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## Cutting Edge: Rapid In Vivo CTL Activity by Polyoma Virus-Specific Effector and Memory CD8<sup>+</sup> T Cells<sup>1</sup>

Anthony M. Byers, Christopher C. Kemball, Janice M. Moser, and Aron E. Lukacher<sup>2</sup>

*For viruses that establish persistent infection, continuous immunosurveillance by effector-competent antiviral CD8<sup>+</sup> T cells is likely essential for limiting viral replication. Although it is well documented that virus-specific memory CD8<sup>+</sup> T cells synthesize cytokines after short term in vitro stimulation, there is limited evidence that these T cells exhibit cytotoxicity, the dominant antiviral effector function. Here, we show that antiviral CD8<sup>+</sup> T cells in mice acutely infected by polyoma virus, a persistent mouse pathogen, specifically eliminate viral peptide-pulsed donor spleen cells within minutes after adoptive transfer and do so via a perforin-dependent mechanism. Antiviral memory CD8<sup>+</sup> T cells were similarly capable of rapidly mobilizing potent Ag-specific cytotoxic activity in vivo. These findings strongly support the concept that a cytotoxic effector-memory CD8<sup>+</sup> T cell population operates in vivo to control this persistent viral infection. The Journal of Immunology, 2003, 171: 17–21.*

**V**iral infections drive vigorous expansion of Ag-specific CD8<sup>+</sup> T cells the dominant antiviral effector activity of which is direct cytolysis of infected cells. After viral clearance, a pool of virus-specific memory CD8<sup>+</sup> T cells survives long term in the host (1). Because of their elevated numbers and reduced activation threshold compared with naive T cells, memory T cells accelerate control of infection (2, 3). Although antiviral memory CD8<sup>+</sup> T cells are induced by reinfection to differentiate to CTL effectors, recent evidence suggests that memory CD8<sup>+</sup> T cells themselves express cytotoxic activity (4, 5).

Memory T cells are heterogeneous in surface phenotype, anatomic localization, and functional capabilities. A CCR7<sup>+</sup>CD62L<sup>high</sup> “central” memory T cell subset resides in secondary lymphoid organs and requires Ag-triggered differentiation to acquire effector competence. Conversely, a CCR7<sup>-</sup>CD62L<sup>low</sup> “effector” memory T cell subset that resides in extralymphoid tissues possesses the capacity to exert effector activity immediately after TCR engagement (6). Experimental support for this dichotomy comes from recent studies showing that virus-specific memory CD8<sup>+</sup> T cells isolated from extra-

lymphoid sites, but not spleen, express cytotoxic activity directly ex vivo (7). Whether such effector-memory CD8<sup>+</sup> T cells express Ag-specific cytotoxic activity in vivo and, if so, how rapidly they can be mobilized to express this critical antiviral effector function on Ag encounter are not known. Moreover, for viruses that establish persistent infection, antiviral effector-memory CD8<sup>+</sup> T cells may prove essential for balancing this host-pathogen relationship (5, 8). Here, we used a novel, highly sensitive in vivo assay to directly investigate the expression of Ag-specific cytotoxic activity by antiviral memory CD8<sup>+</sup> T cells in mice infected by polyoma virus, a persistent mouse pathogen.

### Materials and Methods

#### Mice

C3H/HeNcr and C57BL/6Ncr mice were purchased from the National Cancer Institute (Frederick, MD). B6.MRL-*Tnfrsf6<sup>pr</sup>*, B6.129S-*Tnfrsf1a<sup>tm11mx</sup>*, *Tnfrsf1b<sup>tm11mx</sup>*, and C57BL/6-Pfp<sup>tm15dc</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

#### Viruses and peptides

PCR-based site-directed mutagenesis (Stratagene, La Jolla, CA) was used to change the wild-type middle T (MT)<sup>3</sup> protein 389–397 sequence RRLGRITLL (MT389) to RRLGMILL (M516). This mutant virus, A2.M516, was equivalent to the parental wild-type A2 strain polyoma virus in its capacity to transform rat fibroblasts, productively infect mouse cells in vitro, and disseminate in vivo (data not shown). Adult (6- to 8-wk-old) mice were inoculated s.c. in each hind footpad with  $2 \times 10^6$  PFU of either A2 or A2.M516 virus. Acutely infected mice were used 7 days postinfection (p.i.), unless otherwise noted.

#### <sup>51</sup>Cr release assay

Preparation of <sup>51</sup>Cr-labeled AG104A (H-2<sup>k</sup>) target cells, assay conditions, and calculation of percent specific lysis are previously described (9).

#### In vivo CTL assay

RBC-lysed, single-cell spleen suspensions from naive mice were pulsed at  $1 \times 10^7$  cells/ml with 10 μM peptide (unless otherwise noted) or without peptide in DMEM containing 10% FBS for 30 min at 37°C. Each spleen cell population was stained at  $2 \times 10^7$  cells/ml in Diluent C (Sigma-Aldrich, St. Louis, MO) with 2 μM PKH26 (Sigma-Aldrich) at room temperature for 5 min. PKH26 labeling was stopped by addition of an equal volume of FBS for 1 min. Each cell population was then labeled with a different concentration of CFSE (~1 μM, 0.1 μM, or 1 nM) at  $2 \times 10^7$  cells/ml in HBSS. CFSE labeling was stopped by addition of an equal volume of FBS for 1 min, and  $5 \times 10^6$  cells of each peptide-pulsed population were mixed together and injected i.v. into infected and uninfected syngeneic mice. At designated time points after transfer, mice were

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<sup>3</sup> Abbreviations used in this paper: LT, large T protein; MT, middle T protein; p.i., postinfection.

sacrificed, and organs were harvested. Single-cell suspensions of spleen were prepared, and lymphocytes from lung and liver were isolated as previously described (7) and analyzed by flow cytometry. Percent specific lysis of fluorescent donor spleen cells in each mouse is calculated as follows:  $[(\text{number of unpulsed targets} \times A - \text{number of peptide-pulsed targets}) / \text{number of unpulsed targets} \times A] \times 100$ , where  $A = [\text{number of unpulsed targets} / \text{number of peptide-pulsed targets}]$  in uninfected recipient mice.

#### Flow cytometry

Surface marker analysis, tetramer preparation and staining, and intracellular staining for PE-conjugated granzyme B mAb (clone GB12; Caltag Laboratories, Burlingame, CA), its isotype control Ab (mouse IgG1), or a PE-conjugated rabbit mAb to active caspase 3 (clone CPP-32; BD Pharmingen, San Diego, CA) were conducted as previously described (9).

#### Fluorescence microscopy

RBC-lysed, single-cell spleen suspensions from naive C3H/HeN mice were pulsed with MT389 peptide, labeled with CFSE (5  $\mu\text{M}$ ), and injected i.v. into uninfected and infected syngeneic mice. At 4 h postinjection, organs were snap-frozen in OCT embedding medium, and 5- to 20- $\mu\text{m}$  tissue sections were examined using a Nikon (Melville, NY) Optiphot fluorescence microscope. Cryostat sections of the same organs from different mice were cut at the same thickness.

## Results and Discussion

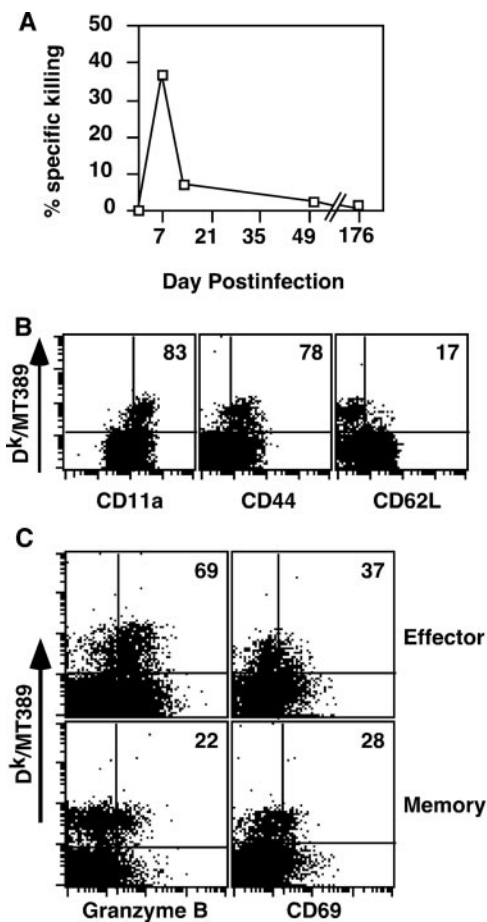
### Undetectable *in vitro* CTL activity by effector phenotype anti-polyoma memory CD8<sup>+</sup> T cells

Freshly explanted spleen cells from adult C3H/HeN mice during acute and persistent phases of polyoma virus infection were tested in a conventional *in vitro* <sup>51</sup>Cr release assay for their ability to lyse syngeneic target cells coated with the dominant D<sup>k</sup>-restricted MT389 peptide derived from the wild-type viral MT protein (10). MT389-specific lysis peaked at day 7 p.i., coincident with maximal MT389-specific CD8<sup>+</sup> T cell expansion (11), was nearly baseline at day 13 p.i. and was undetectable at later time points (Fig. 1A). Although MT389-specific CD8<sup>+</sup> T cells comprise 1/15 of the total CD8<sup>+</sup> T cell population in the spleen of immune C3H/HeN mice (data not shown), the sensitivity of this *in vitro* assay may be limiting for detecting CTL effectors if only a portion of MT389-specific CD8<sup>+</sup> T cells express cytotoxic activity in polyoma-immune mice.

MT389-specific memory CD8<sup>+</sup> T cells in the spleen predominantly express the CD11a<sup>high</sup>CD44<sup>high</sup>CD62L<sup>low</sup> phenotype of effector T cells (Fig. 1B); this is a common phenotype for antiviral memory CD8<sup>+</sup> T cells maintained in the setting of chronic infection (12). Additionally, roughly one-third of memory MT389-specific CD8<sup>+</sup> T cells express CD69, a marker of recent TCR engagement (Fig. 1C); this is consistent with evidence that polyoma viral DNA persists long term in multiple organs, including spleen, of C3H/HeN and C57BL/6 mice (Ref. 13 and data not shown). Interestingly, only a fraction of these effector phenotype memory MT389-specific CD8<sup>+</sup> T cells stain with a granzyme B mAb, compared with >60% of these T cells expressing granzyme B protein in acutely infected mice (Fig. 1C). This phenotypic profile indicates that polyoma-specific memory CD8<sup>+</sup> T cells encounter viral Ags and supports the hypothesis that these T cells actively express effector activities to control this persistent virus infection.

### High specificity and efficiency of anti-polyoma cytotoxicity *in vivo* during acute infection

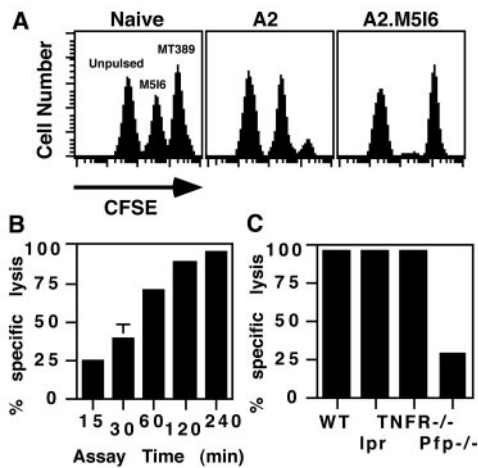
To directly detect polyoma virus-specific CD8<sup>+</sup> T cell cytotoxic activity *in vivo*, we modified a protocol to monitor the ability of infected mice to eliminate fluorescently labeled spleen cells pulsed with MHC class I-restricted viral peptides (14). As



**FIGURE 1.** Ex vivo cytotoxicity and phenotypic analysis of MT389-specific memory CD8<sup>+</sup> T cells. *A*, Spleen cells from polyoma virus-infected C3H/HeN mice were assayed at the indicated day after infection for lysis of <sup>51</sup>Cr-labeled AG104A target cells pulsed with MT389 peptide at an E: T ratio of 400:1. Unpulsed target cells were not lysed (data not shown). Each value represents mean  $\pm$  SEM percent specific lysis at 4 h by spleen cells of two individual mice; results are representative of three experiments. *B*, Plots are gated on CD8<sup>+</sup> T cells, and values are the percentage of splenic D<sup>k</sup>MT389 tetramer<sup>+</sup> T cells that express CD11a, CD44, and CD62L. Data are representative of three experiments, with two 60- to 90-day p.i. mice per experiment. *C*, Plots are gated on CD8<sup>+</sup> T cells, and values are the percent of splenic D<sup>k</sup>MT389 tetramer<sup>+</sup> T cells that express granzyme B, and CD69. Data are representative of three experiments, with two effector mice or two 60- to 90-day p.i. mice (memory) per experiment.

target cells, spleen cells from uninfected mice were pulsed with polyoma peptides or left unpulsed. Each group was then labeled with a single concentration of membrane-integrating dye PKH26 and different concentrations of CFSE, a cytoplasmic dye; these groups were mixed in equal proportions and then injected i.v. into syngeneic mice. The frequency of each peptide-pulsed donor cell population in the spleens of recipient mice was compared against the unpulsed donor cell population, which served as an internal negative control. To assess the efficiency and specificity of this *in vivo* CTL assay in acutely infected C3H/HeN mice, target cells were coated with either MT389 peptide or M516 peptide, an MT389 analog peptide that binds with equivalent affinity to D<sup>k</sup> (9). At 4 h post-transfer to mice infected by wild-type A2 virus, >95% of MT389-pulsed donor cells were cleared from the spleens, whereas M516 peptide-pulsed and unpulsed donor cells were present in equal numbers (Fig. 2A). In spleens of uninfected C3H/HeN mice,





**FIGURE 2.** Characterization of polyoma-specific CTL activity in vivo in acutely infected mice. *A*, Histograms show numbers of CFSE<sup>low</sup> unpulsed, CFSE<sup>intermediate</sup> M516 peptide-pulsed, or CFSE<sup>high</sup> MT389 peptide-pulsed C3H/HeN target cells in the spleens of A2 virus-infected, A2.M516 virus-infected, or uninfected C3H/HeN mice 4 h posttransfer. Results are representative of two experiments, with three mice per experiment. *B*, Unpulsed and MT389 peptide-pulsed C3H/HeN splenic target cells were injected into acutely infected C3H/HeN mice. Values represent mean  $\pm$  SEM specific lysis of target cells at the indicated time points posttransfer of two to three individual mice; results are representative of two to three experiments. *C*, LT360 peptide-pulsed and unpulsed naive C57BL/6, *lpr* (*Fas*<sup>-/-</sup>), or *TNFR*<sup>-/-</sup> *Il*<sup>-/-</sup> spleen cells were injected into C57BL/6 mice at 7 days p.i. LT360 peptide-pulsed and unpulsed naive C57BL/6 spleen cells were injected into uninfected and acutely infected *Pfp*<sup>-/-</sup> mice. Values represent mean  $\pm$  SEM percent specific lysis of peptide-pulsed spleen cells at 4 h of three recipient mice. WT, wild type.

equal frequencies of MT389 peptide-pulsed, M516 peptide-pulsed, and unpulsed donor cells were detected (Fig. 2*A*). C3H/HeN mice infected by the A2.M516 mutant virus mounted an M516-specific CD8<sup>+</sup> T cell response of a magnitude comparable with that of the MT389-specific response during A2 infection, and exhibited no cross-reactivity with MT389, as detected by peptide-stimulated intracellular IFN- $\gamma$  assay (data not shown). As shown in Fig. 2*A*, C3H/HeN mice infected with A2.M516 efficiently eliminated M516 peptide-pulsed, but not MT389 peptide-pulsed, donor spleen cells.

Conventional <sup>51</sup>Cr release assays typically use a 4-h incubation period to reliably quantitate cell-mediated cytotoxicity. This timeframe conflicts with recent data that CTL-induced target cell apoptosis occurs within minutes of T cell-target cell conjugate formation (15). We therefore decided to monitor the kinetics of in vivo CTL activity in acutely infected C3H/HeN mice. Fig. 2*B* demonstrates that substantial Ag-specific cytotoxicity could be detected as early as 15 min after transfer of MT389-pulsed spleen cells into acutely infected mice; MT389-specific killing progressively increased until nearly 100% elimination of the MT389-pulsed donor cells was achieved by 4 h after donor cell transfer. Because the active form of caspase 3, an effector caspase in CTL-mediated apoptosis (16), was not detected by flow cytometry in the remaining MT389 peptide-pulsed targets at 0.5 and 1 h posttransfer (data not shown), serial killing by CTL in vivo likely accounts for this gradual elimination of target cells over time.

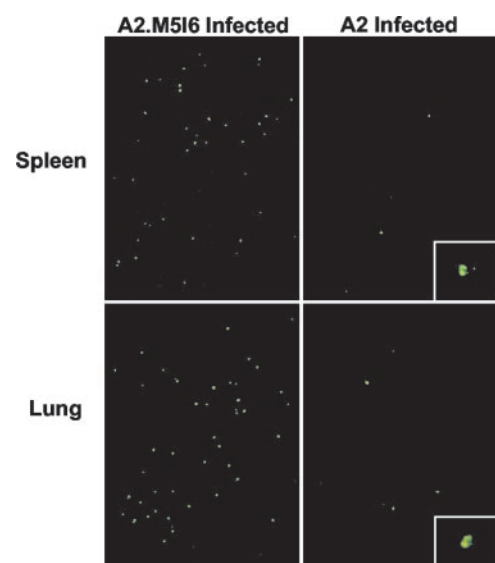
We next investigated the mechanism(s) responsible for this in vivo cytotoxicity using C57BL/6 mice mutated in *Fas* (*lpr*), or carrying targeted disruptions in the perforin gene (*Pfp*<sup>-/-</sup>) or in both

TNF receptor type I and II genes (*TNFR*<sup>-/-</sup>). Using overlapping synthetic peptides covering the six polyoma viral proteins, we identified a strongly dominant D<sup>b</sup>-restricted CD8<sup>+</sup> T cell epitope corresponding to residues 360–368 (AVKNYCSKL) in the polyoma large T (LT) protein, designated LT360. By single-cell assays (MHC class I tetramer staining and intracellular IFN- $\gamma$  production), 15–20% of the CD8<sup>+</sup> T cells recognize the LT360 epitope in C57BL/6 mice at day 7 p.i. (data not shown). As shown in Fig. 2*C*, acutely infected C57BL/6 recipients eliminated LT360 peptide-pulsed spleen cells from *lpr* and *TNFR*<sup>-/-</sup> mice as efficiently as those from wild-type mice. Although D<sup>b</sup>/LT360 tetramers stain similar frequencies of CD8<sup>+</sup> T cells in acutely infected *Pfp*<sup>-/-</sup> and wild-type mice (data not shown), infected *Pfp*<sup>-/-</sup> mice were markedly impaired in their capacity to clear LT360 peptide-pulsed C57BL/6 donor spleen cells (Fig. 2*C*). Taken together, these findings indicate that perforin-granzyme exocytosis is the dominant mechanism for clearance of Ag-pulsed donor spleen cells in vivo.

To exclude the possibility that peptide-pulsed targets were sequestered in the pulmonary vasculature, fluorescence microscopy was used to visualize MT389 peptide-pulsed CFSE-labeled donor spleen cells in cryostat sections of spleen, lung, and liver from infected recipient C3H/HeN mice. At 4 h posttransfer, MT389-pulsed target cells were readily apparent in the spleen and lungs of A2.M516-infected and uninfected (data not shown) recipients, but only rare fluorescent cells were detected in these organs in A2-infected mice (Fig. 3); a similar pattern was seen in the livers of A2-infected mice (data not shown). Moreover, the remaining fluorescent cells in each organ of the A2-infected mice were fragmented and appeared to reside within other cells (Fig. 3, *insets*). These findings further suggest that Ag-specific elimination of target cells takes place in both lymphoid and nonlymphoid sites.

#### Efficient in vivo cytotoxicity by polyoma-specific memory CD8<sup>+</sup> T cells

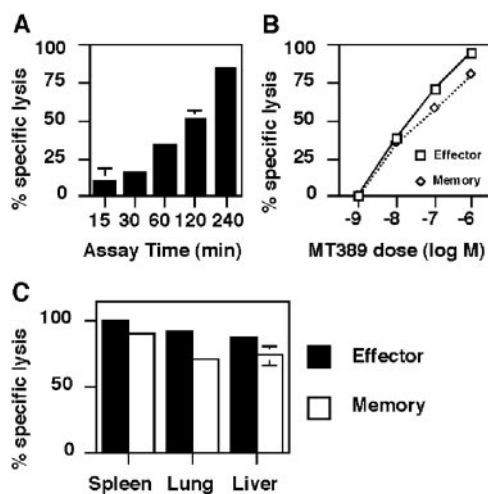
We used the in vivo CTL assay to investigate whether Ag-specific cytotoxicity could be detected in polyoma virus-immune



**FIGURE 3.** In vivo polyoma-specific CTL activity in nonlymphoid organs. MT389 peptide-pulsed, CFSE-labeled (5  $\mu$ M) C3H/HeN target cells were injected into uninfected (data not shown), day 7 p.i. A2.M516 virus-infected, or day 7 p.i. A2 virus-infected C3H/HeN mice. Four hours after transfer, spleens, lungs, and livers (not shown) were snap-frozen, and cryostat sections were examined by fluorescence microscopy. *Large panels*,  $\times 100$ ; *inset*,  $\times 400$ .

mice. As shown in Fig. 4A, substantial MT389-specific killing was observed by 30 min after transfer of MT389-pulsed target cells to immune C3H/HeN mice, with nearly complete lysis by 4 h posttransfer. Parallel D<sup>k</sup>/MT389 tetramer staining of spleen cells at the 4-h posttransfer time point permitted calculation of an in vivo E:T ratio of 7:1 for C3H/HeN mice at day 7 p.i. and 2:1 for immune mice, a difference that likely accounts for the slightly delayed MT389-specific killing in memory (compare Figs. 2B and 4A). Based on peptide titration curves, effector and memory MT389-specific CD8<sup>+</sup> T cells exhibit comparable functional avidity in vivo (Fig. 4B); this finding lends further support for the conclusion that differences in kinetics of target cell elimination by effector and memory CD8<sup>+</sup> T cells at early time points are due to differences in numbers of CTL effectors. We further found that polyoma-immune C57BL/6 mice efficiently eliminated LT360 peptide-pulsed donor spleen cells from normal, *lpr*, and *TNFR*<sup>-/-</sup> mice (data not shown), implicating perforin-granzyme exocytosis as the preferential lytic mechanism used in vivo by anti-polyoma memory CD8<sup>+</sup> T cells. As shown in Fig. 4C, most of the MT389-pulsed donor target cells were selectively cleared from lungs and livers of polyoma-immune, as well as acutely infected, C3H/HeN mice. Fluorescence microscopy confirmed that MT389-pulsed CFSE-labeled target spleen cells were eliminated in the spleen, lung, and liver of immune, but not uninfected, mice (data not shown). Thus, as seen in acutely infected mice, potent and rapid MT389-specific cytotoxicity was detected in vivo in multiple sites in polyoma-immune mice.

Virus-specific memory CD8<sup>+</sup> T cells have been reported to express cytotoxic and cytokine effector activities ex vivo within



**FIGURE 4.** In vivo polyoma-specific memory CTL. *A*, Unimpulsed and MT389 peptide-pulsed C3H/HeN splenic target cells were injected into persistently (>50 days p.i.) infected C3H/HeN mice. Values represent mean  $\pm$  SEM specific lysis of target cells at the indicated time points posttransfer of two to three individual mice; results are representative of two to three experiments. *B*, C3H/HeN splenic target cells pulsed with serial log dilutions of MT389 peptide were assayed by in vivo CTL assay 4 h after transfer into infected C3H/HeN recipients. Values represent the mean  $\pm$  SEM percent specific lysis of target cells in three effector or day 175 p.i. (memory) recipient mice; results are representative of three experiments. *C*, Unimpulsed and MT389 peptide-pulsed C3H/HeN splenic target cells were injected into two effector and two memory (day 45 p.i.) C3H/HeN mice. At 4 h posttransfer, mice were perfused, and lymphocytes were collected from single-cell suspensions of spleen, lung, and liver. Values represent mean  $\pm$  SEM specific lysis of target cells in the indicated organs.

hours after antigenic stimulation (4, 7, 17). In this report, we provide clear evidence that virus-specific memory CD8<sup>+</sup> T cells exert immediate and substantial Ag-specific cytotoxicity in vivo. Because CTL activity in polyoma virus-immune mice is detected as early as 30 min after in vivo encounter with viral peptide-coated target cells, anti-polyoma memory CD8<sup>+</sup> T cells appear poised to express this effector function in vivo. The marked efficiency in eliminating peptide-pulsed Fas- and TNFR-deficient target cells in vivo, coupled with the rapidity of in vivo CTL activity, strongly implicates perforin-granzyme-mediated exocytosis as the most likely cytolytic effector mechanism wielded by polyoma virus-specific memory CD8<sup>+</sup> T cells in vivo. This would further imply that polyoma-specific memory CD8<sup>+</sup> T cells contain either preformed lytic granule proteins or transcripts for these proteins that can be rapidly translated upon TCR engagement. In this connection, recent reports reveal that resting memory CD8<sup>+</sup> T cells contain transcripts for several effector proteins, including granzyme B, perforin, IFN- $\gamma$ , Fas ligand, and RANTES, and that stable cytoplasmic mRNA for RANTES is rapidly translated and secreted after TCR stimulation (18, 19).

It is also conceivable that chronic Ag stimulation might drive a proportion of memory CD8<sup>+</sup> T cell to differentiate to effector cells, which are responsible for eliminating viral Ag-expressing cells during persistent infection. On the basis of the immediacy and efficiency of Ag-specific CTL activity in vivo in polyoma-immune mice, we favor the conclusion that memory CD8<sup>+</sup> T cells themselves play a substantial part in clearing the Ag-pulsed target cells. Additional support for this conclusion is provided by the observation that memory CD8<sup>+</sup> T cells generated in mice infected by the Armstrong strain of lymphocytic choriomeningitis virus, which is completely cleared during acute infection, also rapidly eliminate viral peptide-Ag pulsed target cells in vivo (20). In summary, the findings reported here demonstrate that Ag-specific CD8<sup>+</sup> T cells express potent cytotoxic effector activity in vivo during a persistent viral infection.

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