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Effect of Chronic Viral Infection on Epitope Selection, Cytokine Production, and Surface Phenotype of CD8 T Cells and the Role of IFN-γ Receptor in Immune Regulation

Kavita Tewari, Jonah Sacha, Xiaoyan Gao, and M. Suresh

Regulation of CD8 T cell responses in chronic viral infections is not well understood. In this study, we have compared the CD8 T cell responses to immunodominant and subdominant epitopes during an acute and a chronic lymphocytic choriomeningitis virus (LCMV) infection in mice. The epitope hierarchy of the primary CD8 T cell response was similar in acute and chronic LCMV infections. However, strikingly, the epitope hierarchy of the primary CD8 T cell response was conserved in the T cell memory only in an acute but not in a chronic LCMV infection. Interestingly, in an acute infection, increasing the viral dose caused significant changes in the epitope hierarchy of the LCMV-specific memory CD8 T cell pool, with no effect on the primary CD8 T cell response. Functional and phenotypic analyses revealed that exposure of CD8 T cells to extended periods of antigenic stimulation could lead to long-term defects in cytokine production and alteration in expression of cell surface L-selectin (CD62L). Whereas expression of CD44 was minimally altered, a greater proportion of LCMV-specific memory CD8 T cells were CD62Llow in mice that have recovered from a chronic LCMV infection, compared with acutely infected mice. Mechanistic studies showed that IFN-γR deficiency altered the epitope hierarchy of the pool of LCMV-specific memory CD8 T cells without significantly affecting the immunodominance of the primary CD8 T cell response in an acute infection. Taken together, these findings should further our understanding about the regulation of T cell responses in human chronic viral infections.

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infecting mice with 2 × 10^6 PFU of LCMV-Tib i.v. Infectious LCMV in the tissues and serum of infected mice was quantitated by plaque assay using Vero cell monolayers (17).

**CTL assay**

Primary ex vivo MHC class I (MHC-I)-restricted LCMV-specific cytotoxic activity in the spleens of LCMV-infected mice was measured by ³¹Cr-release assay using MC57 cells as target cells (17).

**Flow cytometry**

LCMV-specific CD8 T cells were visualized using MHC-I tetramers. The development and use of MHC-I tetramers complexed with the LCMV CTL epitope peptides NP396-404 (NP396), GP33-41 (GP33), and GP276-286 (GP276) in the viral nucleoprotein or glycoprotein has been described elsewhere (13). Freshly explanted spleen cells were stained with PerCP-conjugated anti-CD8, PE-labeled anti-CD62L, FITC-conjugated anti-CD44 Abs, and allophycocyanin-labeled MHC-I tetramers for 1 h at 4°C in FACS buffer (PBS containing 2% BSA and 0.1% sodium azide). After staining, cells were fixed in 2% paraformaldehyde and acquired on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). Flow cytometry data were analyzed using CellQuest software (BD Biosciences). Abs were purchased from BD PharMingen (San Diego, CA).

**Intracellular staining for IFN-γ, Bcl-2, Bcl-3, and Bcl-6**

Splenocytes were stained with various LCMV CTL epitope peptides for 5 h in the presence of brefeldin A (13). After culture, cells were stained for cell surface CD8, intracellular IFN-γ, and Bcl-2, Bcl-3, or Bcl-6 using the Cytofix/Cytoperm kit (BD PharMingen) (19). Abs to murine Bcl-2 and IFN-γ were purchased from BD PharMingen. Anti-Bcl-3 and anti-Bcl-6 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Quantitation of CCR7 expression on LCMV-specific CD8 T cells using CCL19-Fc fusion proteins**

CCL19-Fc fusion proteins were kindly provided by J. Cyster (University of California, San Francisco) (20). Splenocytes were first incubated with anti-mouse CD16/CD32 to block Fc receptors and then were stained with CCL19-Fc, PerCP-conjugated anti-CD8, FITC-conjugated anti-CD62L, and allophycocyanin-labeled MHC-I tetramers. CCL19-Fc binding was visualized by a second step staining with PE-labeled anti-human Fcγ (Jackson ImmunoResearch Laboratories, West Grove, PA). As controls for binding specificity of CCL19-Fc, cells were preincubated with recombinant soluble chemokine CCL19 (macrophage-inflammatory protein-3β; R&D Systems, Minneapolis, MN) at a concentration of 5 μg/ml. Stained cells were fixed in 2% paraformaldehyde and acquired on a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed as described above.

**Statistical analysis**

Experimental data were analyzed using commercially available statistical software (SYSTAT, version 8.0; Chicago, IL). Groups were compared by Student’s t test and significance was defined at p ≤ 0.05.

**Results**

**Primary and memory virus-specific CD8 T cell responses during an acute and chronic LCMV infection**

Infection of immunocompetent mice with the Armstrong strain of LCMV results in an acute infection, which is resolved in 8–10 days (13, 17). In striking contrast, infection of mice with T1b strain of LCMV causes a chronic infection, lasting up to 30 days (16, 21). Early in the infection, both LCMV-Arm and LCMV-T1b viruses replicate predominantly in the tissue macrophages found in spleen, lymph nodes, liver, lung, etc. (22). In LCMV-Arm-infected mice, after attaining peak viral titers on day 3 postinfection (PI), viral load in the tissues drops precipitously after day 5 PI and most of the infection is resolved by day 8 PI (21). LCMV-Arm replication is restricted to tissue macrophages with very little spread to the parenchymal cells (22). Unlike LCMV-Arm, which is rapidly controlled, LCMV-T1b spreads rapidly from macrophages to parenchymal cells, resulting in widespread dissemination to most visceral organs (22). Despite being biologically different, LCMV-Arm and LCMV-T1b are >99.8% genetically identical and the amino acid changes are not found in the MHC-I-restricted CTL epitopes (23).

On day 8 PI, we compared MHC-I-restricted cytotoxic activity in the spleen between LCMV-Arm- and LCMV-T1b-infected mice. Consistent with published findings (17, 21), the cytotoxic activity in the spleens of LCMV-T1b-infected mice was substantially lower compared with LCMV-Arm-infected mice (data not shown). As expected, by day 8 PI, all of the LCMV-Arm-infected mice had resolved the infection and no infectious virus was detected in the liver and lungs. On day 8 PI, the viral titers in the liver and lungs of LCMV-T1b-infected mice were 6.5 ± 0.2 and 6.8 ± 0.2 (log_{10} PFU/gram of tissue), respectively. Next, we used MHC-I tetramers to compare the effect of chronic infection with LCMV-T1b on the expansion of CD8 T cells specific to the two D^b-restricted immunodominant epitopes NP396 and GP33. As shown in Fig. 1A, high numbers of LCMV-specific CD8 T cells were detected in the spleens of LCMV-Arm-infected mice. The percentages of NP396- and GP33-specific CD8 T cells in LCMV-T1b-infected mice were significantly reduced, in comparison with LCMV-Arm-infected mice. It is worth emphasizing that a previous study had examined CD8 T cell responses to the immunodominant epitopes only during an LCMV-T1b infection between wild-type and CD4-deficient mice (16). However, this past study did not compare primary CD8 T cell responses with the immunodominant epitopes between LCMV-Arm and LCMV-T1b (chronic) in wild-type mice (16). The present study shows that a chronic LCMV infection reduced the expansion (clonal burst size) of CD8 T cells specific to the two immunodominant LCMV CTL epitopes, as compared with an acute LCMV infection.

The primary CD8 T cell responses to both dominant and subdominant epitopes during an acute LCMV infection have been well studied (13). However, the effect of a chronic LCMV infection on the expansion of CD8 T cells specific to the subdominant epitopes is unclear. To address this issue, on day 8 PI we used intracellular cytokine staining to quantitate CD8 T cell responses to dominant (NP396 and GP33) and subdominant epitopes (GP276, NP205, and GP118) in LCMV-Arm- and LCMV-T1b-infected mice. As shown in Fig. 1B, the percentages of NP396- and GP33-specific CD8 T cells in LCMV-T1b-infected mice were comparable to those in LCMV-Arm-infected mice (Fig. 1B). The reduction in the percentages of NP396- and GP33-specific CD8 T cells in LCMV-T1b-infected mice was not due to functional unresponsiveness (inability to produce IFN-γ; Fig. 1B) because staining with MHC-I tetramers yielded similar results (Fig. 1A). It is noteworthy that the epitope GP33 is presented by both D^b and K^b MHC-I molecules (24). Whereas MHC-I tetramers bind only D^b-restricted GP33-specific CD8 T cells, intracellular cytokine staining detects both D^b- and K^b-restricted GP33-specific CD8 T cells (24). Therefore, percentages of GP33-specific IFN-γ-producing CD8 T cells are more than MHC-I tetramer-binding CD8 T cells (compare Fig. 1, A and B). The total number of CD8 T cells specific to various epitopes in LCMV-Arm- and LCMV-T1b-infected mice is shown in Fig. 1C. In LCMV-T1b-infected mice, the total numbers of CD8 T cells specific to the epitopes NP396, GP33, and GP276 were reduced by 5.4-, 2.4-, and 4-fold, respectively, as compared with LCMV-Arm-infected mice (Fig. 1C). Interestingly, the expansion of NP205- and GP118-specific CD8 T cells was reduced by only ~1.6-fold in LCMV-T1b-infected mice. The epitope hierarchy of the primary CD8 T cell response in LCMV-Arm-infected mice was GP33 ≥
cytometry profiles are gated on total splenocytes, and equally represented (Fig. 1, followed by NP396, epitopes GP276, NP205, and GP118 were altered: although the most dominant epitope was GP33, which was hierarchy of the primary CD8 T cell response was only slightly T1b-infected mice, despite the high antigenic load, the epitope /H11022 cells after an acute and a chronic viral infection.

Expansion of LCMV-specific cells that are specific to the two immunodominant epitopes NP396 and GP33–41 was determined by staining with MHC-I tetramers (D'). The flow cytometry profiles are gated on total splenocytes, and the numbers are the percentages of tetramer-binding CD8 T cells among splenocytes. B, On day 8 PI with LCMV-Arm or LCMV-T1b, the number of CD8 T cells specific to the indicated CTL epitopes was determined by intracellular staining for IFN-γ. The flow cytometry profiles are gated on total splenocytes, and the numbers represent percentages of IFN-γ-producing epitope-specific CD8 T cells of total splenocytes. C, Total number of epitope-specific CD8 T cells in the spleens, and the data are the mean of three to six mice per group and are representative of three independent experiments.

Previous studies have shown that the number of memory CD8 T cells generated is determined by the magnitude of expansion during the primary response (original clonal burst size) to an acute viral infection (13). Accordingly, the epitope hierarchy established in the primary CD8 T cell response is conserved in the pool of memory T cells (13). However, the relationship between epitope hierarchy of the primary T cell response and the memory T cell pool after a chronic viral infection is not well understood. Therefore, we investigated the effect of chronic LCMV infection on epitope composition of CD8 T cell memory. One hundred twenty days after infection with LCMV-Arm or LCMV-T1b, the number of LCMV-specific memory CD8 T cells was determined by intracellular cytokine staining. At this time point, no infectious LCMV was detected in the lungs, liver, and brain of LCMV-Arm- and LCMV-T1b-infected mice (data not shown). LCMV-Arm and LCMV-T1b infection should have been resolved by approximately 10 and 30 days PI, respectively (16, 21). As shown in Fig. 2, A and B, memory CD8 T cells specific to all of the LCMV CTL epitopes were readily detected in both LCMV-Arm- and LCMV-T1b-infected mice. Fig. 2a illustrates the striking differences in the epitope hierarchy of LCMV-specific CD8 T cells between LCMV-Arm- and LCMV-T1b-infected mice. In LCMV-Arm-infected mice, the epitope hierarchy was as follows: GP33 > NP396 > GP276 > NP205 ≥ GP118. In striking contrast, the epitope hierarchy of LCMV-specific memory T cells in LCMV-T1b-infected mice was GP33 ≥ GP276 > NP396 ≥ GP118 > NP205. Most notably, epitope GP276, which was subdominant in the acute infection with LCMV-Arm, emerged as a dominant epitope after a chronic infection with LCMV-T1b. Furthermore, GP118 had become clearly dominant over NP205 in LCMV-T1b-infected mice. To more clearly define epitope hierarchy, we calculated the relative proportions of epitope-specific CD8 T cells among total LCMV-specific CD8 T cells during the primary and memory T cell responses. Data in Fig. 2C show the relative proportions of epitope-specific T cells of total LCMV-specific CD8 T cells on day 8 PI (primary) and day 120 PI (memory) in LCMV-Arm- and LCMV-T1b-infected mice. Consistent with published findings, data in Fig. 2C clearly show that, during an acute infection, the epitope hierarchy of LCMV-specific CD8 T cells in the primary response was conserved in the pool of memory T cells (13). Strikingly, in LCMV-T1b-infected mice, the epitope hierarchy of the LCMV-specific memory T cells was drastically different from the primary CD8 T cell response (Fig. 2C). In the memory phase, note that GP276 is codominant with GP33 in LCMV-T1b-infected mice (Fig. 2C). After an acute LCMV infection, the memory CD8 T cell pool represents a “scaled down” version of the primary response; irrespective of epitope specificity, >95% of the LCMV-specific CD8 T cells are lost after day 8 PI (Figs. 1C and 2B). However, during a chronic LCMV infection, the magnitude of CD8 T cell contraction after day 8 PI seems to be epitope dependent (Figs. 1C and 2B). In LCMV-T1b-infected mice, ~90–95% of CD8 T cells specific to NP396, GP33, GP118, and NP205 epitopes were lost after day 8 PI (Figs. 1C and 2B). However, remarkably only ~70% of GP276-specific CD8 T cells were lost after day 8 PI in LCMV-T1b-infected mice (Figs. 1C and 2B). It is noteworthy that, in the above-described experiments, mice were infected with different doses of LCMV-Arm and LCMV-T1b (2 × 10^3 and 2 × 10^6 PFU of LCMV-Arm and LCMV-T1b, respectively). We questioned whether infection of mice with a “low dose” (2 × 10^6 PFU) of LCMV-T1b would affect the immunodominance hierarchy among LCMV-specific CD8 T cells during the primary and memory
phases of the T cell response. Consistent with published data (25), despite being infected with a lower dose, LCMV-T1b established a chronic infection (data not shown). Similar to data shown in Figs. 1 and 2, this “low-dose” LCMV-T1b infection also led to alteration in the epitope hierarchy among LCMV-specific CD8 T cells in the memory pool, as compared with the primary response (data not shown).

In addition to the prominent alterations in epitope hierarchy, memory CD8 T cells generated after an LCMV-T1b infection were functionally different from memory CD8 T cells in LCMV-Arm-infected mice. Fig. 2A shows that memory CD8 T cells in LCMV-Arm-infected mice produced high levels of IFN-γ, irrespective of epitope specificity. The mean fluorescence intensity of staining for IFN-γ in CD8 T cells from LCMV-Arm-infected mice was generally above 200. In stark contrast, memory CD8 T cells in LCMV-T1b-infected mice produced significantly lower amounts of IFN-γ, and the mean fluorescence intensity of staining for IFN-γ ranged between 95 and 153. CD8 T cells from LCMV-T1b-infected mice also produced lower amounts of TNF-α, as compared with CD8 T cells from LCMV-Arm-infected mice (data not shown). It is worth emphasizing that infectious LCMV-T1b was below detection levels at the time of this analysis for IFN-γ production. Therefore, the observed reduction in IFN-γ production by CD8 T cells from LCMV-T1b-infected mice is distinct from the “stunned” phenotype (functional unresponsiveness) of CD8 T cells, which is associated with high viral load (16, 26).

To further characterize cytokine production, we determined the activation threshold of memory CD8 T cells in LCMV-Arm- and LCMV-T1b-infected mice by measuring IFN-γ production as a function of peptide concentration (day 150 PI). As shown in Fig. 3, LCMV-specific CD8 T cells from both LCMV-Arm- and LCMV-T1b-infected mice were exquisitely sensitive to antigenic stimulation, and the number of IFN-γ-producing cells varied in a dose-dependent manner. The activation threshold of memory CD8 T cells from LCMV-T1b-infected mice was similar to those of LCMV-Arm-infected mice (Fig. 3). These data showed that alterations in epitope hierarchy in LCMV-T1b-infected mice were not associated with any detectable changes in the sensitivities of LCMV-specific memory CD8 T cells to antigenic stimulation in vitro.

Expression of Bcl-2, Bcl-3, and Bcl-6 in Ag-specific CD8 T cells during an acute and chronic LCMV infection

Elegant studies have shown that survival of memory T cells might be dependent upon the induction of antiapoptotic molecules like Bcl-2, Bcl-3, and Bcl-6 (19, 27–28). It was of interest to determine whether epitope-specific differences in the magnitude of CD8 T cell contraction during a chronic LCMV infection were related to the expression patterns of Bcl-2, Bcl-3, and Bcl-6 molecules. The expression of Bcl-2, Bcl-3, and Bcl-6 in LCMV-specific CD8 T cells was determined on day 8 PI, a time point that precedes the onset of the contraction phase of the CD8 T cell response. As shown in Fig. 4A, interestingly, the levels of Bcl-2 expression in...
LCMV-specific CD8 T cells in LCMV-T1b-infected mice were consistently higher than in LCMV-Arm-infected mice. However, Bcl-2 expression in CD8 T cells did not significantly differ between epitopes in either LCMV-Arm- or LCMV-T1b-infected mice. It is worth pointing out that Bcl-2 levels in activated LCMV-specific CD8 T cells in both LCMV-Arm- and LCMV-T1b-infected mice were lower than in CD8 T cells from uninfected naive mice (Fig. 4C). Fig. 4B illustrates that Bcl-3 was readily detected in LCMV-specific CD8 T cells from both LCMV-Arm- and LCMV-T1b-infected mice. However, the levels of Bcl-3 in virus-specific CD8 T cells were similar between LCMV-Arm- and LCMV-T1b-infected mice. Additionally, epitope specificity of CD8 T cells did not affect the expression levels of Bcl-3 in LCMV-Arm- or LCMV-T1b-infected mice. It is noteworthy that, unlike Bcl-2, Bcl-3 levels in CD8 T cells from uninfected naive mice and activated LCMV-specific CD8 T cells were comparable (Fig. 4C). The expression of Bcl-6 was below the level of detection in LCMV-specific CD8 T cells (data not shown). In summary, the expression levels of Bcl-2 and Bcl-3 were not affected by antigenic specificity in LCMV-Arm- or LCMV-T1b-infected mice.

**FIGURE 3.** Activation threshold of memory CD8 T cells after an acute or a chronic LCMV infection. One hundred fifty days after infection with LCMV-Arm or LCMV-T1b, splenocytes were stimulated in vitro with various LCMV CTL epitope peptides at the indicated concentrations, and the number of IFN-γ-producing CD8 T cells was determined by intracellular cytokine staining. The results are expressed as percent of maximum response attained at saturating peptide concentration (2 μg/ml). The plotted data are derived from individual mice infected with LCMV-Arm or LCMV-T1b and are representative of two independent experiments.

**FIGURE 4.** Expression of Bcl-2 and Bcl-3 in virus-specific CD8 T cells after an acute or a chronic LCMV infection. Eight days after infection with LCMV-Arm or LCMV-T1b, splenocytes were stimulated in vitro with LCMV CTL epitope peptides. After culture, cells were stained for cell surface CD8, intracellular IFN-γ, and Bcl-2 (A) or Bcl-3 (B). The histograms are gated on IFN-γ-producing CD8 T cells specific to the indicated epitopes. Splenocytes from naive uninfected mice were stained as controls (C). The dotted lines show staining with an isotype control Ab, and the bold lines represent staining with the Ab against Bcl-2 or Bcl-3. The data are representative of three mice per group from one of two independent experiments. The numbers in A and C are the mean fluorescence intensities of the staining for Bcl-2 ± SD.
FIGURE 5. Surface phenotype of LCMV-specific memory CD8 T cells in an acute and a chronic infection. One hundred twenty days after infection with LCMV-Arm or LCMV-T1b, splenocytes were stained with anti-CD44, anti-CD62L Abs, CCL19-Fc, and MHC-I tetramers. A, Dot plots are gated on the indicated MHC-I tetramer-binding CD8 T cells; the numbers represent percentages of CD62L⁺ and CD62L⁻ among tetramer-binding CD8 T cells. The histograms in B are gated on the indicated MHC-I tetramer-binding CD8 T cells. The numbers in the histograms are the percentages of CCL19-Fc⁺ cells of MHC-I tetramer-binding CD8 T cells. The bold lines in the histogram represent specific staining with CCL19-Fc. The dotted lines in the histograms represent CCL19-Fc staining on LCMV-specific CD8 T cells that were preincubated with recombinant soluble CCL19 before addition of CCL19-Fc fusion protein. The data are representative of six mice per group.

Surface phenotype of memory CD8 T cells after an acute or chronic LCMV infection

We next looked for phenotypic differences in LCMV-specific memory CD8 T cells between LCMV-Arm- and LCMV-T1b-infected mice. Previous studies have shown that short-term TCR signaling induces rapid loss of CD62L expression followed by re-expression within 24–48 h (29). However, extended TCR stimulation might lead to transcriptional silencing of the CD62L locus and reduced expression of cell surface CD62L for long periods (29). On day 8 PI, at the peak of the T cell response, all of the LCMV-specific CD8 T cells in both LCMV-Arm- and LCMV-T1b-infected mice expressed greatly reduced levels of cell surface CD62L, as compared with naive T cells (data not shown). However, differences were notable in the proportions of CD62L-expressing memory CD8 T cells between LCMV-Arm- and LCMV-T1b-infected mice (Fig. 5A). Whereas 60–70% of LCMV-specific memory CD8 T cells in LCMV-Arm-infected mice expressed CD62L, only 20–40% of memory CD8 T cells were CD62L⁻ in LCMV-T1b-infected mice. These data suggested that CD62L expression on activated CD8 T cells might be regulated by duration of antigenic stimulation in vivo. However, it is worth noting that LCMV-specific memory CD8 T cells both LCMV-Arm- and LCMV-T1b-infected mice were uniformly CD44^high (Fig. 5A).

Based on the expression of CCR7, memory T cells have been classified into “central memory” and “effector memory” (30). According to this paradigm, central memory CD8 T cells express the chemokine receptor CCR7, lack immediate effector function, and traffic through the secondary lymphoid tissues. In contrast, effector memory T cells lack CCR7, but possess immediate effector function and traffic through extralymphoid tissues (30). A recent report has challenged this paradigm of classifying memory T cells into central memory and effector memory based on CCR7 expression (31). We were interested to examine whether a chronic viral infection would affect CCR7 expression on LCMV-specific memory CD8 T cells. The expression of CCR7 on CD8 T cells was determined by flow cytometry using CCL19-Fc fusion protein (20). CCR7 is the only known receptor for CCL19. Data in Fig. 5B show the levels of CCR7 expression on LCMV-specific CD8 T cells, as measured by binding to CCL19. The specificity of CCL19-Fc binding was confirmed by preincubating cells with recombinant chemokine CCL19 before staining with CCL19-Fc. The staining of CD8 T cells was completely blocked by preincubation with soluble CCL19, which illustrated that staining was most likely a consequence of binding to CCR7 (20). Data in Fig. 5B show that CCR7 expression was detected in ~60% of LCMV-specific memory CD8 T cells in LCMV-Arm-infected mice. The proportion of CCR7 expressing LCMV-specific memory CD8 T cells was slightly lower (ranged from 40–50%) in LCMV-T1b-infected mice, as compared with LCMV-Arm-infected mice.

Effect of viral dose on epitope hierarchy of CD8 T cell responses to LCMV

Next, we investigated the effect of viral dose on epitope hierarchy of the primary and memory pool of virus-specific CD8 T cells by infecting C57BL/6 mice with three different doses of LCMV-Arm by i.v. injection: 2 x 10⁴ PFU (low dose), 2 x 10⁵ PFU (medium dose), or 2 x 10⁶ PFU (high dose). As shown in Fig. 6, in mice infected with a low or medium dose of LCMV-Arm, the epitope hierarchy of the primary and memory CD8 T cell pool was GP33 > NP396 > GP276. In striking contrast, in high-dose-infected mice, the epitope hierarchy of the primary and memory CD8 T cell pool was different (Fig. 6). In high-dose LCMV-Arm-infected mice, the epitope hierarchy of the primary CD8 T cell response was GP33 > NP396 > GP276 (Fig. 6), but during the memory phase it changed to GP33 > GP276 > NP396 (Fig. 6). Furthermore, although not dramatic, it is worth noting that the percentages of GP276-specific memory CD8 T cells in the spleen increased in a virus dose-dependent fashion. These data suggested that the viral dose might play an important role in the emergence of GP276 as a codominant epitope during an acute viral infection.

Role of IFN-γ/IFN-γR interactions in regulating epitope hierarchy during acute and chronic LCMV infection

Several steps in the pathway of Ag processing and presentation are sensitive to alterations by effects of cytokines like IFN-γ and TNF-α (11). IFN-γ and TNF-α affect Ag processing and presentation by up-regulating the expression of molecules like TAP, MHC-I, and proteosomal subunits (11). Loss of IFN-γ has been shown to alter the immunodominance of the primary CD8 T cell response to Listeria monocytogenes (LM) in mice (32). Furthermore, DNA immunization-induced alterations in immunodominance during an acute LCMV infection are regulated by IFN-γ (33). In this study, we investigated the role of IFN-γ/IFN-γR interactions in regulating the epitope hierarchy of the CD8 T cell responses during an acute or a chronic LCMV infection. To determine the role of IFN-γ in regulating the CD8 T cell responses to an acute viral infection, groups of wild-type C57BL/6 (+/+) and IFN-γR⁻/⁻ mice were infected with LCMV-Arm. On day 8
IFN-γ cytotoxic CD8 T cells in the spleens of infected mice were quantitated by intracellular cytokine staining on days 8 (primary) and 40 (memory) PI. The data are mean of three independent experiments (three mice per group).

**FIGURE 6.** Effect of virus dose on the immunodominance hierarchy of CD8 T cell response to LCMV. C57BL/6 mice were infected with the indicated doses of LCMV-Arm, and virus-specific CD8 T cell responses were quantitated by intracellular cytokine staining on days 8 (primary) and 36 (memory) PI. The dot plots are gated on viable splenocytes, and the numbers represent percentages of epitope-specific CD8 T cells of total splenocytes. Data are representative of two independent experiments (three mice per group).

**FIGURE 7.** Effect of IFN-γR deficiency on CD8 T cell responses to an acute LCMV infection. Groups of wild type (+/+) and IFN-γR-deficient (IFN-γR−/−) mice were infected with LCMV-Arm, and the number of CD8 T cells specific to the indicated epitopes was determined by intracellular staining for IFN-γ on days 8 (primary) and 40 (memory) PI. A. Dot plots of IFN-γ-producing CD8 T cells from IFN-γR−/− mice; dot plots are gated on total viable splenocytes. The numbers are the percentages of IFN-γ-producing CD8 T cells that are specific to the indicated epitopes among total splenocytes. B. Total number of epitope-specific CD8 T cells in the spleens of +/+ and IFN-γR−/− mice on day 8 (primary) and day 40 (memory) PI. The data are mean of three mice per group at each time point and are representative of three independent experiments.

PI, primary LCMV-specific CD8 T cell responses were analyzed in the spleen using intracellular staining for IFN-γ. As expected, LCMV-Arm-infected +/+ mice exhibited potent CD8 T cell responses. The epitope hierarchy of the anti-LCMV CD8 T cell response in +/+ mice was GP33 > NP396 > GP276 > NP205 (Fig. 7B), which was identical with the data shown in Fig. 1B. Upon LCMV-Arm infection, IFN-γR−/− mice mounted robust CD8 T cell responses directed against both dominant and subdominant LCMV CTL epitopes (Fig. 7, A and B). IFN-γ receptor deficiency had minimal effects on the epitope hierarchy of the primary anti-LCMV CD8 T cell response. The immunodominance hierarchy of the primary LCMV-specific CD8 T cell response in IFN-γR−/− mice was NP396 > GP33 > GP276 > GP118 > NP205 (Fig. 7). However, it is noteworthy that the expansion of LCMV-specific CD8 T cells (primary response) was significantly impaired in IFN-γR−/− mice, as compared with +/+ mice (Fig. 7B). To examine the importance of IFN-γ in regulation of immunodominance hierarchy among the pool of LCMV-specific memory CD8 T cells, +/+ and IFN-γR−/− mice were infected with LCMV-Arm, and CD8 T cell responses were analyzed 40 days later. As expected, the immunodominance hierarchy among LCMV-specific memory CD8 T cells in +/+ mice was GP33 > NP396 > GP276 > NP205 > GP118 (Fig. 7B), which was the same as the primary response. On day 40 PI, memory CD8 T cells specific to all of the LCMV CTL epitopes were readily detected in the spleens of IFN-γR−/− mice (Fig. 7A). As shown in Fig. 7, A and B, strikingly, in IFN-γR−/− mice the epitope hierarchy of the memory CD8 T cells (day 40 PI) was different from that of the primary CD8 T cell response (day 8 PI). The immunodominance hierarchy among LCMV-specific memory CD8 T cells in IFN-γR−/− mice was GP33 > NP396 > GP276 > GP118 > NP205 (Fig. 7, A and B). In contrast with the primary response, GP33 is clearly dominant over all of the epitopes and, remarkably, GP276 and NP396 have become “equidominant” in the memory T cell
Discussion

It is well recognized that polyclonal CD8 T cell responses in several viral infections of humans and mice are directed against immunodominant and subdominant epitopes. The regulatory mechanisms underlying the immunodominance hierarchy of the CD8 T cell response is a subject of intense investigation because of its implications in vaccine development and in immunotherapy of patients with cancer or chronic viral infections. In this manuscript, we have documented the effect of acute vs chronic LCMV infection on the epitope hierarchy during primary and memory phases of the CD8 T cell response. Furthermore, we have investigated for the first time the role of IFN-γ/IFN-γR interactions in regulating the immunodominance hierarchy of the virus-specific CD8 T cell response in an acute or a chronic LCMV infection.

As mentioned before, it is well documented that the epitope hierarchy of the primary CD8 T cell response is conserved during the phase of memory in a setting of acute viral infection (12, 13). In this study, we confirmed these findings and extended our knowledge about regulation of immunodominance hierarchy during chronic viral infection. Our studies showed that, unlike an acute LCMV infection, the immunodominance hierarchy of the primary response is not maintained in the memory CD8 T cell pool in mice that have resolved a chronic infection. After attaining a peak on day 8 PI, CD8 T cells undergo a contraction phase when large numbers of LCMV-specific CD8 T cells are eliminated, presumably by apoptosis (13). Our studies show that during a chronic LCMV infection, the contraction of GP276-specific CD8 T cells seems to be substantially attenuated, as compared with CD8 T cells specific to other LCMV CTL epitopes. Therefore, the alteration in the immunodominance hierarchy during a chronic LCMV infection is likely to result from differential susceptibility of CD8 T cells to deletion mechanisms based on epitope specificity. It was recently reported that the contraction phase of the CD8 T cell response might be programmed early after infection (35). Our studies suggest that programmed contraction of the CD8 T cell response during a chronic LCMV infection might be epitope dependent. Why is immunodominance hierarchy changing during the course of a chronic LCMV infection and not after an acute infection? Regulation of immunodominance in CD8 T cell responses is complex and might be affected by multiple factors, including the affinity of the antigenic peptide to the MHC, efficiency of processing of the antigenic epitope, temporal sequence of protein expression in infected cells, relative abundance of protein Ag in the APC, nature of APC, the presence of costimulatory interactions between
T cell and APC. T cell repertoire, and clonal competition between T cells to interact with the APC (11). In the context of the present study, the factors that could be affecting the immunodominance are the antigenic load and/or the duration of infection. During an acute LCMV infection, it is possible that CD8 T cells specific to the dominant epitopes respond rapidly and reduce the antigenic load, thereby limiting the activation and expansion of subdominant epitope-recognizing CD8 T cells. However, during a chronic infection, contraction of CD8 T cells specific to the dominant epitopes might provide opportunities for activation and expansion of subdominant epitope-specific CD8 T cell clones. The alteration in epitope hierarchy seen in the memory phase after a chronic infection and non acute infection might be due to differences in cell tropism between LCMV-Arm and LCMV-T1b (25). However, increasing the dose of LCMV-Arm resulted in a significant change in the CD8 T cell epitope hierarchy of the memory T cell pool. Taken together, our studies show that the viral dose and/or duration of infection are critical parameters that regulate immunodominance hierarchy of the memory CD8 T cell pool during an acute LCMV infection. While this manuscript was in preparation, it was reported that persistently infected perforin-deficient mice (per−/−) exhibit alteration in epitope hierarchy during the course of a persistent infection (36). Although this report is consistent with our data, the mechanisms underlying the alteration in immunodominance hierarchy in persistently infected per−/− mice could be different. Our results on changing immunodominance during a chronic LCMV infection are largely in agreement with another recent report (37). The kinetics of Ag-specific CD8 T cell responses have also been studied in mice persistently infected with murine cytomegalovirus (38). Unlike in our studies, murine cytomegalovirus-infected mice do not show altered immunodominance but tend to accumulate virus-specific CD8 T cells over time.

Several studies have documented functional impairment of CD8 T cells during persistent viral infections of mice and humans (8, 16, 26, 36, 37). In these studies, the dysfunctional status of the CD8 T cells was associated with conditions of high viral load in the infected host. Several LCMV strains can cause a chronic infection in immunocompetent mice, but the duration of infection varies from one strain to another (21–23, 25). Unlike LCMV-T1b, which is resolved in ~30 days, infection of mice with clone 13 strain of LCMV (LMV-clone 13) results in a protracted infection lasting up to 6 mo (21). Both LCMV-T1b and LCMV-clone 13 were isolated from the lymphoid tissues of LCMV carrier mice infected at birth with LCMV-Arm (22, 23, 25), and both exhibit >99% genetic identity (23). Using LCMV-clone 13, it was recently reported that high viral load in the tissues is associated with the failure of a large proportion of virus-specific CD8 T cells to produce detectable levels of IFN-γ, TNF-α, and IL-2 (37). This state of impairment in cytokine producing ability is termed as “full functional exhaustion.” In LCMV-clone 13-infected mice, in some instances, this functional exhaustion can be incomplete (partial exhaustion), wherein CD8 T cells are able to produce low levels of IFN-γ but are unable to produce TNF-α and IL-2 (37). In the present study, the functional impairment of CD8 T cells seen in LCMV-T1b-infected mice is distinct from the partially exhausted phenotype seen in LCMV-clone 13-infected mice. In LCMV-T1b-infected mice, CD8 T cells were able to produce both IFN-γ and TNF-α, albeit at lower levels, as compared with LCMV-Arm-infected mice. Therefore, the functional status of CD8 T cells in LCMV-T1b-infected mice might reflect a “milder” form of partial exhaustion. It is worth emphasizing that, unlike the LCMV-clone 13 study, which was done in animals with high viral load (37), our experiments were performed several months after viral clearance. Hence, it is possible that CD8 T cells with a similar functional phenotype (milder exhaustion) can be detected in LCMV-clone 13-infected mice several months after viral clearance. Nonetheless, our findings show that chronic antigenic stimulation could lead to long-term functional defects in memory CD8 T cells. Further studies to investigate the processes of TCR signaling and cytokine gene transcription and translation in CD8 T cells from LCMV-T1b-infected mice might shed some light on the underlying defect in cytokine production.

We also examined whether memory CD8 T cells generated after the clearance of a chronic LCMV infection were phenotypically different from memory CD8 T cells in acutely infected mice. In our studies, memory CD8 T cells generated after an acute or chronic LCMV infection were uniformly CD44high. Occasionally, NP396-specific memory CD8 T cells showed slightly reduced expression of CD44 in LCMV-T1b-infected mice, as compared with LCMV-Arm-infected mice. In contrast with our studies, virus-specific CD8 T cells in LCMV-clone 13-infected mice consistently expressed low levels of CD44, as compared with LCMV-Arm-infected mice (37). The underlying mechanisms and biological significance of altered CD44 expression in chronic LCMV infections needs further investigation. Interestingly, in the present study, there were notable differences in the fraction of CD62L expressing LCMV-specific memory CD8 T cells between acute and chronic infections. Although a large fraction of memory CD8 T cells in acutely infected mice were CD62Lhigh, the majority of memory CD8 T cells were CD62Llow after chronic infection. At the peak of the T cell response (day 8 PI) in both acute and chronic LCMV infection, LCMV-specific CD8 T cells are CD62Llow (data not shown). After viral clearance, these CD62Llow cells gradually revert to a CD62Lhigh phenotype (39). The rate of reversal of CD8 T cells from a CD62Llow to a CD62Lhigh phenotype seems to be determined by the duration and magnitude of antigenic stimulation (39). The persistence of CD62Llow phenotype of memory CD8 T cells in mice that have recovered from a chronic infection is reminiscent of prolonged antigenic stimulation in LCMV-T1b-infected mice, as compared with acutely infected mice. In acutely infected mice, there was an excellent correlation between CCR7 and CD62L expression on memory CD8 T cells. However, in mice that have recovered from a chronic infection, there was no correlation between CCR7 and CD62L expression in LCMV-specific memory CD8 T cells.

Similar to LCMV infection, the CD8 T cell response to intracellular bacteria LM is also directed against dominant and subdominant epitopes (32). In LM infection of mice, the immunodominance hierarchy of the primary CD8 T cell response seems to be regulated by IFN-γ (32). Under conditions of IFN-γ deficiency, in LM-infected mice, the CD8 T cells recognizing the subdominant epitope exhibit increased expansion reaching levels comparable to that of the dominant epitope and subsequent loss of epitope hierarchy (32). These data suggest that IFN-γ could suppress the activation and expansion of subdominant epitope-specific CD8 T cells, at least in the LM infection of mice. In striking contrast to this report, our studies showed little to no alteration in the immunodominance hierarchy of the primary CD8 T cell response to LCMV in IFN-γ−/− mice, as compared with +/+ mice. The differences in pathogenesis (cell tropism, antigenic load, temporal pattern of Ag expression in APCs, kinetics of CD8 T cell activation, the role of factors of innate immunity, etc.) between LM and LCMV infection might dictate the sensitivity of the immunodominance hierarchy of the primary CD8 T cell response to regulation by IFN-γ. In LCMV-Arm-infected +/+ mice, the epitope hierarchy of the primary CD8 T cell response and the pool of memory CD8 T cells were remarkably similar. In striking contrast, in IFN-γ−/− mice, the epitope hierarchy of the
pool of LCMV-specific memory CD8 T cells was substantially altered as compared with the primary response. IFN-γ or IFN-α/β deficiency causes a slight delay in clearing an acute LCMV infection, as compared with +/+ mice (32, 40). Therefore, the observed alteration of immunodominance hierarchy among LCMV-specific memory CD8 T cells in IFN-α/β−/− mice could be a consequence of loss of IFN-γ signaling and/or longer duration of antigenic stimulation, which needs further investigation.

In summary, we have demonstrated that increasing the viral dose or duration of infection can have significant effects on the CD8 T cell responses in vivo with special reference to: 1) regulation of immunodominance hierarchy during the phase of T cell memory, 2) functional competence of memory T cells, and 3) alterations in the expression of cell surface molecules on memory CD8 T cells. These findings might have significant implications in vaccine design and targeting of epitopes for immunotherapy during chronic viral infections.

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References