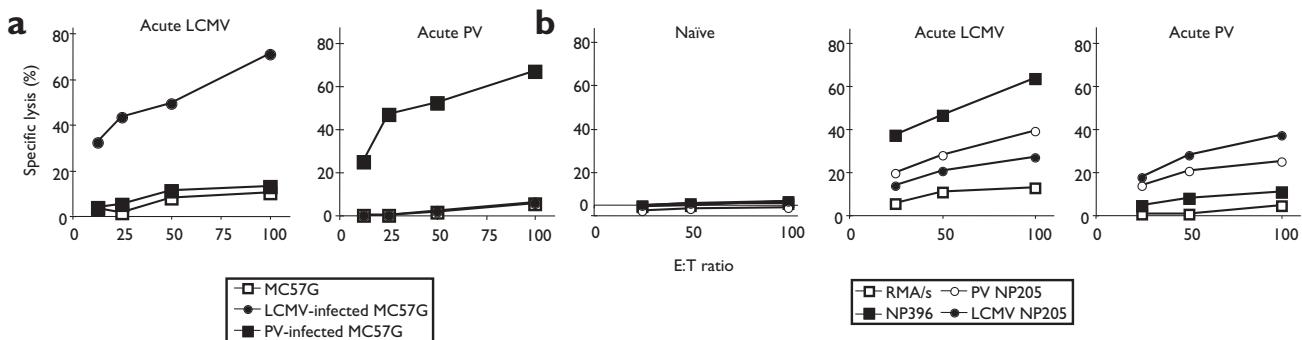


Figure 2. Cross-reactivity between LCMV and PV is not evident with virus-infected targets but is detectable with LCMV NP(205–212) and PV NP(205–212). (a) Splenocytes from B6 mice that were acutely infected with either LCMV (8 days after challenge) or PV (7 days after challenge) were examined with a standard cytotoxicity assay against virus-infected MC57G target cells. (b) Splenocytes from naïve B6 mice or B6 mice that were acutely infected with either LCMV or PV were examined with a standard cytotoxicity assay against peptide-pulsed RMA target cells. E:T, effector to target ratio.

elicited a higher frequency of CD8⁺ T cells specific for PV NP(205–212) than for LCMV NP(205–212). There was an average 1.7-fold increase for PV NP(205–212) in five experiments, as determined by intracellular IFN- γ staining.

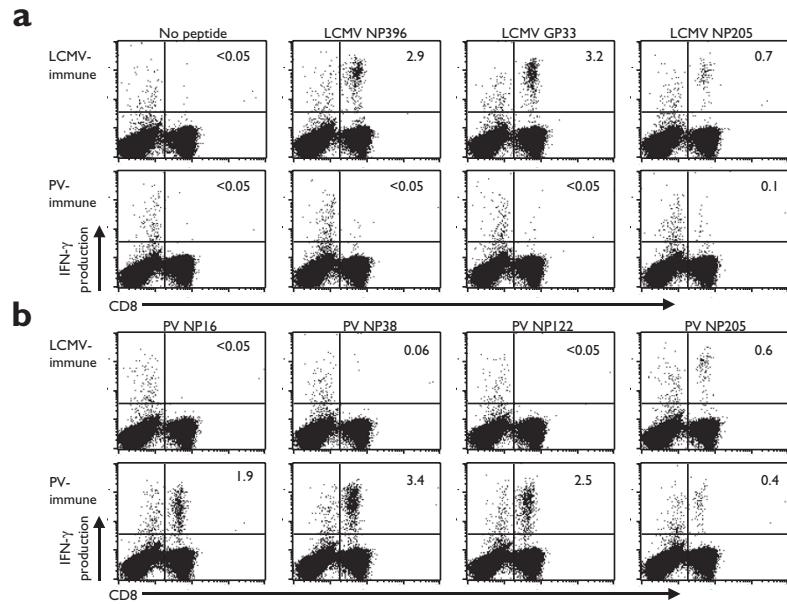
Cross-reactivity of CD8⁺ T cells on NP(205–212)

To assess the cross-reactive nature of PV NP(205–212), the acute responses elicited by infection with either LCMV or PV were compared for their ability to lyse virus-infected target cells (Fig. 2a) or NP(205–212)-pulsed target cells (Fig. 2b). Splenocytes from mice infected with either LCMV or PV lysed target cells infected with the homologous virus but not with the heterologous virus (Fig. 2a), confirming the concept that any cross-reactivity between the viruses is very low⁴. In cytotoxicity assays, cells pulsed with high concentrations of peptide are more sensitive targets than virus-infected cells, allowing for the detection of epitope-specific CD8⁺ T cells present at lower frequencies. We found that splenocytes from mice infected with either PV or LCMV lysed target cells pulsed with either LCMV NP(205–212) or PV NP(205–212) (Fig. 2b). Infection of mice with PV failed to elicit a response against LCMV NP(396–404), whereas effector cells induced by LCMV efficiently lysed NP(396–404)-pulsed target cells. Splenocytes from naïve mice did not show cytotoxicity against any target cells above background amounts. *In vitro* stimulation of splenocytes from LCMV-immune mice with either LCMV NP(205–212) or PV NP(205–212) generated T cell cultures with comparable lytic activity against target cells pulsed with either of the two peptides but not with NP(396–404) (data not shown). Finally, using intracellular IFN- γ staining, we showed that T cells induced by either virus recognized cells coated with NP(205–212) encoded by either virus (Fig. 1). Together these studies suggest that LCMV NP(205–212) and PV NP(205–212) induce cross-reactive CTL responses.

Figure 3. NP(205–212)-specific T cells are present in both LCMV-immune and PV-immune mice. Splenocytes from either PV-immune or LCMV-immune mice were examined by intracellular IFN- γ staining. The numbers represent the percentage of IFN- γ -producing CD8⁺ T cells. (a) LCMV-encoded peptides and (b) PV-encoded peptides were used. Data are representative of three separate experiments.

Low frequency of memory NP(205–212)-specific T cells

LCMV-immune mice are more resistant to PV challenge than naïve mice⁵. Previous infection with PV also affords some protection against LCMV challenge. It was proposed that this protection was mediated by the presence of cross-reactive CD8⁺ T cells in the memory population of these immune animals, but cross-reactive epitopes were not identified. The frequency of NP(205–212)-specific memory T cells in PV-immune mice was on average less than that in LCMV-immune mice; it ranged from undetectable to 0.6% of the CD8⁺ T cells in PV-immune mice. Memory CD8⁺ T cells specific for the LCMV immunodominant epitopes NP(396–404) and GP(33–41) were present at high frequencies in LCMV-immune mice but were undetectable in PV-immune mice (Fig. 3a). Memory CD8⁺ T cells specific for the PV immunodominant epitopes NP(16–25), NP(38–45) and NP(122–132) were present at high frequencies in PV-immune mice but were undetectable in LCMV-immune mice (Fig. 3b). However, a very low frequency of cross-reactive NP(205–212)-specific T cells was found in both LCMV-immune and PV-immune mice, and we questioned whether this would allow for their early expansion after heterologous challenge and result in protection.



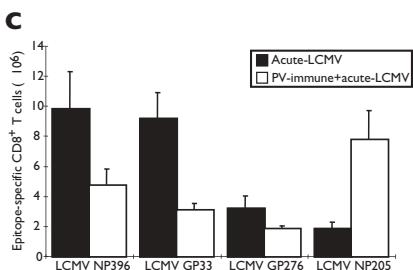
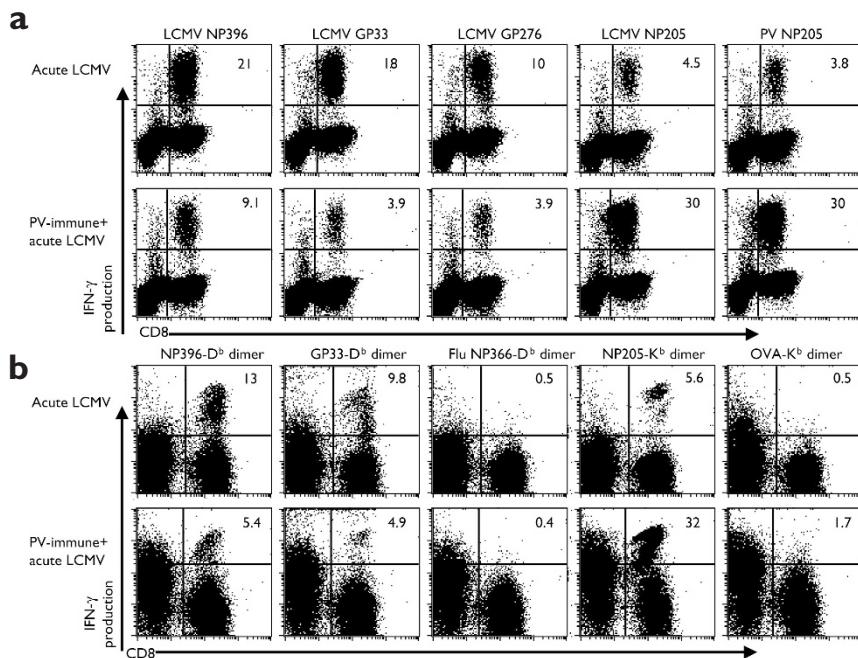


Figure 4. Previous infection with PV alters the hierarchy of the CD8⁺ T cell response elicited by acute LCMV infection. **(a)** Splenocytes from naïve or PV-immune mice acutely infected with LCMV (8 days after challenge) were examined by intracellular IFN- γ staining. The numbers represent the percentage of IFN- γ -producing CD8⁺ T cells. **(b)** Splenocytes from naïve or PV-immune mice acutely infected with LCMV were stained with the indicated peptide-loaded MHC-IgG1 dimers. Data are representative of three separate experiments. **(c)** Numbers of IFN- γ -secreting, epitope-specific T cells generated in naïve mice infected with LCMV or in PV-immune mice infected with LCMV.

Dominant LCMV epitope in PV-immune mice

The data above show that LCMV and PV share a subdominant epitope, and that mice infected with either virus maintain memory T cells specific for the cross-reactive peptides. To explore the impact of T cell cross-reactivity on the induction of virus-specific CD8⁺ T cells, we used intracellular cytokine staining to measure IFN- γ production by epitope-specific T cells generated in either age-matched naïve or PV-immune mice that were acutely infected with LCMV (Fig. 4a). The lack of cross-reactive neutralizing antibody responses between LCMV and PV allows for individual mice to be sequentially infected with these two viruses without a reduction in viral antigen by antibodies³⁰. Naïve mice infected with LCMV mounted a CD8⁺ T cell response with a normal epitope hierarchy. In contrast, the hierarchy of the LCMV-specific T cell response generated in PV-immune mice was altered, with a marked increase in the frequency of NP(205–212)-specific T cells. Additionally, the T cell responses specific for the normally immunodominant LCMV epitopes were decreased compared to those in naïve mice.

Intracellular IFN- γ staining is dependent on the ability of individual T cells to secrete IFN- γ in response to peptide stimulation. To exclude the possibility that nonfunctional LCMV-specific T cells were present in PV-immune mice infected with LCMV, splenocytes from PV-immune or naïve mice that were infected with LCMV were stained with peptide-loaded MHC-Immunoglobulin G1 (IgG1) dimers³¹. Both the suppression of NP(396–404)- and GP(33–41)-specific T cell responses and the amplification of NP(205–212)-specific T cell responses in PV-immune mice infected with LCMV were confirmed with the MHC-IgG1 dimers (Fig. 4b). The control MHC-IgG1 dimers—Influenza NP(366–374) for D^b and ovalbumin peptide OVA(257–264) for K^b—stained in background amounts. These results indicated that the reduction of CD8⁺ T cells specific for the LCMV immunodominant epitopes cannot be attributed to the presence of nonfunctional or non-IFN- γ -producing T cells. The increase in NP(205–212)-specific T cells and decreases in the immunodominant-specific responses were also reflected in the total numbers of CD8⁺ T cells (Fig. 4c).

Because studies of antiviral T cell responses in humans generally use peripheral blood, it was of interest to determine whether alterations in immunodominance hierarchies would be detectable in the peripheral blood of mice exposed to multiple viruses (Fig. 5). Naïve mice infected with LCMV mounted a CD8⁺ T cell response with a normal epitope hierarchy in the peripheral blood (Fig. 5a). As shown above with splenocytes, the frequency of NP(205–212)-specific T cells was markedly elevated in the peripheral blood of the four PV-immune mice infected with LCMV, ranging between 7–28% of the CD8⁺ T cells. In two of the four PV-immune mice infected with LCMV (Fig. 5b,d), T cell responses specific for the normally immunodominant LCMV epitopes were markedly decreased. The immunodominant epitope-specific T cells generated in the remaining two PV-immune mice infected with LCMV (Fig. 5e,f) were affected less, but were still lower in frequency than the responses in naïve mice infected with LCMV. These results showed that previous virus infections can greatly alter immunodominance hierarchies during subsequent infections, with increases in the frequency of cross-reactive T cells and suppression of T cell responses against non-cross-reactive epitopes. The variability in responses elicited by secondary viral infection was consistent with the concept that genetically identical mice develop distinctive TCR repertoires³², which may promote variations in cross-reactivity between individual mice.

Early LCMV response in PV-immune mice

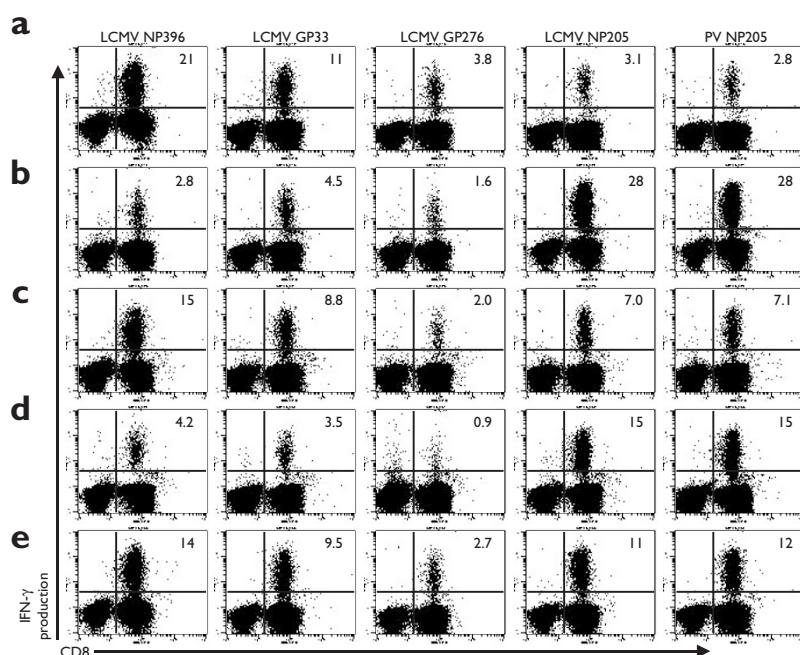
Studies of heterologous immunity have shown that previous infection with either LCMV or PV provides partial protection against heterologous viral infection as early as 4 days after challenge⁵. In the spleens of LCMV-immune mice compared to naïve mice challenged with PV, there was a significant 97% reduction in the viral titer (LCMV-immune, 3.2 ± 0.3 log PFU/ml; naïve, 4.7 ± 0.4 log PFU/ml; $P < 0.05$, $n = 5$ mice per group). There was a more modest, but still significant, 50% reduction in LCMV splenic titers upon challenge of PV-immune mice compared to naïve mice (PV-immune, 5.3 ± 0.2 log PFU/ml; naïve, 5.6 ± 0.2 log PFU/ml; $P < 0.05$, $n = 5$ mice per group). Based on these observations,

Figure 5. Lymphocytes in peripheral blood from PV-immune mice infected with LCMV display alterations in immunodominance hierarchies. Lymphocytes in peripheral blood isolated from either (a) naïve or (b–d) PV-immune mice that were acutely infected with LCMV 8 days after challenge were examined by intracellular IFN- γ staining. The numbers represent the percentage of IFN- γ -producing CD8 $^{+}$ T cells.

we speculated that the cross-reactive NP(205–212) T cell response would develop earlier than the response to other immunodominant epitopes. Therefore, we examined CD8 $^{+}$ T cell responses on days 4, 5 and 6 after challenge in groups of three or four mice by intracellular IFN- γ staining (**Web Fig. 1** online). In naïve mice infected with LCMV, NP(396–40), GP(33–41)- and NP(205–212)-specific T cells were detected directly *ex vivo* by intracellular IFN- γ staining 6 days after challenge and had a standard hierarchy of dominance (**Web Fig. 1a–c** online). In contrast, PV-immune mice infected with LCMV preferentially mounted an NP(205–212)-specific response that began to expand on day 5 after challenge and became dominant on day 6. The frequencies of NP(396–404)- and GP(33–41)-specific responses were reduced on day 6 relative to the numbers in infected naïve mice. CD8 $^{+}$ T cells specific for PV NP(38–45) were not detectable in naïve mice infected with LCMV (**Web Fig. 1d** online). These results showed that alterations in the hierarchy of CD8 $^{+}$ T cells after LCMV infection of PV-immune mice were evident early during the immune response, which was consistent with the earlier anamnestic-like response of memory T cells. The cross-reactive T cells would be, therefore, available more rapidly to clear the second virus.

Dominant PV epitope in LCMV-immune mice

Acute PV infection of naïve C57BL/6 (B6) mice was dominated by responses against the PV peptides NP(16–25), NP(38–45) and NP(122–132), with a low but detectable response against NP(205–212) (**Fig. 6a**). In contrast, acute PV infection of LCMV-immune mice induced enhanced, 10- to 20-fold increased, responses against LCMV NP(205–212) and PV NP(205–212) (**Fig. 6a–d**). In LCMV-immune mice challenged with PV the responses against the three immunodominant peptides were markedly decreased compared to PV infection in naïve mice (**Fig. 6b,c**). The reduced frequency of the normally immunodominant responses correlated with the expansion of the cross-reactive



NP(205–212)-specific T cells. The total number of NP(205–212)-specific CD8 $^{+}$ T cells also increased during acute PV infection of LCMV-immune mice compared to the numbers in naïve mice infected with PV (**Fig. 6d**). These results demonstrated that previous infection with a virus encoding a cross-reactive epitope shifted the normally subdominant NP(205–212)-specific T cell response generated by infection into a dominant response and suppressed responses against the non-cross-reactive epitopes.

Homologous virus challenge

Our data showed that previous viral infection markedly altered the immunodominance hierarchy of T cells responding to a secondary challenge with a heterologous virus encoding a cross-reactive peptide. To determine whether the same alterations in hierarchies could be observed after homologous virus challenge, we used the intracellular IFN- γ assay to examine T cell responses generated in either LCMV-immune or PV-immune mice challenged with the homologous virus. Rechallenge of LCMV-immune mice with LCMV focused the T cell response on the

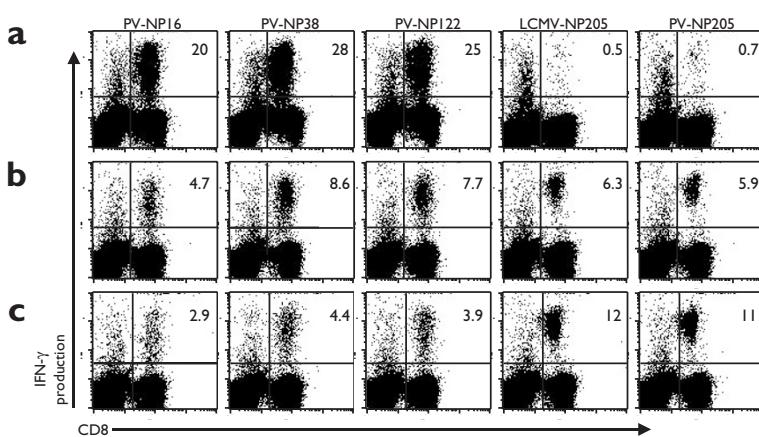


Figure 6. Previous infection with LCMV alters the hierarchy of the CD8 $^{+}$ T cell response elicited by acute PV infection. Splenocytes from (a) naïve mice that were acutely infected with PV or (b,c) LCMV-immune mice that were acutely infected with PV were examined by intracellular IFN- γ staining (7 days after challenge). Data are representative of three separate experiments. The numbers represent the percentage of IFN- γ -producing CD8 $^{+}$ T cells. (d) Numbers of epitope-specific T cells generated in naïve mice infected with PV or in LCMV-immune mice infected with PV. The results are average data from four individual mice.

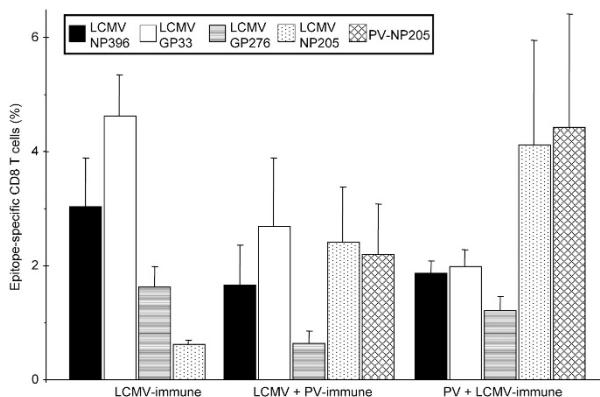


Figure 7. NP(205–212)-specific CD8⁺ T cells are preferentially maintained in memory after secondary infection with cross-reactive viruses. Splenocytes from LCMV-immune, LCMV + PV-immune or PV + LCMV-immune mice were examined by intracellular IFN- γ staining. Mice infected with two viruses were generated by infecting them with the first virus and waiting at least 6 weeks before secondary challenge. After the secondary challenge, mice were left to rest for at least 6 weeks before use. The percentages of IFN- γ -producing cells stimulated by each peptide are shown. The results are mean data from five individual mice.

immunodominant NP(396–404) peptide, with only a weak response against NP(205–212) (*Web Fig. 2a* online). A similar trend was seen with the rechallenge of PV-immune mice with PV (*Web Fig. 2b* online); in this case, the NP(38–45)-specific T cell response was dominant. Thus, the results indicated that this disruption of T cell immunodominance occurred only with heterologous virus challenge.

Cross-reactivity in maintaining and establishing memory
Infection of LCMV-immune mice with PV and other viruses decreases the overall magnitude of the LCMV-specific CD8 memory pool⁷, with variations in memory cell loss being dependent on epitope specificity. We hypothesized that cross-reactivity between the virus-specific T cell responses might contribute to this variability in T cell loss. Having now defined a cross-reactive peptide between LCMV NP(205–212)- and PV NP(205–212)-specific T cells, we examined the maintenance of both cross-reactive and non-cross-reactive memory CD8⁺ T cells in LCMV-immune mice that were infected with PV and then allowed to reach a memory state again (LCMV + PV-immune mice) (**Fig. 7**). A normal immunodominant hierarchy for the LCMV-specific memory T cells was seen in the LCMV-immune mice. In LCMV + PV-immune mice the normally low frequency of the cross-reactive NP(205–212)-specific T cells in LCMV-immune mice was increased fourfold, becoming codominant with NP(396–404) and GP(33–41). The frequencies of the memory CD8⁺ T cells specific for what we now know are non-cross-reactive immunodominant LCMV epitopes were decreased, in agreement with published data⁷.

Previous infection with LCMV markedly altered the hierarchy of T cells elicited by a subsequent PV infection during the primary response (**Fig. 7**). To examine the impact of previous PV infection on the establishment of LCMV-specific memory CD8⁺ T cells, PV-immune mice were infected with LCMV and then allowed to reach a memory state again (that is, PV + LCMV-immune). In PV + LCMV-immune mice, NP(205–212)-specific T cells dominated the LCMV-specific memory repertoire; there was a statistically significant increase in the magnitude of their response compared to that of LCMV-immune mice; $P < 0.02$ for LCMV NP(205–212) and $P < 0.005$ for PV NP(205–212).

The frequency of non-cross-reactive memory T cells specific for the normally dominant LCMV epitopes was diminished. Our findings indicate that the observed attrition of memory CD8⁺ T cells caused by multiple viral infections occurs only for the non-cross-reactive T cells, whereas the cross-reactive T cells are increased in frequency. These data also showed that previous viral infections alter the establishment of virus-specific memory CD8⁺ T cells for subsequent infections, with cross-reactive T cells being maintained and comprising a larger proportion of the memory pool.

Discussion

Our findings show that cross-reactivity between heterologous viruses may be a key factor that influences both the hierarchy of CD8⁺ T cell responses and the shape of memory T cell pools. A heterologous virus infection can elicit a strong immunodominant T cell response to a cross-reactive epitope that is otherwise weak and subdominant, even after repeated challenge with homologous virus. Evidence that virus-specific CD8⁺ T cells are degenerate in their ability to recognize antigen^{23–37} suggests that many cross-reactive T cell epitopes between heterologous viruses remain to be identified. The epitope-specific CD8⁺ T cell responses that are generated in mice by virus infection generally have distinct hierarchies of dominance⁹. However, studies in both humans and mice show that the immunodominance hierarchies of virus-specific T cells may be more variable than previously thought^{23,26,27}. Although some parameters that influence the hierarchies of virus-specific T cells have been described⁹, the complex nature of immunodominance is not fully explained by these factors. We show here that immunodominance hierarchies of T cells elicited by a viral infection can be markedly affected by a previous encounter with a heterologous pathogen. The data show that strongly cross-reactive memory T cells are maintained after secondary viral infections, while non-cross-reactive T cells are lost simultaneously from the memory pool. Our findings suggest that the viruses encountered during an individual's lifespan will constantly transform the T cell repertoire and have a lasting effect on the induction of CD8⁺ T cell responses against future viral infections.

LCMV and PV are members of the *Arenaviridae* family; LCMV is classified as an Old World arenavirus, whereas PV is a New World arenavirus³⁸. These viruses are only distantly related, as shown by nucleotide and amino acid comparisons between the two viruses³⁹. The genetic distance between LCMV and PV is further emphasized by the limited serologic cross-reactivity between the two viruses and the absence of cross-reactive neutralizing antibodies^{30,40}. The LCMV immunodominant (H-2^b) T cell epitopes share limited sequence similarity with the corresponding sequences in PV. The NP(205–212) peptide, however, is highly conserved between LCMV and PV, with variations at only two amino acid residues. In fact, the NP(205–212) peptide sequence is highly conserved among all Old World (Lassa and Mopeia) and New World (Guanarito, Junin, Machupo, Oliveros, Pirital, Sabia and Tacaribe) arenaviruses analyzed³⁹. CD8⁺ T cell cross-reactivity within the closely related Old World arenaviruses, but not between the distantly related Old and New World arenaviruses, has been demonstrated³⁶.

The NP(205–212) epitope is a subdominant epitope during the acute LCMV-specific response, and low frequencies of memory NP(205–212)-specific T cells are maintained¹⁵. In mice whose TCR repertoire had been altered by previous infection with either LCMV or PV, the normally subdominant NP(205–212)-specific T cell response was markedly enhanced after secondary challenge with the heterologous virus but not by the homologous virus. This amplification of the NP(205–212)-specific response in virus-immune mice may



be attributed to the higher precursor frequency of NP(205–212)-specific T cells present and to the fact that memory CD8⁺ T cells respond more vigorously than naïve T cells to antigen⁴¹. Epitope-specific memory T cells appear to have a selective advantage during the establishment of immunodominance⁴², as shown with TCR-transgenic CD8⁺ T cells⁴¹.

CD8⁺ T cell cross-reactivity between unrelated viruses may only become readily detectable after secondary heterologous viral challenge, which preferentially expands the number of cross-reactive T cells⁴. In agreement with this, published data show that a low degree of cross-reactivity between influenza strain-specific CD8⁺ T cells was greatly enhanced after sequential infection with the influenza strains³⁵. Together these findings suggest that potential cross-reactive CD8⁺ T cell responses can be easily overlooked when examining viral infections in naïve mice.

Whereas PV-immune mice infected with LCMV generated strong NP(205–212)-specific T cell responses, responses against the LCMV immunodominant epitopes were reduced in frequency. This reduction in the dominant LCMV-specific epitopes is reminiscent of the immunodomination phenomenon⁴³, which has been documented for T cell responses specific for influenza¹², OVA⁴⁴, LCMV^{45,46} and minor histocompatibility antigens⁴⁷. Immunodomination is characterized as the ability of T cells specific for immunodominant epitopes to suppress responses against other peptides⁹. Proposed mechanisms for immunodomination include the early accumulation of peptide-specific T cells that may reduce the antigen load prior to optimal activation of T cells for other epitopes⁴⁶, the competition for antigen-presenting cells and/or essential cytokines^{44,47} or suppression of T cells by a global effect potentially mediated by cytokines. In contrast, generation of memory CD8⁺ T cells specific for a H-2^d-restricted subdominant LCMV epitope induced by DNA immunization did not suppress the induction of T cells specific for an immunodominant epitope after infection with LCMV⁴⁵. These findings may be explained by the strong immunogenicity of the immunodominant epitope for H-2^d mice NP(118–126) peptide⁴⁸ and suggest that the ability of T cells to suppress is variable.

In mice, previous viral infections can have profound effects on host responses to subsequent infections with potentially cross-reactive heterologous viruses, leading to protective immunity and enhanced immunopathology^{6,28}. The human immune system is continually bombarded with infectious agents, so the likelihood for the induction of cross-reactive responses between heterologous viruses is high. In fact any immunodominant epitope defined for a human viral pathogen may only be immunodominant because of previous exposure of the host to a ubiquitous agent encoding a cross-reactive peptide. In many viral infections, such as infection with Epstein-Barr virus and varicella zoster virus, disease manifests with more severe immunopathological lesions in teenage and young adult patients than in younger children^{49,50}. It is likely that a host's immune repertoire is altered with each successive infection and that the type of responses generated against each subsequent challenge reflect their past exposures to immunogens.

Methods

Mice. Male mice B6 (H-2^d) were from the Jackson Laboratory (Bar Harbor, ME) and were aged 2–18 months. All experiments were done in compliance with institutional guidelines, as approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Viruses. LCMV (Armstrong strain) and PV (AN3739 strain) stocks were prepared in the baby hamster kidney cell line BHK21 as described⁴. For the generation of acute virus-specific T cell responses (7 to 9 days after infection), mice were infected intraperitoneally with 5–10⁴ plaque-forming units (PFU) of LCMV or 2–10⁷ PFU of PV. The stocks of PV were

purified through a sucrose gradient and diluted in Hanks' balanced salt solution. Mice were considered immune 6 weeks or later after infection.

Synthetic peptides. Synthetic peptides were generated by either Genemed Synthesis (San Francisco, CA) or American Peptide Company (Sunnyvale, CA) and were purified with reverse phase-high pressure liquid chromatography to 90% purity. Final products were analyzed by mass spectroscopy. Different sources of PV NP(16–25) showed diverse abilities to stimulate CD8⁺ T cells during intracellular IFN- γ assays, but this was compensated for, in part, by increased concentrations of peptide in the assay. The reasons for this diversity, which could reflect peptide stability or sensitivity to competition from contaminants, are not known. The different sources yielded consistent data for all other peptides examined.

Assay for cell-mediated cytotoxicity. The ⁵¹Cr-release assay was used to quantify virus-specific CTLs as described⁷. Virus-infected target cells were prepared by infecting MC57G cells with virus for 48 h and then labeling with ⁵¹Cr. To prepare peptide-pulsed target cells, ⁵¹Cr-labeled RMA cells were incubated with 100 nM of synthetic peptide for 1 h at 37 °C and then washed three times. For effector cells, single-cell suspensions were prepared from spleens, and erythrocytes were removed by lysis with a 0.84% NH₄Cl solution. Effector cells were then added in various graded effector:target ratios. The amount of radioactivity released in the supernatants was quantified with a liquid scintillation counter (Wallac, Turku, Finland). Percent specific lysis was calculated as [(E - S)/(M - S)] × 100, where E equals the counts per minute released from targets incubated with lymphocytes, S equals the counts per minute released from target cells incubated with no lymphocytes and M equals the counts per minute released from cells after lysis with 1% Nonidet P40 (USB, Cleveland, OH).

Intracellular IFN- γ staining. IFN- γ -producing CD8⁺ T cells were detected with the Cytofix-Cytoperm Kit Plus (with GolgiPlug, BD Pharmingen, San Diego, CA), as described⁷. Splenocytes were prepared as described above. Peripheral blood was prepared by lysing red blood cells with 0.84% NH₄Cl solution for 10 min at 37 °C. Briefly, cells were incubated with 5 nM synthetic peptide, 10 U/ml of human recombinant IL-2 (BD Pharmingen) and 0.2 l of GolgiPlug for 5 h at 37 °C. The samples were analyzed with a FACS-Calibur and CellQuest Software (both from Becton Dickinson, San Diego, CA). The LCMV GP(33–41) sequence contains two overlapping peptides: K^a-restricted GP(34–41) and D^b-restricted GP(33–41)¹⁴. Thus, T cells specific for either GP(33–41) or GP(34–41) will recognize the GP(33–41) peptide in most functional assays, including intracellular IFN- γ assays. In our functional studies both the GP(33–41) and GP(34–41) peptides were used, but for simplicity only results with the GP(33–41) epitope were shown because this reflects the magnitude of the T cell responses against both peptides. When shown, error bars are representative of the s.e.m.

Virus titration. The amount of splenic virus was quantified by plaque assay with a 10% tissue homogenate from individual mice as described⁵. The Student's *t*-test was used for statistical analysis.

MHC-IgG1 dimer staining. MHC-IgG1 dimers were prepared as described³¹. D^b-IgG and K^a-IgG1 dimers (BD Pharmingen) were incubated with 600-fold molar excess peptide and 0.15 g of β_2 -microglobulin per μ g dimer for 7 days. The peptide-loaded MHC dimers were used to stain CD8⁺ T cells at 1 μ g/10⁶ cells. Cells were incubated for 1.5 h at 4 °C in FACS buffer. Samples were washed and stained with anti-CD8 α and biotinylated anti-mouse IgG1 (clone A85-1, BD Pharmingen) for 20 min. Samples were then washed and stained with phycoerythrin-streptavidin (BD Pharmingen) for 20 min. Stained samples were analyzed as described above.

Statistical analyses. The Mann-Whitney test was used for data analysis where indicated.

Note: Supplementary information is available on the *Nature Immunology* website.

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Competing interests statement

The authors declare competing financial interests: see the *Nature Immunology* website (<http://immunology.nature.com>) for details.

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