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Cutting Edge: MHC Class II-Restricted Killing In Vivo during Viral Infection

Evan R. Jellison, Sung-Kwon Kim, and Raymond M. Welsh

Class II-restricted CD4 T cell-mediated killing of target cells has previously been documented in vitro but not in vivo. In this study, we demonstrate CD4-dependent MHC class II-restricted killing in lymphocytic choriomeningitis virus-infected mice in vivo using an in vivo cytotoxicity assay that features class II-expressing B cells as targets. The Journal of Immunology, 2005, 174: 614–618.

The ability of “killer” CD8+ T cells to lyse virus-infected cells has been well characterized in vitro and in vivo, but the “helper” CD4+ T cell subset is usually associated with the production of cytokines and with the provision of secondary activation or survival signals to APCs, B cells, and CD8 T cells. Some 20 years ago, however, CD4 T cells cultivated in vitro were found to have cytolytic potential and to lyse targets in an MHC class II-dependent manner (1–4). Cytotoxic CD4 T cell clones have been generated, for example, against influenza virus in the mouse and against measles virus, HIV, and EBV in humans. For a period of time it was uncertain whether CD4 T cell cytotoxicity was an artifact of long-term in vitro culture (5), but eventually CD4 killer cell activity was found immediately ex vivo in cells that had been activated in vitro with keyhole limpet hemocyanin (6) or during HIV infection (7). In contrast to the killing mediated by CD8 T cells, however, CD4 T cell-mediated killing has not been convincingly shown to occur in vivo.

In vitro cytotoxicity studies have suggested that CD4 killer T cells, when present, may employ similar mediators of cytotoxicity as CD8+ CTL, using TNF-α (8), Fas ligand (FasL) (9), and perforin-dependent mechanisms (10). Initially, it was thought that CD4 T cell killing was mediated exclusively by TNF-α or FasL and that perforin was only expressed in CD8 T cells and NK cells. However, CD4 T cell clones were eventually shown to sometimes express perforin (11), and ex vivo studies from HIV-infected patients showed that CD4-mediated cytotoxicity could occur through a granule exocytosis mechanism involving perforin (7). Other mechanisms may also be involved. Two studies have reported the involvement of TRAIL in the killing of tumor cells by cytototoxic CD4 T cells (12, 13). It is also notable that CD4 killer T cell activity in vitro has been described as a potential function of CD4+ CD25+ regulatory T cells (14).

Studies by Lukacher et al. (2) showed that, after adoptively transferring an influenza-specific CD4 T cell clone into mice infected with two different strains of influenza virus, only the strain of virus recognized by the clone was cleared. This was taken as evidence that the antiviral effect of the clone may not involve release of soluble mediators but instead was due to exquisite recognition of cells expressing the cognate ligand, consistent with a direct cytotoxicity mechanism. What remains, however, is a clear demonstration of Ag-dependent, class II-dependent CD4 T cell-mediated killing in vivo.

In the present study, we provide definitive evidence for MHC class II-restricted killing in vivo by doing in vivo cytotoxicity assays in lymphocytic choriomeningitis virus (LCMV)-infected C57BL/6 mice (15). The LCMV infection stimulates strong CD8 and CD4 T cell responses against a number of defined MHC class I- (16, 17) and MHC class II- (18) presented epitopes. The two MHC class II I-A-restricted epitopes for LCMV are NP309–328 and GP61–80 (18), and up to 10% of CD4 T cells can be specific to these epitopes during an acute infection (19). Because of this high frequency, we reasoned that the LCMV-infected mouse might be a good system to demonstrate MHC class II-restricted killing in vivo, if indeed it does exist.

Materials and Methods

Mice and infections
C57BL/6J, Tnfsf14Mtmgtg (TNFR-1–/– p55–/–), and B6Smn.C3-Tnfsf6gld mice were purchased from The Jackson Laboratory. B6.SJL (Ly5.1) mice were purchased from Taconic Farms. B6.129P2-TCRB (aβTCR–/–), B6.MRL-Tnfrsf6gld (lpr), and B6 × 129 TNF-α–/– and +/+ mice were bought from The Jackson Laboratory and have been maintained in our animal colony. Mice were inoculated with 4 × 10^3 PFU of LCMV Armstrong i.p.

In vivo cytotoxicity assay

In vivo cytotoxicity was determined using B6 splenocytes coated with peptide epitopes and differentially labeled with the fluorescent dye CFSE (Molecular Probes) as previously described (15). Splenocytes from naive mice were separated and labeled with indicated peptides (0.5 μg/ml; 45 min at 37°C, 5%
CO2). Populations were then labeled with different concentrations of CFSE (1 and 0.4 μM for two targets or 1, 0.4, and 0.15 μM for three targets), combined, and injected i.v. into mice. Spleen lymphocytes were isolated 18 h later, and the ratio of CFSElow/CFSEhigh cells was determined by flow cytometry. To identify the type of target cells for some assays, surface stains were performed as described below using fluorescently labeled mAbs specific for Ly5.1 (A20), I-Ab (AP6-120.1), B220 (RA3-6B2), and CD19 (1D3), all purchased from BD Pharmingen. The percentage of specific killing was calculated as follows: (1 − ratio immune/ratio naive) × 100. Ratio = number of events LCMV peptide-coated target/number of events reference target. GP61–80 peptide has the amino acid sequence GLNGPDIYKGVYQFKSVEFD; NP 309–328 is SGEGWPIYACTRTSVVGRWE; and HA192–207 (20) is SLYVQASGRV TVSTRR (D. Woodland, personal communication).

Intracellular staining

Single-cell lymphocyte suspensions were prepared from spleens. The erythrocytes were lysed using a 0.84% NH4Cl solution. LCMV peptide-specific, IFN-γ-secreting CD4+ and CD8+ T cells were detected using the CytoTox/CytoPerm Kit Plus (with GolgiPlug; BD Pharmingen), as described previously (21). Briefly, cells were incubated with 5 μM synthetic peptide, 10 U/ml human rIL-2 (BD Pharmingen), and 0.2 μl of GolgiPlug for 5 h at 37°C. To visualize overall functionality of CD4+ and CD8+ T cells, cells were incubated with 1 μg of purified anti-mouse CD3ε mAbs (145-2c11; BD Pharmingen), following preincubation with 1 μg of Fc block (2.4G2) in 96-well plates containing 100 μl of FACS buffer (HBBS, 2% FCS, and 0.1% NaN3), the cells were stained for 30 min at 4°C with combinations of fluorescently labeled mAbs specific for CD4 (L3T4), CD8α (53-6.7), or CD8β (Ly-3) and IFN-γ (XMG1.2), all purchased from BD Pharmingen, and for granzyme B (GB12), purchased from Caltag Laboratories. freshly stained samples were analyzed using a BD Biosciences LSRII and FACS Diva Software or FACSCalibur and CellQuest Pro Software.

Cell depletion

CD4+ cells were depleted in vivo by i.p. inoculation of 0.5 mg of mAb GK1.5 (22) on both 2 and 1 days before target cell transfer. Depletion of CD4+ T cells was confirmed by cell surface stain for L3T4. CD8α cells were depleted using 2.5 mg mAb Lyt-2 (2.43; Ref. 23) on both 5 and 1 days before target cell transfer. Depletion of CD8+ T cells was confirmed by cell surface stain for CD8β (Ly-3). mAb GK1.5 (anti-CD4) was given to us by Dr. K. Rock (University of Massachusetts Medical School, Worcester, MA) and mAb Lyt-2 (2.43) was prepared by us from ascites.

Results and Discussion

MHC class II-restricted killing in vivo

We first examined killing of GP61-coated targets in vivo by transferring Ly5.1+ congenic C57BL/6 (Ly5.2) mice. After 20 h in vivo, ~52% of the MHC class IIhigh donor cells (lymphocyte gated) labeled with GP61 peptide were lost when compared with the reference influenza A HA192 peptide-coated donor population (Fig. 1A and Table IA). In contrast to these results, GP61–coated MHC class IIlow donor cells in the same recipient mouse were not eliminated (Fig. 1B and Table IA). In vivo cytotoxicity against class IIhigh GP61–coated targets was also observed at day 9 postinfection (Table IB). As a second approach for doing in vivo cytotoxicity assays, we used donor cells from αβ T cell knockout (KO) mice in our assay. Because of the absence of T cells, most of the donor cells from αβ T cell KO mice constitutively expressed MHC class II and could be evaluated directly without staining for MHC class II in the in vivo cytotoxicity assay. This technique demonstrates a similar loss of the GP61–coated population when compared with the reference population (Fig. 1C and Table I, C and D). This killing was also seen when the CFSE label was reversed such that the GP61–coated population was CFSEhigh and the reference population was CFSElow (48 vs 47%). A reduced level of cytotoxicity (16%) was seen after incubation for 6 h in vivo compared with 48% killing seen after 20 h. Lower levels of in vivo cytotoxicity were directed against the LCMV-encoded subdominant MHC class II epitope NP309 at day 14 postinfection (Table IC). When αβ T cell KO donor cells were stained for expression of CD19 and B220, 80% of the donor lymphocytes and 60% of the donor leukocytes were double positive, which is characteristic for B

![Image](http://www.jimmunol.org/)
cells. By comparing the cytotoxicity between these B cells and all other lymphocyte-gated donor cells, B cells revealed a 53% loss of the GP61-coated population whereas none (≥ 2%) of the other donor cells were killed (Table I). Taken together, these data demonstrate the existence of viral Ag-specific MHC class II-restricted killing in vivo.

Depletion of CD4 T cells ablates MHC class II-restricted killing in vivo
Since MHC class II complexes are recognized by CD4 T cells, we determined whether CD4 T cells were acting as the effectors by depleting hosts of CD4 cells using the mAb GK1.5 before injection of the donor populations. CD4 depletion was highly effective, as shown in Fig. 2. In naive mice, equal numbers of

Table I. Specific killing of LCMV target population in vivo

<table>
<thead>
<tr>
<th>Donor Cell Type</th>
<th>n</th>
<th>% Killing</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: MHC class II&lt;sup&gt;high&lt;/sup&gt; targets vs MHC class II&lt;sup&gt;low&lt;/sup&gt; targets</td>
<td>Ly 5.1</td>
<td>3</td>
<td>52 ± 0.06</td>
</tr>
<tr>
<td>B: Day 9 hosts vs Day 14 hosts</td>
<td>Ly5.1</td>
<td>3</td>
<td>38 ± 9</td>
</tr>
<tr>
<td></td>
<td>Ly5.1</td>
<td>3</td>
<td>41 ± 15</td>
</tr>
<tr>
<td>C: GP&lt;sub&gt;61&lt;/sub&gt; targets vs NP&lt;sub&gt;309&lt;/sub&gt; targets</td>
<td>αβ T cell KO</td>
<td>25</td>
<td>31 ± 7</td>
</tr>
<tr>
<td></td>
<td>αβ T cell KO</td>
<td>3</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>D: CD19&lt;sup&gt;+&lt;/sup&gt; B220&lt;sup&gt;+&lt;/sup&gt; targets vs CD19&lt;sup&gt;+&lt;/sup&gt; B220&lt;sup&gt;−&lt;/sup&gt; targets</td>
<td>αβ T cell KO</td>
<td>6</td>
<td>53 ± 6</td>
</tr>
<tr>
<td></td>
<td>αβ T cell KO</td>
<td>6</td>
<td>−2 ± 9</td>
</tr>
<tr>
<td>E: PBS treated hosts vs anti-CD4 treated hosts</td>
<td>αβ T cell KO</td>
<td>3</td>
<td>41 ± 5</td>
</tr>
<tr>
<td>F: PBS treated hosts vs anti-CD8&lt;sup&gt;+&lt;/sup&gt; treated hosts</td>
<td>Ly5.1</td>
<td>2</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>G: Wild-type targets day 14 vs lpr targets day 14</td>
<td>Ly5.1</td>
<td>5</td>
<td>26 ± 13</td>
</tr>
<tr>
<td>H: Wild-type targets day 9 vs lpr targets day 9</td>
<td>Ly5.1</td>
<td>3</td>
<td>38 ± 9</td>
</tr>
<tr>
<td>I: Wild-type hosts vs gld hosts</td>
<td>αβ T cell KO</td>
<td>10</td>
<td>31 ± 7</td>
</tr>
<tr>
<td>J: Wild-type targets vs TFNR 1 KO targets</td>
<td>Ly5.1</td>
<td>3</td>
<td>40 ± 7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Summary of in vivo cytotoxicity assays for class II-restricted killing. Results based on the percent killing of GP<sub>61</sub>-coated targets except where NP<sub>309</sub> was specifically tested. In A, Ly5.1 splenocytes were tested as targets and analyzed by FACS using coexpression of class II Ags. In B, E, F, G, and I, only Ly5.1 MHC class II<sup>low</sup> cells are tested. In C, D, and H, αβ T cell KO targets that constitutively express MHC class II were used.

<sup>b</sup> Paired t test, two tailed.

<sup>c</sup> Versus uninfected animals.

FIGURE 2. Depletion of CD4<sup>+</sup> T cells by treatment with GK1.5 ablates MHC class II-restricted killing. A, αβ T cell KO donor cells in naive or day 14 LCMV-infected hosts treated with either PBS or anti-CD4 (GK1.5) Ab. Values of p were generated using a paired t test assuming unequal variances comparing experimental animals to naive animals. These are representative figures from the data listed in Table I.
the injected GP₆₁-coated and reference peptide-coated populations of αβ T cell KO splenocytes were observed by flow cytometry (Fig. 2, day 0). At day 14, a selective loss of the GP₆₁-coated population was observed in LCMV-infected mice with intact CD4 cell population (Fig. 2, day 14 LCMV, and Table I). In infected mice depleted of CD4 T cells 2 days before transfer, the GP₆₁-coated targets were not eliminated and their profiles resembled those in naive mice in that the GP₆₁-coated population was about equal in size to the reference population (Fig. 2, day 14 LCMV-CD₄, and Table I). The amount of killing was reduced 5-fold, from 41% in controls to 8% in CD₄-depleted animals (Table I). In a separate experiment, depletion of CD₄κε cells in vivo did not prevent the elimination of GP₆₁-coated targets (Table IF). These experiments indicate that the killing of GP₆₁-coated αβ T cell KO splenocytes is dependent on CD4 T cells.

MHC class II-restricted killing is partially mediated by Fas-FasL

Several effector mechanisms have been demonstrated in the lysis of target cells in vitro by CD4 T cells, including FasL, TNF-α, and perforin. To implicate a mechanism for CD4-directed MHC class II-restricted killing in vivo during the LCMV infection, we used splenocytes from Fas mutant lpr mice mixed with Ly5.1 splenocytes as donors and transferred these into either naive or LCMV-infected mice. We were thus able to distinguish killing of Fas mutant lpr targets from wild-type targets in the same mouse based on the congenic marker. The lpr targets were killed less efficiently than wild-type targets (20 vs 41%, respectively; Table II). A similar pattern was seen on day 9 of infection (Table IH). These results suggested that engagement of Fas-FasL may be required for some but not all of the class II-restricted killing in vivo. To examine the role of FasL, we used αβ T cell KO splenocytes as donors and either C57BL/6 or FasL mutant gld mice as hosts in our assay. αβ T cell KO splenocytes were killed less efficiently in LCMV-infected gld hosts compared with LCMV-infected C57BL/6 hosts (16 vs 31%, respectively; Table II). These results complement the results using lpr targets and further indicate a role for FasL in some, but not all, of the MHC class II-restricted killing. Analysis of the intracellular cytokine profiles of gld mice revealed no deficiency in the frequencies of CD4 T cells producing IFN-γ (Fig. 3) or TNF-α (data not shown) in response to stimulation by GP₆₁ when compared with wild-type mice.

FasL and TNF-α can sometimes exert complementary and even redundant functions, and both have been implicated in CD4 T cell killing in vitro (8, 9). We therefore performed the class II killing assay using TNFR1 KO splenocytes as donors or TNF-α KO mice as hosts. TNFR1 KO target splenocytes were killed at the same level of their wild-type counterparts (Table II), suggesting that TNF was not required for this in vivo cytotoxicity. Killing of wild-type targets was observed in LCMV-infected TNF-α KO mice (15 ± 6%, n = 5), although it was not as robust as in littermate controls (29 ± 8%, n = 5), but this difference could be attributed to significant differences in the number of GP₆₁-specific T cells in vivo (1.6 × 10⁶, n = 2 in wild type vs 7.6 × 10⁵, n = 2 in TNF-α KO). We also examined this killing in perforin KO mice and found virtually no killing in six of six KO mice at day 9, despite the presence of CD4 T cells that produced IFN-γ in response to GP₆₁ (3.5 ± 1% of CD4 T cells, n = 6) and only one of four surviving mice at day 14 had >7% killing. These experiments, however, were complicated by the fact that perforin is needed for viral clearance, and the resulting high Ag load in vivo may cause immune suppression or may compete for the cytotoxic CD4 T cells and result in reduced clearance of the transferred cell population. To reduce viral load in those assays, we transferred 5 × 10⁵ GP₃₃⁺ specific P14-transgenic T cells into perforin KO mice 1 day before infection and assayed for class II-restricted killing on day 14 postinfection. This treatment resulted in a 15-fold increase in leukocyte number over the normally lymphopenic perforin KO spleen, but failed to reveal in vivo class II-restricted cytotoxicity (−12 ± 3%, n = 3). These results suggest a role for perforin in vivo cytolyis, but a complete clarification of the role of perforin would be better analyzed in another system.

Taken together, our results show that CD4-dependent MHC class II-restricted killing occurs in vivo. As with previously published in vitro studies (8–10), it cannot be explained solely by one mechanism, but here we identify Fas-FasL as one of the mechanisms. The importance of this CD4-dependent cytotoxicity remains unclear, but the studies showing requirements for CD4 T cells or for MHC class II in the control of viral infections in vivo must now take this cytotoxic function into account.
Acknowledgments

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References