Uncovering Subdominant Cytotoxic T-Lymphocyte Responses in Lymphocytic Choriomeningitis Virus-Infected BALB/c Mice

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The cytotoxic T-lymphocyte response against lymphocytic choriomeningitis virus (LCMV) in BALB/c mice is predominantly directed against a single, Ld-restricted epitope in the viral nucleoprotein (residues 118 to 126). To investigate whether any Kd/Dd-restricted responses were activated but did not expand during the primary response, we used a BALB/c mutant, BALB/c-H-2dm2, which does not express the Ld molecule. Splenocytes from LCMV-infected BALB/c mice were transferred into irradiated BALB/c-H-2dm2 mice and rechallenged with LCMV. Thus, they were exposed to an antigenic stimulus without the involvement of the immunodominant Ld-restricted epitope. In this adoptive transfer model, the donor splenocytes protected the recipient mice against chronic LCMV infection by mounting a potent Kd- and/or Dd-restricted secondary antiviral response. Analysis of a panel of Kd binding LCMV peptides revealed that residues 283 to 291 from the viral glycoprotein (GP283–291) comprise a major new epitope in the adoptive transfer model. Because the donor splenocytes were first activated during the primary infection in BALB/c mice, the GP283–291 epitope is a subdominant epitope in BALB/c mice that becomes dominant after rechallenge in BALB/c-H-2dm2 mice. This study makes two points. First, it shows that subdominant CTL responses can be protective, and second, it provides a general experimental approach for uncovering subdominant CTL responses in vivo. This strategy can be used to identify subdominant T-cell responses in other systems.

Infection of mice with the arenavirus lymphocytic choriomeningitis virus (LCMV) results in a vigorous antiviral immune response, in which CD8+ cytotoxic T lymphocytes (CTLs) are crucial for elimination of the infection (2, 7, 13, 18). CD8+ CTLs recognize short antigenic peptides associated with major histocompatibility complex (MHC) class I molecules. In BALB/c mice, the primary antiviral CTL response is almost exclusively directed against a single peptide from the LCMV nucleoprotein (NP) (residues 118 to 126 [NP118–126]; RPQASGVYM) that is presented by the Ld MHC class I molecule (26, 32). Few, if any, Dd- or Kd-restricted responses are usually detected (5, 10, 21), and the response against the Ld-restricted NP118–126 epitope is therefore considered immunodominant. However, the lack of detectable Dd- and/or Kd-restricted responses does not necessarily imply that CTLs recognizing putative Dd- and/or Kd-restricted epitopes do not exist in BALB/c mice. It is, for instance, possible that such CTLs are primed during the early stages of the antiviral response but that they do not expand because the dominant Ld-restricted response is activated more rapidly and clears the infection. In this view, decreasing levels of antigen would limit putative Dd- or Kd-restricted responses. To determine whether Dd- and/or Kd-restricted antiviral responses are indeed primed during an LCMV infection in BALB/c mice, we designed an adoptive transfer model. The BALB/c mutant c-H-2dm2 (which will be referred to as “dm2”) has a deletion in the gene for the Ld class I molecule (17, 24, 28) and cannot, therefore, present the immunodominant NP118–126 epitope. Thus, these mice offer the unique opportunity to analyze antiviral CTL responses in the absence of the immunodominant Ld-restricted response. By transferring splenocytes from acutely LCMV-infected BALB/c mice into irradiated dm2 mice, followed by an LCMV infection, a population of largely Ld-restricted antiviral BALB/c CTLs is rechallenged in the absence of the Ld class I molecule. The Ld-restricted epitope is not presented, and any subdominant, non-Ld-restricted antiviral responses present in the BALB/c splenocyte pool will be amplified when the dm2 recipients are challenged with LCMV. Here we show that under these conditions, the antiviral CTL response switches to epitopes presented by different restriction elements. This new antiviral response is effective, because the donor splenocytes protect the recipient dm2 mice against chronic LCMV infection. The BALB/c CTL response in the dm2 recipients is Dd- and/or Kd-restricted, implying that the BALB/c splenocytes harbored Dd- and/or Kd-restricted, antiviral CTL precursors. Analysis of a panel of Kd binding peptides from the viral NP and glycoprotein (GP) led to the identification of a subdominant CTL epitope that becomes dominant in the adoptive transfer model. Thus, antigenic stimulation in the absence of the Ld class I molecule induces a shift in the antiviral CTL response with non-Ld-restricted epitopes becoming dominant.

MATERIALS AND METHODS

Mice. Five- to eight-week-old female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) or bred at our colony at the University of California at Los Angeles. BALB/c-H-2dm2 mice (dm2 mice) were bred at our colony at the University of California at Los Angeles.
**Virus and cells.** LCMV Armstrong (4) and its derivative clone 13 were used in this study (1, 4, 16). Primary infections in BALB/c or dm2 mice were done by intraperitoneal (i.p.) injection of 2 × 10^6 PFU of LCMV Armstrong. For rechallenge experiments, mice were inoculated intravenously (i.v.) with 2 × 10^6 PFU of LCMV clone 13. Vero cells were used for virus titration. BALB clone 7 cells, Ld/Kd cells (L cells transfected with the Ld/Kd MHC I molecule) (11), and dm2 cells were used as target cells in 31Cr release assays. Cell lines were maintained in Eagle’s minimal essential medium (Vero cells) or RPMI medium supplemented with 10% fetal bovine serum, 4 mM l-glutamine, and antibiotics.

Adoptive transfer. dm2 mice were irradiated (550 to 600 rads) 1 day before transfer. Single-cell suspensions of splenocytes (5 × 10^7 cells) from LCMV Armstrong-infected BALB/c mice were transferred into irradiated dm2 mice by i.v. injection. Recipient mice were simultaneously infected i.v. with LCMV clone 13. Mice were sacrificed at day 8 after the transfer and rechallenge, and single-cell suspensions of splenocytes were used in 31Cr release assays to measure cytotoxic activity.

Peptides. Peptides were either synthesized at Cytel as previously described (25) or purchased as crude material from Chiron Mimotopes (San Diego, Calif.). Peptides synthesized at Cytel were purified to >95% homogeneity by reverse-phase high-performance liquid chromatography. The purity of the synthetic peptides was ascertained by amino acid analysis, sequencing, and/or mass spectrometry analysis.

**RESULTS**

**BALB/c donor splenocytes generate a protective antiviral CTL response upon transfer into irradiated dm2 mice.** The experimental model used in this study is based on the transfer of splenocytes from acutely infected BALB/c mice into irradiated dm2 mice, followed by an LCMV challenge with clone 13. dm2 mice do not express the Ld class I molecule. Therefore, the BALB/c antiviral CTLs (harboring mostly Ld-restricted effectors) are exposed to a strong antigenic stimulus that is presented by Kd/Dd-expressing (i.e., dm2) antigen-presenting cells.

To obtain donor spleen cells, BALB/c mice were first infected with 2 × 10^6 PFU of LCMV Armstrong. This infection induces a potent antiviral CTL response that is largely directed against the dominant NP_{118–126} epitope. At day 8 postinfection, 5 × 10^7 splenocytes from LCMV-infected BALB/c mice were injected i.v. into irradiated dm2 mice; at the same time, the recipient mice were challenged with 2 × 10^6 PFU of LCMV clone 13. LCMV clone 13 is an LCMV Armstrong derivative that readily establishes a persistent infection in mice, unless primed LCMV-specific CD8+ T cells in the irradiated dm2 mice were able to protect the recipients against the LCMV clone 13 challenge. We determined virus titers in sera and in the spleens of the recipient mice at day 8 after transfer and LCMV challenge. The results clearly show that recipient mice controlled the LCMV infection (Table 1). Since CD8+ CTLs are critical for viral clearance (2, 7, 13, 18), this result implies that the donor CD8+ T cells in the irradiated recipient dm2 mice were capable of mounting an effective antiviral CTL response. Control dm2 mice that were irradiated but had not received donor spleen tissue did not clear the infection, proving that the protection was completely dependent on the BALB/c donor spleen cells. Similar results were obtained with irradiated BALB/c recipients: only the mice that received donor spleen tissue could control the LCMV infection. Transfer of splenocytes from naive BALB/c mice into either BALB/c or dm2 mice did not protect the recipients against chronic infection (reference 4 and data not shown), demonstrating that primed CTLs are required for protection.

**BALB/c splenocytes mount a Dd- and/or Kd-restricted antiviral response in the recipient dm2 mice.** To investigate how the donor CTLs protected the recipients against chronic infection, we determined the class I restriction of the BALB/c CTL response after transfer and rechallenge in dm2 mice. Direct ex vivo CTL activities were measured in a standard 51Cr release assay against syngeneic LCMV-infected target cells that expressed different MHC class I molecules and, therefore, presented different epitopes. The following target cells were used: (i) BALB clone 7 cells (expressing Ld, Kd, and Dd), (ii) L cells transfected with Ld (expressing Ld), and (iii) dm2 cells (expressing Kd and Dd). As shown in Fig. 1A, BALB/c mice infected with LCMV Armstrong generate a predominantly Ld-restricted, primary antiviral CTL response at day 8 postinfection. LCMV-specific lysis of Ld cells (Ld) was comparable to killing of LCMV-infected BALB clone 7 cells (Dd Kd Ld) and lysis of LCMV-infected dm2 cells (Dd Kd Ld) was negligible (Fig. 1A). Splenocytes from these BALB/c mice were then transferred into irradiated dm2 mice, which were then challenged with LCMV clone 13. The LCMV infection induced a potent secondary CTL response from the donor splenocytes. Direct ex vivo analysis of antiviral CTL responses in the recipients at day 8 after transfer and rechallenge revealed that only the donor CTLs recognized epitopes presented by LCMV-infected BALB clone 7 and dm2 cells but no longer lysed infected L cells that presented the Ld-restricted immunodominant epitope (Fig. 1B). Thus, the BALB/c CTLs were not recognized by either Dd- or Kd-restricted epitopes instead of the Ld-restricted epitope. From this result, it can be inferred that the BALB/c donor splenocytes contain Dd- and/or Kd-restricted CTLs and that Dd/Kd-restricted responses are uncovered when the immunodominant epitope is not presented.

Normal dm2 mice infected with LCMV Armstrong generate a primary antiviral response that is also Dd- and/or Kd-restricted (Fig. 1C), confirming previous observations (10). This CTL response is relatively weak, consistent with several studies that indicate that dm2 mice eliminate virus less rapidly (10), are more susceptible to establishment of chronic infection (19), and are less susceptible to T-cell-mediated immunopathology (5). Finally, when BALB/c effectors are transferred into irradiated BALB/c mice and rechallenged with LCMV, the antiviral CTL response is, as expected, Ld restricted (data not shown).

The antiviral CTL response is directed against a subdominant, Kd-restricted epitope in the viral glycoprotein. We have...
recently identified several peptides, derived from the LCMV GP and NP, that bind the $K^d$ and $D^d$ class I molecules (30). These peptides were identified with the allele-specific sequence motifs that have been described for $K^d$ and $D^d$ binding peptides (8, 9, 22, 23), combined with sensitive MHC binding assays (27). As summarized in Table 2, this strategy yielded five peptides (8, 9, 22, 23), combined with sensitive MHC binding (IC50, 50 to 500 nM) affinity. No high- or intermediate-affinity peptide. Also, LCMV-infected and uninfected targets were received 5 similar to the one described above, irradiated dm2 mice re-

Because the antiviral response in the recipient dm2 mice switches to $K^d$, and/or $D^d$-restricted epitopes, it seemed plausible that one or more of the $K^d$ binding peptides were in fact new epitopes in the adoptive transfer model. To verify this, we studied whether any of these peptides could sensitize target cells for lysis by BALB/c effectors after their transfer and secondary stimulation in dm2 mice. In an experimental setup similar to the one described above, irradiated dm2 mice received $5 \times 10^5$ splenocytes from LCMV-infected BALB/c mice and were inoculated with LCMV clone 13. At day 8 postinfection, the recipient mice were sacrificed and the spleen cells (containing $K^d/D^d$-restricted antiviral CTLs) were used (direct ex vivo) as effector cells in a $^{51}Cr$ release assay. To determine whether the antiviral CTL would recognize any of the peptides, BALB clone 7 target cells were coated with each of the five $K^d$-restricted peptides or the $L^d$-restricted immunodominant peptide. Also, LCMV-infected and uninfected targets were included in the experiment. As a control, direct ex vivo primary antiviral CTL activities in LCMV-infected BALB/c and dm2 mice (i.e., without transfer) were measured. As shown in Fig. 2A, our results confirm that the primary antiviral activity in BALB/c mice is mainly directed against the immunodominant NP118–126 epitope (compare lysis of LCMV-infected cells versus that of NP118–126-coated cells [Fig. 2A]). When this population of effector cells is transferred and rechallenged in dm2 mice, the $L^d$-restricted immunodominant epitope NP118–126 is no longer recognized, demonstrating that the original $L^d$-re-

In this study, we provide evidence that BALB/c antiviral CTLs recognize $K^d$ and/or $D^d$-restricted viral epitopes when the $L^d$-restricted immunodominant epitope is not presented. $K^d/D^d$-restricted antiviral responses that are subdominant in BALB/c mice are uncovered in the absence of the immunodominant response and protect the recipient dm2 mice against chronic LCMV infection. The $K^d/D^d$-restricted CTLs were first activated during the primary antiviral response in BALB/c mice: without primed antiviral CD8+ CTLs, mice cannot control the strong LCMV clone 13 challenge used in this study. After transfer into dm2 mice and antigenic (re)stimulation, they expanded to levels that were readily detectable in our assays. One of the new $K^d$-restricted epitopes comprises residues 283 to 291 from the viral GP. Significant responses against this epitope were detected after adoptive transfer and rechallenge of BALB/c spleen cells, as well as in LCMV-infected dm2 mice. However, transferred splenocytes lysed LCMV-infected targets more efficiently than GP283–291-coated targets, indicating that there may be yet additional epitopes. Such other epitopes could be $D^d$ restricted, since a $D^d$-re-

### TABLE 2. LCMV GP- and NP-derived peptides conforming to the $K^d$ and $D^d$ motifs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Motif</th>
<th>Binding affinity IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP16–126</td>
<td>RPOASGVYM</td>
<td>$L^d$</td>
<td>1.3</td>
</tr>
<tr>
<td>NP114–322</td>
<td>PYSACRTSI</td>
<td>$K^d$</td>
<td>4.8</td>
</tr>
<tr>
<td>GP283–291</td>
<td>GYCLTKWMI</td>
<td>$K^d$</td>
<td>73</td>
</tr>
<tr>
<td>GP99–108</td>
<td>HYNSMGTGL</td>
<td>$K^d$</td>
<td>85</td>
</tr>
<tr>
<td>GP35–43</td>
<td>VYNFATCGI</td>
<td>$K^d$</td>
<td>220</td>
</tr>
<tr>
<td>GP442–350</td>
<td>LFKTTLVSNL</td>
<td>$K^d$</td>
<td>314</td>
</tr>
<tr>
<td>GP44–51</td>
<td>FALISFELL</td>
<td>$D^d$</td>
<td>1,194</td>
</tr>
</tbody>
</table>

*As described previously (30), LCMV NP and GP amino acid sequences were screened for motif-fitting peptides. Peptides were synthesized and tested for MHC binding. Binding affinities are expressed as IC50.

1 Immunodominant NP118–126 epitope.
major epitope in the adoptive transfer model and in dm2 mice is consistent with our previous study in which we showed (i) that GP\textsubscript{283–291} and GP\textsubscript{99–108} are subdominant epitopes in BALB/c mice, (ii) that the response against GP\textsubscript{99–108} was less vigorous than the response directed against GP\textsubscript{283–291} after secondary in vitro stimulation with peptide, and (iii) that immunization with GP\textsubscript{283–291} protected BALB/c mice against chronic infection (30). In the adoptive transfer model described in this paper, the hierarchy found is slightly different: responses against GP\textsubscript{283–291} are clearly dominant, and responses against GP\textsubscript{99–108} can hardly be detected, demonstrating that the difference between GP\textsubscript{283–291} and GP\textsubscript{99–108} is magnified. In addition, whereas our previous report established the existence of CTLps specific for the subdominant epitopes (30), the present study describes a subdominant effector response and defines conditions under which such subdominant responses become dominant.

Allan and Doherty first showed that the absence of the L\textsuperscript{d} molecule in dm2 mice did not prevent T-cell-mediated immunopathology following intracerebral LCMV inoculation (5). They concluded that dm2 mice mounted a weak antiviral CTL response that is K\textsuperscript{d} and to a lesser extent D\textsuperscript{d} restricted. More recently, Gegin and Lehmann-Grube showed that dm2 mice could control an LCMV infection and generated a weak K\textsuperscript{d}/D\textsuperscript{d}-restricted antiviral CTL response (10). Here, we confirm these previous findings and extend them by identifying one of the major epitopes in dm2 mice. However, Gegin and Lehmann-Grube also observed that transfer of LCMV-specific CTLs (mostly L\textsuperscript{d} restricted) from BALB/c mice into dm2 mutant mice resulted in viral clearance from dm2 mice in the absence of any K\textsuperscript{d}- or D\textsuperscript{d}-restricted CTL effectors and they concluded that killing per se was not essential for clearance of LCMV infection (10). Our study shows very clearly that subdominant CTL effectors are substantially enhanced and readily detectable in this adoptive transfer model. These different results could be due to the fact that in our study, the recipient dm2 mice were infected with 2 × 10\textsuperscript{6} PFU of LCMV clone 13, which provides a much stronger antigenic stimulation than the challenge used by Gegin and Lehmann-Grube (10\textsuperscript{3} PFU of LCMV WE). In addition, we analyzed antiviral CTL responses at day 8 after transfer, whereas Gegin and Lehmann-Grube measured CTL responses at 48 h after transfer (10).

In the adoptive transfer model, CTLs recognizing GP\textsubscript{283–291}
were primed by LCMV in BALB/c mice. As shown previously, GP<sub>283-291</sub> specific CTLs could also be primed by peptide immunization (30). GP<sub>283-291</sub> specific CTLs appear to be (at least in part) responsible for viral clearance in dm2 mice (this study) and provide protection against an LCMV challenge after peptide immunization (30). These findings are particularly interesting in the light of future studies of therapeutic vaccination, i.e., immunization to fight an established, chronic infection. One of the most striking characteristics of a chronic LCMV infection is that the antiviral CD8<sup>+</sup> CTLs disappear before the virus is cleared (4, 6, 20). This is thought to result (in part) from the exhaustion (deletion) of LCMV-specific CTLs: mass- antigenic stimulation activates all virus-specific CTLs, leading to anergy and death of these CTLs (12, 20). Because subdominant epitopes do not play a major role in the primary response in BALB/c mice (Fig. 2A), immunization with a subdominant epitope, such as GP<sub>283-291</sub>, might be used to trigger a new antiviral response in chronically infected animals.

Our data show that in the absence of the MHC class I molecule that presents the immunodominant epitope, the antiviral LCMV CTL response can be “forced” towards different epitopes. The plasticity of the H-2<sup>d</sup>-restricted antiviral CTL response has also been demonstrated by von Herrath et al., who developed transgenic mice expressing the LCMV NP<sub>1</sub> gene in the thymus (31). These mice did not generate any high-affinity NP-specific CTLs but instead generated a weak H-2<sup>d</sup>-restricted antiviral CTL response to one or more epitopes in the N-terminal part of the viral GP. It would be interesting to analyze CTL responses against our set of peptides in the transgenic mouse model.

In conclusion, our data demonstrate that in vivo activation of subdominant LCMV epitopes can occur, that CTLs directed against these epitopes are capable of controlling infection, and that epitopes that were previously subdominant can become dominant under different experimental conditions or in a different cellular environment.

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