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Naive Precursor Frequencies and MHC Binding Rather Than the Degree of Epitope Diversity Shape CD8+ T Cell Immunodominance

Maya F. Kotturi,* Iain Scott,† Tom Wolfe,† Bjoern Peters,* John Sidney,* Hilde Cheroutre,† Matthias G. von Herrath,† Michael J. Buchmeier,‡ Howard Grey,* and Alessandro Sette2‡*

The primary CD8+ T cell response of C57BL/6J mice against the 28 known epitopes of lymphocytic choriomeningitis virus (LCMV) is associated with a clear immunodominance hierarchy whose mechanism has yet to be defined. To evaluate the role of epitope competition in immunodominance, we manipulated the number of CD8+ T cell epitopes that could be recognized during LCMV infection. Decreasing epitope numbers, using a viral variant lacking dominant epitopes or C57BL/6J mice lacking H-2Kb, resulted in minor response increases for the remaining epitopes and no new epitopes being recognized. Increasing epitope numbers by using F1 hybrid mice, delivery by recombinant vaccinia virus, or epitope delivery as a pool in IFA maintained the overall response pattern; however, changes in the hierarchy did become apparent. MHC binding affinity of these epitopes was measured and was found to not strictly predict the hierarchy since in several cases similarly high binding affinities were associated with differences in immunodominance. In these instances the naive CD8+ T cell precursor frequency, directly measured by tetramer staining, correlated with the response hierarchy seen after LCMV infection. Finally, we investigated an escape mutant of the dominant GP33–41 epitope that elicited a weak response following LCMV variant virus infection. Strikingly, dominance loss likely reflects a substantial reduction in frequencies of naive precursors specific for this epitope. Thus, our results indicate that an intrinsic property of the epitope (MHC binding affinity) and an intrinsic property of the host (naive precursor frequency) jointly dictate the immunodominance hierarchy of CD8+ T cell responses. The Journal of Immunology, 2008, 181: 2124–2133.

A viral pathogen encodes thousands of potentially immunogenic determinants, yet during the course of an infection CD8+ T cells only recognize and respond to a minute fraction of potential viral epitopes. This phenomenon, known as immunodominance, is a fundamental feature of T cell responses. Several factors have been suggested to contribute to CD8+ T cell immunodominance (1, 2). Of known importance are the efficiency of Ag processing and presentation (3–5), binding affinity of peptides to MHC class I (6, 7), stability of the peptide–MHC complex (8), and epitope abundance on the surface of APCs (9, 10). Immunodominance hierarchies have also found to be influenced by viral load and the kinetics of viral protein expression (11–14). Although less well defined, the kinetics of the CD8+ T cell response to Ag (15), TCR avidity (16), variations in the naive CD8+ T cell repertoire (9, 17, 18), and immunoregulatory mechanisms are also thought to play critical roles (19). In particular, immunodomination, a process whereby a T cell response of one specificity is suppressed by the response of another, contributes to shaping immunodominance (20–24). How these factors are coordinated to determine CD8+ T cell immunodominance has been only partially defined.

Lymphocytic choriomeningitis virus (LCMV)3 inoculation of mice is an informative experimental model for studying CD8+ T cell immunodominance (25). Immunization of immunocompetent C57BL/6J (H-2b) adult mice with the Armstrong strain of LCMV results in a strong and protective CD8+ T cell response directed against as many as 28 H-2b-restricted epitopes (26). These responses are associated with a clear hierarchy (26). Almost 30% of the overall CD8+ T cell response to LCMV is dominated by three major viral epitopes: GP33–41 restricted by Db, GP34–41 restricted by Kb, and nucleoprotein (NP) NP396–404 restricted by Db (27). The remainder of the response is accounted for by 7 intermediate and 18 minor epitopes derived from GP, NP, and the viral RNA L polymerase.

Since the complete repertoire of Ag-specific CD8+ T cells to LCMV has been defined in H-2b mice, we decided to manipulate the number of epitopes seen by CD8+ T cells during acute LCMV infection to investigate the role of epitope complexity in determining the immunodominance hierarchy. Surprisingly, we found the immunodominance hierarchy is unaffected by epitope deletion or addition, and instead is largely shaped by intrinsic properties such as the MHC binding affinity of viral epitopes and the size of the naive CD8+ T cell precursor repertoire. Thus, immunodominance appears, to some extent, to be a predetermined feature of Ag-specific CD8+ T cells.

3 Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; VACV, vaccinia virus; ICCS, intracellular cytokine staining; BFA, brefeldin A.

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Materials and Methods

**Mice**
C57BL/6J, B6AF1/J, B6D2F1/J, B6C3F1/J, and B6JLFl/J mice were purchased from The Jackson Laboratory. H-2k<sup>+</sup>/<sup>-</sup> mice (28) were purchased from Taconic Farms. Stephen Schoenberger (La Jolla Institute for Allergy and Immunology) provided OT-1 Rag<sup>2</sup> mice, bearing transgenes for expression of an OVA-specific TCR. Mice (6- to 10-wk-old females) were housed in the animal facilities at the La Jolla Institute for Allergy and Immunology (La Jolla, CA). All mouse studies followed guidelines set by the National Institutes of Health and Institutional Animal Care and Use Committee-approved animal protocols.

**Viruses and immunizations**
The Armstrong clone 53b strain of LCMV and the variant GPVNPV virus were used in this study (29, 30). Stock viruses were prepared in BHK-21 cells, and viral titer was determined through plaque assays on Vero E6 cells in three independent experiments. Mutations in the epitopic regions of GPVNPV were confirmed by RT-PCR using a high-fidelity polymerase, followed by sequencing analysis. GPVNPV contains three single point mutations, encoding changes from F to L at aa 403 in NP396–404/Db, F to L at aa 38 in GP33–41/Db and GP34–41/K<sub>d</sub>, and V to A at aa 278 in GP276–286/Db. For acute LCMV infection, mice were injected i.p. with 1 × 10<sup>5</sup> pfu of LCMV Armstrong or GPVNPV in PBS. For vaccinia virus (VACV) infection, mice were injected i.p. with either 2 × 10<sup>5</sup> PFU of VACW-Western Reserve strain or 1 × 10<sup>5</sup> PFU of VACV-containing one of the LCMV genes. On day 8 postinfection for LCMV and day 7 postinfection for VACV, mice were sacrificed and CD8<sup>+</sup> splenocytes were analyzed ex vivo by intracellular cytokine staining (ICCS) assays for IFN-γ. For peptide immunization, mice were immunized s.c. with a peptide pool (10 μg) plus 50 μl/ml GolgiPlug (BD Immunocytometry Systems) and analyzed using FlowJo software. To determine the total number of naive tetramer-specific cells in an individual mouse, the percentage of tetramer-positive events was multiplied by the total number of viable cells in the enriched fraction.

**Results**
**Magnitude and breadth of CD8<sup>+</sup> T cell responses against an LCMV variant lacking dominant epitopes**
We recently defined a set of 28 CD8<sup>+</sup> T cell epitopes that seemingly accounts for the totality of the Ag-specific response in LCMV-immunized H-2<sup>b</sup> mice (26). We arbitrarily classify these epitopes as major (>5 × 10<sup>5</sup> responding CD8<sup>+</sup>IFN-γ<sup>+</sup> T cells), intermediate (1–5 × 10<sup>5</sup> cells), or minor (<1 × 10<sup>5</sup> cells) based on the magnitude of the response directed against each epitope following an LCMV infection. We reasoned that if immunodominance is a major factor determining this hierarchy, then deleting or adding epitopes should affect the overall hierarchy. To investigate the influence of the major epitopes on regulating immunodominance of CD8<sup>+</sup> T cells during acute LCMV infection, we examined responses in C57BL/6J (H-2<sup>b</sup>) mice immunized with a variant virus of the Armstrong strain of LCMV, termed GPVNPV. This variant virus was generated by growing LCMV Armstrong-injected H-2<sup>b</sup>-restricted cells under selective pressure with individual CTL clones specific for either the major dominant epitope GP33–41/Db or NP396–404/Db, or the intermediate dominant epitope GP276–286/Db (29, 30). The resultant GPVNPV virus was shown to carry point mutations in the GP33–41/Db, GP34–41/K<sub>d</sub>, NP396–404/Db, and GP276–286/Db wild-type epitopes that resulted in single amino acid changes. Through chromium release CTL assays, the variant virus was shown to evade wild-type epitope-specific CD8<sup>+</sup> T cell responses (29); however, examining responses to GPVNPV using the more sensitive IFN-γ ICCS assay was not performed. Herein, we measured by IFN-γ ICCS assays the numbers of CD8<sup>+</sup>IFN-γ<sup>+</sup> T cells responding to all 28 H-2<sup>b</sup>-restricted epitopes in LCMV Armstrong- and GPVNPV-immunized H-2<sup>b</sup> mice at the peak of the T cell response (8 days after infection).

To demonstrate that GPVNPV lacks responses to the four dominant epitopes, we enumerated the number of CD8<sup>+</sup> IFN-γ<sup>+</sup> T cells from GPVNPV-immunized H-2<sup>b</sup> mice responding to either the variant or wild-type epitopes. We found that CD8<sup>+</sup> splenocytes made reduced amounts of IFN-γ in response to the variant epitopes in GPVNPV-immunized H-2<sup>b</sup> mice (Fig. 1). The NP396–404/Db, GP33–41/Db, GP34–41/K<sub>d</sub>, and GP276–286/Db variant epitopes produced, respectively, 3-, 12-, 105-, and 37-fold less IFN-γ relative to the amount produced by CD8<sup>+</sup> T cells to the wild-type epitopes in LCMV-immunized H-2<sup>b</sup> mice. Similarly, CD8<sup>+</sup> splenocytes from GPVNPV-inoculated H-2<sup>b</sup> mice also made significantly diminished amounts of IFN-γ in response to the NP396–404/Db, GP33–41/Db, GP34–41/K<sub>d</sub>, and GP276–286/Db wild-type epitopes (Fig. 1).

When the responses to the remaining epitopes were measured, there was no emergence of a major immunodominant response that...
was similar in magnitude to the responses observed to the three major dominant wild-type epitopes (GP33–41/Dα, GP34–41/Kk, or NP396–404/Dβ) (Fig. 2A). A range of 1.3- to a maximum of 9.3-fold compensatory increases to both Dβ- and Kk-restricted epitopes was observed in variant virus-immunized mice. The possibility that novel epitopes, previously unidentified, might be recognized in GPVNVPV-immunized mice was examined by testing the antigenicity of overlapping 15-mer peptides spanning the entire LCMV Armstrong proteome (26). However, we were unable to detect any new epitopes (data not shown).

Next, the total CD8+IFN-γ response against all epitopes was calculated by summation of all the individual responses. We found that the total response against the 28 known epitopes was 3.5 × 10^6 cells in the case of LCMV, while the total response against the same 28 epitopes (including the variant peptides) was only 2.2 × 10^6 cells in the case of the GPVNVPV variant virus (Table I). Removal of the four dominant epitopes resulted in a decrease of 1.8 × 10^5 CD8+IFN-γ cells, leaving 1.7 × 10^5 responding cells. Therefore the cumulative response of 2.2 × 10^6 cells in GPVNVPV-infected mice represents only a ~30% overall compensation. These results demonstrate that the elimination of four of the dominant epitopes leads to an overall decreased response to the variant virus, with some compensatory increased responses to intermediate and minor epitopes and no emergence of detectable responses against new epitopes.

CD8+ T cell response to LCMV in H-2Kb−/− mice

As an alternative approach to study the effect of epitope loss on the immune response to LCMV, we next examined the CD8+ T cell response in H-2Kb−/− mice immunized with either LCMV Armstrong or GPVNVPV. Deletion of the Kk allele in the absence of compensatory responses and in conjunction with the mutations in four dominant epitopes (three of which are Dβ restricted) would be expected to result in a >75% decrease in the response to LCMV. When examining the IFN-γ response to the individual epitopes in LCMV-immunized H-2Kb−/− mice, we found that as expected CD8+ T cells recognized the known Dβ-restricted major dominant epitopes (Fig. 2B). However, deletion of the Kk allele did not increase responses against any of the Dβ-restricted epitopes, either major, intermediate, or minor (Fig. 2B, arrows indicate Dβ-restricted epitopes). In fact, the responses to most Dβ-restricted epitopes were actually decreased in H-2Kb−/− mice.

We also observed a CD8+ T cell response to the Kk-restricted GP34–41 epitope in LCMV-immunized H-2Kb−/− mice. Recognition of this epitope could be due to low-affinity binding of the GP34–41 peptide to the Dβ molecule, thus inducing a weak Dβ-restricted response. Three other Kk-restricted epitopes (L1369–377, L1878–1885, and L743–751) were also still recognized at appropriate levels in H-2Kb−/− mice, suggesting these particular epitopes might be dually restricted.

When the responses in the H-2Kb−/− mice inoculated with the GPVNVPV virus were examined, the results were remarkably similar to what was observed in the case of wild type H-2b mice. CD8+ T cell responses to the major dominant epitopes were absent or decreased compared with those observed with the wild-type
Table I. Summary of total CD8+ IFNγ+ T cell response against 28 LCMV-specific epitopes in various systems analyzed

<table>
<thead>
<tr>
<th>Mouse H-2 haplotype</th>
<th>Immunogen</th>
<th>Total Sum Response ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-2b</td>
<td>LCMV</td>
<td>35 ± 20 × 10⁶</td>
</tr>
<tr>
<td>H-2b</td>
<td>GPVNPV</td>
<td>22 ± 10 × 10⁶</td>
</tr>
<tr>
<td>H-2K b−/−</td>
<td>LCMV</td>
<td>8.1 ± 6.3 × 10⁶</td>
</tr>
<tr>
<td>H-2K b−/−</td>
<td>GPVNPV</td>
<td>3.2 ± 2.4 × 10⁶</td>
</tr>
<tr>
<td>H-2b×x</td>
<td>LCMV</td>
<td>10 ± 2.1 × 10⁶</td>
</tr>
<tr>
<td>H-2b×d</td>
<td>LCMV</td>
<td>22 ± 11 × 10⁶</td>
</tr>
<tr>
<td>H-2b×x</td>
<td>LCMV</td>
<td>20 ± 12 × 10⁶</td>
</tr>
<tr>
<td>H-2b×d</td>
<td>LCMV</td>
<td>29 ± 19 × 10⁶</td>
</tr>
<tr>
<td>H-2b</td>
<td>rVACV-LCMV GPC/NP/L</td>
<td>1.7 ± 0.71 × 10⁶</td>
</tr>
<tr>
<td>H-2b</td>
<td>Peptide pool</td>
<td>0.08 ± 0.05 × 10⁶</td>
</tr>
</tbody>
</table>

* Cumulative CD8+ IFNγ+ T cell responses to 28 LCMV-specific epitopes generated by individual peptide stimulation in IFN-γ ICCS assays. The total sum response represents an average value from individually analyzed mice (±SD) from at least two independent experiments. The statistical analysis compares values significantly different from those for the wild-type H-2b mice infected with LCMV. *, p < 0.05 (Mann-Whitney t test).

Similar pattern of immunodominance of LCMV H-2b-restricted epitopes in H-2b homozygous and heterozygous mice

Next, we investigated the effect of increasing the number of epitopes available for recognition during LCMV infection. To this end, we immunized different H-2b F1 hybrid mice with LCMV Armstrong, thus increasing epitope complexity through the presence of additional MHC alleles, without changing the context of acute LCMV infection. Eight days after virus inoculation, IFN-γ responses to each of the known 28 H-2b-restricted epitopes in B6Af1/J (H-2b×x), B6D2F1/J (H-2b×d), B6C3F1/J (H-2b×k), and B6SJLF1/J (H-2bxx) mice were analyzed by IFN-γ ICCS assays.

We found that the immunodominance hierarchy of responding CD8+ T cells in H-2b-immunized mice was similar to the hierarchy in H-2b F1 hybrid-immunized mice (Fig. 3). Major dominant epitopes, such as NP396–404/Db, GP33–41/Db, and GP34–41/Db, were still elicited robust T cell responses, whereas responses to intermediate or minor dominant epitopes were weaker or completely lost.

Interestingly, the NP396–404/Db-specific response was less dominant in H-2b×x and H-2b×d mice compared with the other mouse strains analyzed. This might be due to additional CD8+ T cells dominated by the NP396 epitope, such as the Ld-restricted NP118–126 in H-2b×d mice, or alternatively a negative influence on the generation of the T cell repertoire to the major dominant H-2b-restricted epitopes. The overall sum of the responses directed against the 28 epitopes was between 1.0 and 2.9 × 10⁷ CD8+ IFN-γ+ T cells (Table I), suggesting that the compensatory phenomena, presumably related to responses restricted by other MHC, are the responses that expand in the F1 hybrids and, in turn,
dampen the H-2b-restricted responses. Overall, these findings demonstrate that augmenting epitope complexity through increased MHC diversity does not alter, in any dramatic fashion, CD8+ T cell responses to major dominant epitopes but does weaken responses to other epitopes in the H-2b system.

**Immunodominance pattern of LCMV H-2b-restricted epitopes in the context of a different viral infection**

We next examined the effect of increasing epitope complexity by altering the context in which the LCMV Ags are presented to the immune system. We evaluated the CD8+ T cell responses to the 28 known LCMV-specific epitopes in H-2b mice immunized separately with different rVACV expressing the LCMV GPC, NP, or L protein. CD8+ splenocytes from rVACV-immunized H-2b mice were analyzed by IFN-γ ICCS assays 7 days after immunization.

Fig. 4 shows the total number of responding CD8+ IFN-γ+ T cells to each of the known LCMV-specific epitopes when the response to the GP-, NP- and L-specific epitopes were evaluated in rVACV-LCMV GPC, rVACV-LCMV NP, and rVACV-LCMV L-immunized H-2b mice, respectively. In rVACV-immunized mice, the LCMV-specific response remained focused on the major dominant epitopes, NP396–404/Db, GP33–41/Db, and GP34–41/Kb (Fig. 4), albeit the magnitude of the NP396–404/Db-specific response was lower than that of GP33–41/Db and GP34–41/Kb. A similar response pattern is also found if the individual epitope-specific responses are represented as a fraction of the overall total response (data not shown). With the exception of GP276–286/Db, the responses to the intermediate and minor dominant epitopes were virtually eliminated. In this new context, the magnitude of the entire LCMV-specific response in rVACV-immunized H-2b mice was significantly reduced ~20-fold compared with the response in mice immunized with LCMV Armstrong (Table I).

In the same set of experiments, we also examined the VACV-specific response by measuring the total number of CD8+ IFN-γ+ T cells responding to 10 VACV-specific H-2b-restricted epitopes in either wild-type VACV-Western Reserve or rVACV-immunized mice 7 days after immunization (35). The magnitude of the epitope-specific responses to VACV was not substantially altered by co-expression of LCMV proteins (data not shown).

**MHC binding affinity partially accounts for immunodominance hierarchy**

Although there are certain changes to the hierarchy, overall it appears that the pattern of LCMV-specific responses is maintained after epitope deletion or addition or delivery by a different viral system. This suggests the possibility that immunodominance might be shaped by intrinsic properties of LCMV antigenic proteins or by LCMV-specific epitopes. To address this hypothesis, we first examined the relationship between the MHC binding affinity of the 28 H-2b-restricted epitopes and the total number of epitope-specific CD8+ IFN-γ+ T cells seen after acute LCMV infection (see Table II for reference binding affinities (26)). The IC50 values were plotted against the total number of CD8+ IFN-γ+ T cells specific for each epitope (Fig. 5, filled circles). The vertical lines in Fig. 5 represent thresholds of responding cells, separating major intermediate and minor epitopes.
the epitope-specific CD8+ T cell populations into major, intermediate, and minor.

We noted that the three major epitopes all bound with an affinity of 25 nM or less. Of the seven intermediate epitopes, four bound with affinities similar to the major epitopes and three epitopes bound with affinities in the 25–500 nM range. Thus, although, on average, intermediate epitopes had a somewhat lower binding affinity than did major epitopes, it is clear that other factors besides MHC binding must dictate the lower place of these intermediate epitopes in the immunodominance hierarchy.

Likewise, in the case of the minor epitopes, lower binding affinities were detected overall, with three epitopes falling above the 500 nM threshold, having an average binding affinity of ~26,200 nM. However, it was noted that five of the minor epitopes did have affinities of 25 nM or less, again indicating that factors other than MHC binding also are important in determining their lower place in the overall hierarchy of immunodominance.

In the same set of experiments, we also measured the binding capacity of the variant epitopes contained in the GPVNPV variant virus to their appropriate MHC molecule. We observed that all four variant peptides bound 1.8- to 28-fold less efficiently to either D8 or K8 compared with the wild-type peptides (Table II and Fig. 5, open circles). While the decreased responses to NP396–404, GP34–41, and GP276–286 variant peptides might be explained by their corresponding decreased binding affinities, this was not the case for the GP33–41 variant peptide. It was noted that the GP33–41 variant peptide was associated with a minor CD8+ response even though the variant peptide still bound with high (22 nM) affinity. Thus, in the case of the GP33–41 variant peptide, factors other than lower and insufficient MHC binding are responsible for the loss of dominance of this epitope.

**Immunodominance hierarchy following peptide immunization**

We next examined the contribution of other intrinsic properties of the LCMV-derived epitopes in shaping the observed epitope hierarchy. To completely eliminate the context of acute LCMV infection and the Ag of origin, we immunized H-2b mice with a pool of the 28 LCMV-specific peptides. Eleven days after peptide immunization, the total number of responding CD8+ IFN-γ+ T cells was measured by IFN-γ ICCS assays.

Overall, the total response to the panel of 28 epitopes was $8.0 \times 10^6$ cells, representing a significant ~430-fold decrease compared with the response following LCMV infection, and a ~22-fold decrease compared with the response observed in the case of the same epitopes delivered by the rVACV vectors (Table I). As shown in Fig. 6, following peptide pool immunization, the major epitopes, NP396–404/D8, GP33–41/D8, and GP34–41/K8, were relatively strongly recognized, suggesting

![FIGURE 5. Comparison between MHC binding affinity and the total number of epitope-specific CD8+ T cells producing IFN-γ after acute LCMV infection. Each filled circle represents the IC50 value and total number of CD8+ IFN-γ+ T cells for the wild-type 28 LCMV-specific epitopes, while the open circles represent corresponding values for the variant epitopes. The horizontal lines correspond to IC50 values of 25 and 500 nM, while the vertical lines are thresholds for either the major, intermediate, or minor epitopes.](http://www.jimmunol.org/)

![FIGURE 6. Peptide-specific CD8+ T cell responses following peptide pool immunization. H-2b mice were immunized s.c. with a peptide pool containing all 28 LCMV-specific peptides. Eleven to 12 days after immunization, splenocytes were stimulated with the indicated peptides and stained for CD8 and intracellular IFN-γ. Total number (±SD, n = 6) of CD8+ IFN-γ+ T cells responding to each of the known LCMV-specific epitopes was measured. Each mouse is analyzed individually, and data represent average values from two independent experiments.](http://www.jimmunol.org/)
that the context of LCMV infection does not significantly contribute to the dominance of these epitopes. However, the response to NP396–404/Db was considerably reduced in comparison to GP33–41/Db and some of the intermediate (NP238–248/Kb), and minor (L689–697/Db and L455–463/Db) dominant epitopes were recognized to a similar degree as NP396–404/Db and GP34–41/Kb. A similar pattern of responses is also found if the individual epitope-specific responses are represented as a fraction of the total response (data not shown).

**Naive CD8+ T cell precursor frequency correlates to immunodominance hierarchy**

The data presented above clearly illustrate that factors other than MHC binding affinity must also play key roles in shaping the immunodominance hierarchy. Since the CD8+ T cell response was to a large extent focused on the major dominant epitopes after peptide pool immunization, lacking the context of a viral infection, we questioned whether the immunodominance hierarchy might be already imprinted in the naive T cell repertoire. To address this point we studied seven peptides in more detail. All seven peptides bound with similarly high affinity, but they were associated with either major, intermediate, or minor immunodominant status (see Table II and Fig. 5). We examined whether the CD8+ T cell response to these epitopes following acute LCMV infection was proportional to the naive T cell precursor frequency using a tetramer-based enrichment method to enumerate naive epitope-specific T cell populations from individual mice (33). We analyzed the naive T cell populations specific for two major, three intermediate, and two minor dominant LCMV epitopes.

As shown in Fig. 7, individual naive H-2b mice on average contained 449 GP33–41/Dd-specific, 117 NP396–404/Dd-specific, 90 L2062–2069/Kb-specific, 57 NP205–212/Kb-specific, 43 GP118–125/Kb-specific, 24 L156–163/Kb-specific, and 15 L338–346/Dd-specific CD8+ T cells. We confirmed that the tetramer-enriched CD8+ T cells were naive based on their CD44highCD62l– phenotype (data not shown). We found that the size of each naive T cell population was significantly greater (Mann-Whitney U test, p < 0.01) than the number of irrelevant RAG-deficient OT-1 TCR transgenic T cells stained with the same tetramers (Fig. 7, open symbols). We also attempted to detect naive precursors specific for two additional minor epitopes with high binding affinities, L689–697 and GP221–228. The tetramer staining for these two populations, however, was poor.

Overall, each naive epitope-specific CD8+ T cell population analyzed was roughly proportional to the corresponding expanded populations found in mice acutely infected with LCMV (see Fig. 2A for comparison). Thus the two major determinants had the highest precursor frequencies, followed by the precursor frequencies to the three intermediate epitopes, with the lowest precursor frequencies being found for the two minor epitopes. The 4-fold greater frequency of the GP33–41/Dd-specific precursors compared with the NP396–404/Dd-specific precursors correlated best to the responses observed following peptide and rVACV immunization, whereas following LCMV infection the response to NP396–404/Dd was consistently equal to or better than that to GP33–41/Dd.

**FIGURE 8.** Immunogenicity and naive T cell precursor frequencies specific for the GP33–41/Dd wild type and variant epitopes. A, Total number (±SD, n = 6) of CD8+IFN-γ+ T cells responding to the GP33–41/Dd wild type and variant epitopes following individual peptide immunization of H-2b mice. Each mouse is analyzed individually, and data represent average values from two independent experiments. B, Total number of GP33–41/Dd wild-type and GP33–41/Dd variant-specific CD8+ T cells in spleen and lymph nodes from naive H-2b mice (filled symbols; n = 15–19) and from OT-1 Rag−/− TCR transgenic mice (open symbols; n = 3–5) following tetramer-based enrichment. Each symbol represents an individual mouse, whereas horizontal bars represent average precursor values. When no tetramer-specific CD8+ T cells were detected in OT-1 Rag−/− mice, precursor values were set at a detection threshold of 10 cells.
Poor recognition of GP33–41/D\textsuperscript{b} viral epitope correlates with low precursor frequency

At least for GP33–41/D\textsuperscript{b}-specific CD8\textsuperscript{+} T cells, it appears that naive precursor frequency significantly contributes to the dominance of this T cell population following LCMV infection. As a further test of this conclusion, we also enumerated the number of precursor GP33–41/D\textsuperscript{b} variant epitope-specific CD8\textsuperscript{+} T cells in H-2\textsuperscript{b} mice. This analysis was prompted by the findings that the GP33–41 variant peptide was a very poor immunogen in the context of infection with the GPVNPV variant virus (Fig. 2A), yet had a similarly high binding affinity to MHC as the wild-type GP33–41/D\textsuperscript{b} epitope. When the wild-type and variant GP33–41 peptides were used as immunogens, the relatively poor immunogenicity of the variant peptide was confirmed (Fig. 8A). Note also that in Fig. 8A the wild-type GP33–41 peptide-specific response was \sim 2-fold greater than the response shown in Fig. 6. This difference likely reflects that the peptide immunization in Fig. 6 is using a pool of all 28 peptides, while Fig. 8A represents a single GP33–41 peptide immunization. The analysis of the naive T cell precursor frequencies for these epitopes demonstrates that the differences in the immunogenicity could be fully explained by the almost 10-fold difference in precursors specific for the two epitopes (Fig. 8B). On average, individual naive H-2\textsuperscript{b} mice contained 56 GP33–41/D\textsuperscript{b} variant-specific CD8\textsuperscript{+} T cells. Thus, the loss of dominance of this variant epitope likely reflects a diminished T cell repertoire capable of recognizing the mutated epitope.

Discussion

Herein we investigated the effect on CD8\textsuperscript{+} T cell responses of altering epitope complexity in the context of acute LCMV Armstrong infection. By decreasing the number of epitopes capable of being recognized by the host, an overall decreased CD8\textsuperscript{+} T cell response was observed without any detectable responses directed against new epitopes. As outlined in the summary of total CD8\textsuperscript{+} T cell responses (Table I), with each decrease in epitope complexity, there is a corresponding reduction in the absolute CD8\textsuperscript{+} T cell response. Therefore, epitope loss results in a new lower \"set point\" for the anti-LCMV CD8\textsuperscript{+} T cell response. However, some compensatory responses were also observed, particularly in the case of H-2\textsuperscript{b} mice immunized with GPVNPV. Still, the total amount of compensation present in GPVNPV-inoculated H-2\textsuperscript{b} mice (\sim 9.0 \times 10\textsuperscript{5} CD8\textsuperscript{+} IFN-\gamma\textsuperscript{+} T cells) was far from complete and only made up for roughly half of the response lost by the deletion of the four dominant epitopes (\sim 1.8 \times 10\textsuperscript{7} CD8\textsuperscript{+} IFN-\gamma\textsuperscript{+} T cells).

A previous study also reported that removal of immunogenic epitopes was associated with increases in other T cell responses (12). However, whether this resulted in compensation of overall wild-type virus responses was not determined since CD8\textsuperscript{+} responses against only 10 LCMV-specific epitopes were examined. Secondary challenge of H-2\textsuperscript{b} mice with an influenza A variant virus lacking CD8\textsuperscript{+} T cell responses to dominant epitopes also resulted in compensation to multiple epitopes as well as minor responses to new epitopes (21, 22). Similarly, these responses did not numerically compensate for the loss of wild-type virus responses (21, 22), suggesting that at least for viruses with small RNA genomes, epitope complexity does not dictate immunodominance and epitope hierarchy.

We also demonstrated that increasing the number of epitope-specific CD8\textsuperscript{+} T cell responses did not greatly affect LCMV-specific CD8\textsuperscript{+} responses to major epitopes, but did dampen responses to both intermediate and minor epitopes. Presumably, compensatory responses, either restricted by other MHC alleles in the F\textsubscript{1} hybrids or the VACV-specific CD8\textsuperscript{+} response in rVACV-inoculated mice, might play a role in diminishing these responses. Similarly, in a previous study, the H-2\textsuperscript{b}-restricted influenza-specific CD8\textsuperscript{+} T cell response in H-2\textsuperscript{b}\textsuperscript{nu/nu} F\textsubscript{1} hybrid mice was dampened compared with the response in H-2\textsuperscript{b} mice, but overall there were only subtle changes to the immunodominance hierarchy (36). Collectively, these findings have important implications for future vaccine design, particularly when using VACV as a vehicle for immunization. In support of this, it has been previously shown that a VACV recombinant expressing the major GP33–43/D\textsuperscript{b} epitope induces greater protective immunity in H-2\textsuperscript{b} mice compared with a rVACV expressing either the intermediate NP205–212/K\textsuperscript{b} or minor GP92–101/D\textsuperscript{b} epitope (32). This phenomenon of epitope dominance has also been observed in rVACV vaccination strategies targeting simian HIV. Several studies have demonstrated that rhesus monkeys vaccinated with recombinant modified VACV Ankara vectors elicit a strong CD8\textsuperscript{+} T cell response to immunodominant epitopes but only a weak response to subdominant epitopes (37–39).

It is clear that manipulating epitope complexity results in varying levels of compensation and suppression. However, these changes do not dramatically alter the pattern of the H-2\textsuperscript{b}-restricted response directed against LCMV. To explain our findings we hypothesized that properties intrinsic to the LCMV-specific epitopes might be the key factors in determining the hierarchy of epitopes recognized. We first examined the contribution of MHC binding affinity. We observed a positive correlation between binding affinity and the magnitude of the epitope-specific CD8\textsuperscript{+} T cell response following LCMV infection, and all major epitopes bound with affinities of 25 nM or less. However, we also observed that nine epitopes with an equally high binding capacity (IC\textsubscript{50} < 25 nM) only induced an intermediate or minor T cell response. Likewise, the GP33–41/D\textsuperscript{b} variant epitope also only generated a minor T cell response in GPVNPV-immunized mice despite its binding with high affinity. These data imply that although MHC binding affinity is important, it does not, by itself, determine the immunodominance hierarchy. Other MHC binding parameters, such as the stability of the MHC-peptide complex might also contribute to immunodominance (8); however, we recently showed that complex stability did not correlate significantly better with immunodominance compared with the binding affinity (19).

In cases where MHC binding affinity is approximately equivalent, competition for MHC binding might play a role in determining the epitope hierarchy. Indeed, L2062–2069, GP118–125, and L156–163 peptides with high MHC binding affinity were quite immunogenic following single peptide immunization (data not shown), but not after immunization with a peptide pool containing all 28 LCMV-specific epitopes. A previous study also demonstrated the loss of dominance of major epitopes when immunized as a peptide mixture (40). Likewise, L689–697/D\textsuperscript{b} and L455–463/D\textsuperscript{b} are significantly higher in the immunodominance hierarchy after peptide pool immunization than they are following LCMV infection. Additional factors that might influence immunogenicity levels following peptide immunization could be the intrinsic stability of peptides and the effective Ag dose.

Our study presents the first direct evidence that CD8\textsuperscript{+} T cell immunodominance hierarchies are to a major extent determined by the naive T cell repertoire. This is consistent with various previous studies (9, 13, 17, 18) that suggest the size of naive CD8\textsuperscript{+} T cell populations might partially account for the immunodominance hierarchy. In our study, the number of GP33–41/D\textsuperscript{b}-specific naive CD8\textsuperscript{+} T cells is \sim 2.5- to 5-fold higher than the previous estimate of 100–200 precursors (41). Earlier work on determining LCMV-specific precursor frequencies relied on an indirect method of titrating known quantities of TCR transgenic T cells into naive
recipients (41, 42). While tetramer binding gives a direct measurement of epitope-specific precursor T cells, it has its own weaknesses. On the one hand it might not detect T cells with a low-affinity TCR. Conversely, detection of precursors by tetramer binding does not provide information on the functionality of the positive cells and thereby might overestimate precursor frequency.

Although the link between naive precursor frequency and the magnitude of the CTL response is striking, we emphasize that our data also show that precursor frequency is not the only determinant of immunodominance. Besides the above-mentioned difference of MHC binding affinity, other factors also clearly play a role. For example, while precursor frequencies specific for the major dominant epitopes were larger than the values obtained for other epitopes, we found that there were significantly more naive CD8\(^+\) T cells specific for the GP33–41/D\(^b\) epitope compared with the NP396–404/D\(^b\) epitope. Interestingly, it has been demonstrated that the kinetics of LCMV protein expression and presentation aid in shaping the immunodominance hierarchy in H-2\(^b\) mice (13). Specifically, in the presence of high viral loads, NP-specific CD8\(^+\) T cells are thought to be more prone to functional exhaustion than are GP-specific CD8\(^+\) T cells owing to earlier NP expression (43), as well as faster kinetics of NP-derived epitope presentation compared with the GP (13). These results suggest that the naive precursor frequency might play a prominent role in contributing to the immunodominance of GP33–41/D\(^b\), but that other mechanisms relating to protein expression are also important for NP396–404/D\(^b\). Not only was a lower precursor frequency for NP396–404/D\(^b\) observed, but we also found a lower response to this peptide following rVACV-LCMV NP infection and peptide pool immunization compared with the response seen after LCMV infection. One possible explanation for the lower NP396–404/D\(^b\)-specific response might be that the NP396–404 peptide is not efficiently processed following rVACV-LCMV NP infection. Alternatively, following LCMV infection, it is plausible that cross-reactive T cells, in part, contribute to the large CD8\(^+\) T cell response directed against the NP396–404 peptide.

Although not directly addressed in this study, epitope abundance on the surface of APCs might also influence the immunodominance hierarchy of LCMV-specific responses. By eluting CTL epitopes from LCMV-infected cells, a previous study demonstrated that the magnitude of CD8\(^+\) T cell responses after LCMV infection correlated with peptide abundance on the cell surface (10). Interestingly, we found that delivery of LCMV Ags by rVACV resulted in a dramatic reduction in the total LCMV-specific response. Because LCMV replicates more efficiently than rVACV in mice, we would expect that the rVACV system would deliver less protein and therefore potentially fewer epitopes than wild-type LCMV. The 20-fold reduction in CD8\(^+\) T cells responding to the LCMV-specific epitopes in the context of rVACV infection suggests that epitope abundance might be important in establishing absolute CD8\(^+\) T cell numbers.

Our results also for the first time demonstrate that the frequency of naive precursors plays a role in pathogen escape from immune recognition. The GPVNPV variant virus was developed by selective pressure from CTL clones that only recognized the GP33–41/D\(^b\), GP34–41/K\(^a\), GP276–286/D\(^b\), or NP396–404/D\(^b\) epitopes (29, 30). However, these findings indicate the precise mechanism of CTL escape was not elucidated. Herein we found that CTL escape was not solely attributable to decreased MHC binding affinity (as was the case for GP34–41/K\(^a\), GP276–286/D\(^b\), and NP396–404/D\(^b\) epitopes), but also directly due to a diminished naive CD8\(^+\) T cell repertoire capable of recognizing the GP33–41/D\(^b\) variant epitope. Likewise, during HIV and SIV infection, viral variants are selected that escape immune recognition. In many cases, CTL escape seems to be a direct consequence of substantially reduced MHC binding affinity of the mutated epitopes (44–46). However, there are other cases where the variant epitopes still bind with high affinity to MHC and yet evade CTL recognition (44, 47). In these instances, an insufficient precursor frequency might be the mode of CTL escape. Taken together, these data suggest that precursor frequency not only is relevant for shaping the immunodominance hierarchy but its importance also extends and reverberates into mechanisms of viral escape and immunopathology.

In summary, our study demonstrates that intrinsic properties of viral epitopes, such as MHC binding affinity, and intrinsic properties of the host, such as the naive T cell repertoire, significantly contribute to shaping the immunodominance hierarchy. Based on these data, it seems reasonable to propose that naive precursor frequencies could be used as a diagnostic measure of the effectiveness of a particular epitope or Ag in a vaccination setting. This would alleviate the need for obtaining lymphocytes from exposed or immunized individuals, which, depending on the pathogen, can be logistically difficult. We have also previously established that LCMV epitopes differ in their protective capacity (26, 32) and, interestingly, all epitopes with a naive precursor frequency of >100 cells or more per mouse are capable of protecting mice against LCMV infection. Finally, our data might have important implications for rational vaccine design, especially when Ags are delivered by a VACV vector. Our results indicate that the LCMV-specific responses were limited to major dominant epitopes with high precursor frequencies and that the response to even these epitopes was much lower than that achieved in the course of an LCMV infection.

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Disclosures

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