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*J Immunol* (2003) 171 (1): 27–31.

<https://doi.org/10.4049/jimmunol.171.1.27>

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## CUTTING EDGE

Cutting Edge: Rapid In Vivo Killing by Memory CD8 T Cells<sup>1</sup>Daniel L. Barber, E. John Wherry, and Rafi Ahmed<sup>2</sup>

*In this study, we examined the cytotoxic activity of effector and memory CD8 T cells in vivo. At the peak of the CTL response following an acute lymphocytic choriomeningitis virus infection, effector CD8 T cells exhibited extremely rapid killing and started to eliminate adoptively transferred target cells within 15 min by a perforin-dependent mechanism. Although resting memory CD8 T cells are poorly cytolytic by in vitro <sup>51</sup>Cr release assays, there was rapid elimination (within 1–4 h) of target cells after transfer into immune mice, and both CD62L<sup>high</sup> and CD62L<sup>low</sup> memory CD8 T cells were able to kill rapidly in vivo. Strikingly, when directly compared on a per cell basis, memory CD8 T cells were only slightly slower than effector cells in eliminating target cells. These data indicate that virus specific memory CD8 T cells can rapidly acquire cytotoxic function upon re-exposure to Ag and are much more efficient killers in vivo than previously appreciated. The Journal of Immunology, 2003, 171: 27–31.*

The ability of Ag-specific CD8 T cells to seek out pathogen-infected cells or tumor cells and kill them in a highly specific manner constitutes a powerful immune effector function (1, 2). Although CD8 T cells can use several distinct mechanisms to kill (3), studies using knockout mice suggest that the perforin-granzyme pathway might be the most effective in vivo, at least in terms of controlling microbial infections (1, 2).

Naive CD8 T cells are incapable of immediately killing infected cells, but after their initial encounter with Ag, naive CD8 T cells begin a proliferative program and differentiate into activated effector cells. These effector CD8 T cells express perforin and granzymes and display very high levels of cytotoxic activity. During acute viral infections, such as infection of mice with lymphocytic choriomeningitis virus (LCMV),<sup>3</sup> vaccinia, or vesicular stomatitis virus, the CD8 T cell response peaks ~1 wk postinfection, and it is at this time that the highest level of direct ex vivo cytotoxic activity is detected with <sup>51</sup>Cr release as-

says. The lytic activity then gradually decreases as the infection is cleared, and the effector CD8 T cells differentiate into memory cells (4, 5). Indeed, in many systems memory CD8 T cell cytotoxic activity, as measured by <sup>51</sup>Cr release assays, is detectable only after the cells have been reactivated and expanded in culture. However, a recent study has shown that memory CD8 T cells present in nonlymphoid tissues can display direct ex vivo cytotoxicity (6).

Although CTL-mediated cytotoxicity has been extensively studied for many years, most of the studies have relied on in vitro assays to measure killing. Consequently, the in vivo cytotoxic potential of these cells is not well understood. To address this issue, we have used an in vivo cytotoxicity assay to examine the lytic potential of effector and memory CD8 T cells.

## Materials and Methods

## Mice and infections

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). B6.MRL-*Tnfrsf6* (Fas deficient) and B6;129S-*Tnfrsf1a*<sup>tm11mc</sup>*Tnfrsf1b*<sup>tm11mc</sup> (*TNFR*<sup>-/-</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-*Ppftm1Sdz* (*pfr*<sup>-/-</sup>) and P14 TCR-transgenic mice are maintained in our animal colony. Mice were infected with  $2 \times 10^7$  PFU of LCMV-Armstrong i.p.

## In vivo cytotoxicity assay

Splenocytes from naive mice were costained with PKH26 (Sigma-Aldrich, St. Louis, MO) and either 1  $\mu$ M, 100 nM, or 1 nM CFSE (Molecular Probes, Eugene, OR). These labeled cells were then coated with the indicated peptides (1  $\mu$ M) and transferred i.v. ( $5 \times 10^6$  cells of each population) into the indicated groups of mice. At the indicated time points, lymphocytes were isolated from liver, lung, or spleen as previously described (6). Target cells were distinguished from recipient cells based on PKH26 staining and from one another based on CFSE staining. Gating on PKH26<sup>+</sup> cells, the percent killing was calculated as follows:  $100 - [( \% \text{ peptide pulsed in infected} / \% \text{ unpulsed in infected}) / ( \% \text{ peptide pulsed in uninfected} / \% \text{ unpulsed in uninfected})] \times 100$ .

## Fluorescence microscopy

CFSE-labeled (1  $\mu$ M), NP396 peptide-pulsed (1  $\mu$ M) target cells were injected into uninfected or LCMV-Armstrong day 8 infected mice. After 2 h, tissues were harvested and frozen in OCT medium, and 7- $\mu$ m sections were examined for CFSE fluorescence.

## Adoptive transfer experiments

Effector and memory CD8 T cells were generated as previously described (5).

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Received for publication March 5, 2003. Accepted for publication May 2, 2003.

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<sup>1</sup> This work was supported by National Institutes of Health Grants AI00348 and AI44644. E.J.W. was supported by a Cancer Research Institute postdoctoral fellowship.

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<sup>3</sup> Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; T<sub>EM</sub>, effector memory cells; T<sub>CM</sub>, central memory cells.

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

Chromium release assays were performed as previously described (5).

*In vitro killing by caspase detection*

Caspase-based killing assays were performed as previously described (7) using Cytolix kits (OncoImmunin, Gaithersburg, MD). Percent killing was calculated for the PKH26<sup>+</sup> target cells as:  $[(\% \text{ peptide pulsed caspase}^+ - \% \text{ unpulsed caspase}^+)/ (100 - \% \text{ unpulsed caspase}^+)] \times 100$ .

## Results and Discussion

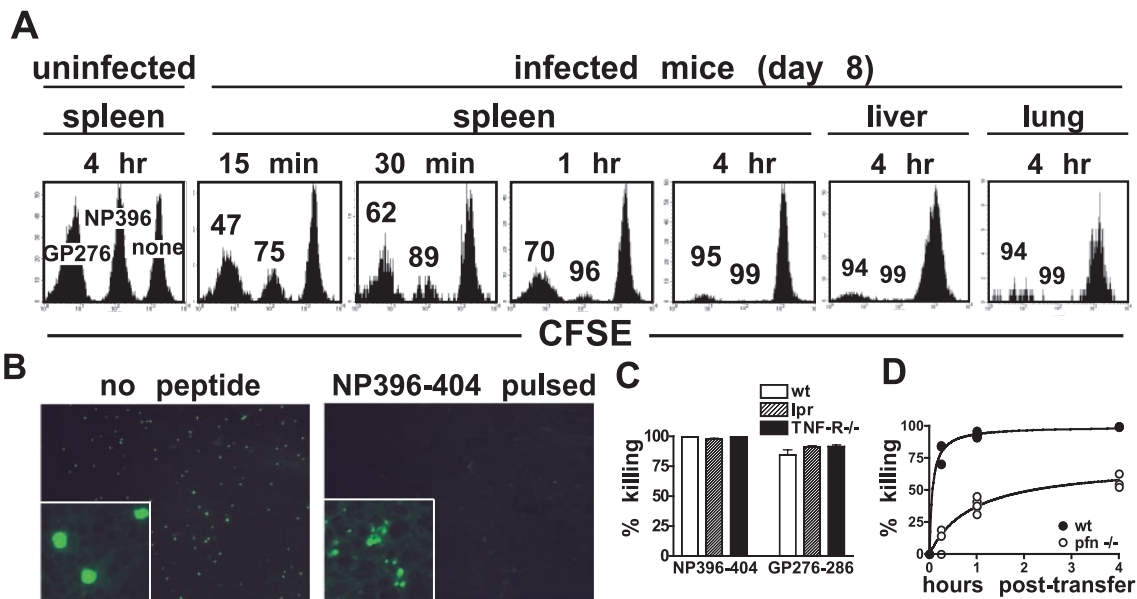
### *Kinetics and mechanism of target cell destruction by effector CD8 T cells in vivo*

We first examined killing in vivo by both NP396- and GP276-specific effector CD8 T cells by transferring targets into mice 8 days after acute LCMV infection. At this time point, NP396-specific T cells represented ~14% of total splenic CD8 T cells, and GP276 specific T cells represented ~6% (data not shown). After transfer of targets, 75% of the NP396 pulsed targets and 47% of the GP276 pulsed targets were eliminated in only 15 min, and most of the targets were killed within 1 h (Fig. 1A). Rapid killing of target cells was also detected in the liver and the lung (Fig. 1A). This indicates that the peptide-pulsed target cells were not selectively sequestered in the periphery.

To confirm our flow cytometric analysis of in vivo cytotoxicity, we also examined in vivo killing histologically. In these experiments, target cells were labeled with CFSE and then either pulsed with NP396 peptide or left unpulsed. As expected, uncoated targets were detectable in the spleens of day 8 mice. In contrast, NP396-pulsed targets were not detected in day 8 mice (Fig. 1B). However, under higher magnification we could observe rare cells displaying dim, punctate CFSE staining (Fig. 1B). Very similar staining in dendritic cells that have engulfed dying cells has also been reported (8). Therefore, this punctate fluorescence may be engulfed dying target cells. In contrast, uncoated targets appeared healthy and had normal morphology.

This confirms our flow cytometric determinations, and taken together these data demonstrate that this assay allows us to measure peptide-specific cytotoxic activity in vivo. Furthermore, it indicates that killing can occur in vivo in <15 min.

We next investigated the mechanism of CD8 T cell killing in vivo. To test the role of Fas, we prepared target cells from +/+ mice or *lpr* mice. We found no difference in the killing of +/+ and *lpr* targets 8 days postinfection, indicating that LCMV-specific CD8 T cells do not require Fas/Fas ligand engagement to rapidly kill in vivo (Fig. 1C). We also examined the role of TNF- $\alpha$  by offering +/+ or *p55*<sup>-/-</sup>*p75*<sup>-/-</sup> targets to normal mice. There was no difference in the ability of LCMV-specific CD8 T cells to kill +/+ or *p55*<sup>-/-</sup>*p75*<sup>-/-</sup> targets 8 days postinfection, indicating that TNFR signals were not required (Fig. 1C). To investigate the contribution of the perforin pathway, we infected *pf1*<sup>-/-</sup> or +/+ mice with LCMV-Armstrong and determined the level of in vivo killing 8 days postinfection. Perforin<sup>-/-</sup> mice were severely compromised in their ability to kill peptide-coated targets (Fig. 1D). This was not due to a difference in the number of NP396- and GP276-specific T cells, because both groups contained similar numbers of Ag-specific T cells (data not shown). The low level killing detected in the absence of perforin presumably represents the collective contribution of perforin-independent killing mechanisms and may be TNFR or Fas dependent as has been previously observed (1). However, in such a case, our data indicate that the small role of Fas- and TNFR-mediated killing are masked in the presence of perforin-mediated killing. Therefore, in vivo killing by LCMV-specific CD8 T cells was largely mediated by the perforin-dependent granule exocytosis pathway. This is in contrast to another report concluding that LCMV-specific CD8 T cells do not require perforin to kill in in vivo CTL assays (9), but it is consistent with the observation that perforin-deficient animals cannot control LCMV (1, 2).



**FIGURE 1.** Kinetics and mechanism of effector CD8 T cell killing in vivo. *A*, Target cells were transferred into mice 8 days post-LCMV infection or into uninfected mice. Histograms are gated on PKH26<sup>+</sup> target cells in the spleen, liver, or lung. Numbers represent the percentage of target cells killed. *B*, NP396-pulsed or unpulsed CFSE-labeled targets were transferred into day 8 mice. Spleens were harvested 2 h later, and sections were analyzed for CFSE fluorescence. *C*, Wild-type (wt), *lpr*, or *TNFR*<sup>-/-</sup> targets were transferred into day 8 mice, and percent killing determined at 4 h. *D*, Target cells were transferred into wild-type or *pf1*<sup>-/-</sup> mice 8 days postinfection.

## Memory CD8 T cells are potent killers in vivo

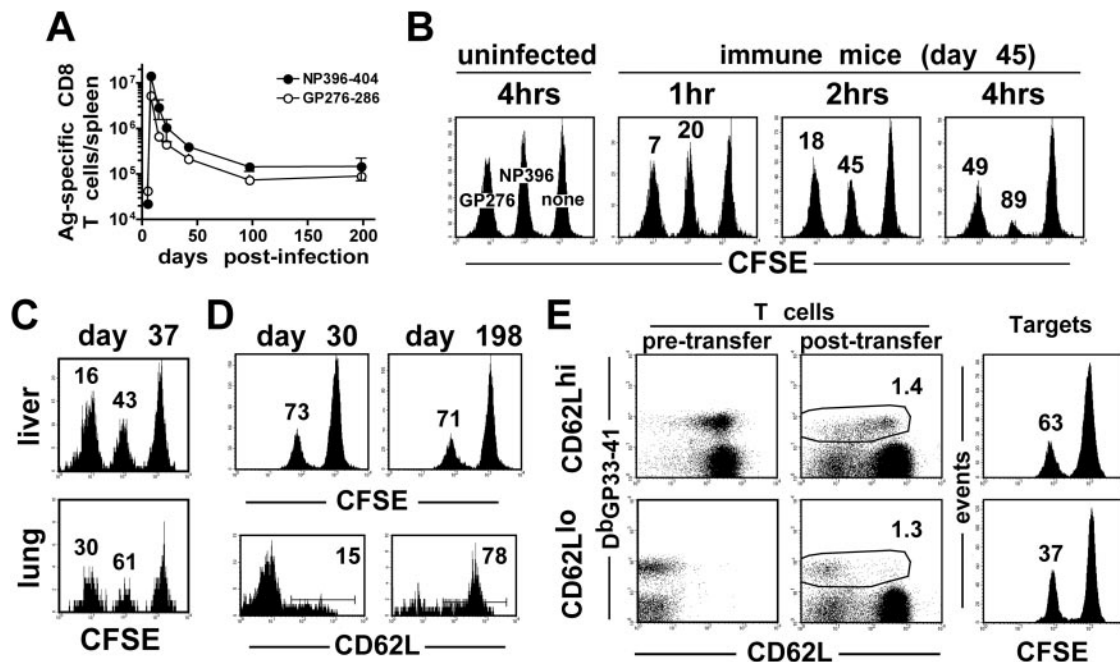
After the peak of the CD8 T cell response at day 8, there is a contraction phase where ~90–95% of the Ag-specific cells die by apoptosis, and then a stable number of memory cells persists for the life of the animal (Fig. 2*A* and Refs. 10 and 11). We next measured the kinetics of memory CD8 T cell killing in vivo by transferring target cells into LCMV-immune mice. Strikingly, memory CD8 T cells displayed very rapid cytolytic activity in vivo. Killing first became detectable in ~1 h (Fig. 2*B*). At 4 h post-transfer of targets, 89% of the NP396-coated targets and 49% of the GP276-coated targets were killed. We also examined cytolytic activity in peripheral tissues. There were 10- to 20-fold fewer control target cells in the liver and lung than in the spleen (data not shown), and the peptide-pulsed target cells that trafficked into these tissues were also eliminated (Fig. 2*C*).

We next measured the cytolytic activity of memory cells at late time points postinfection. The ability to rapidly eliminate target cells in vivo was remarkably stable over time (Fig. 2*D*). Furthermore, lytic activity was stable despite other phenotypic changes. Between days 30 and 198, although the in vivo lytic activity did not change, the percentage of CD62L-expressing cells increased from 15% to 78% of the NP396-specific memory CD8 T cells (Fig. 2*D*). Memory CD8 T cells undergo a gradual differentiation process during which effector memory ( $T_{EM}$ ) cells convert to central memory ( $T_{CM}$ ) cells (4, 5). These data indicate that even though the memory pool converts from predominantly CD62L<sup>low</sup>  $T_{EM}$  at day 30 to mostly CD62L<sup>high</sup>  $T_{CM}$  at day 198, the ability to rapidly acquire cytotoxic activity

in vivo remains relatively constant during this  $T_{EM}$  to  $T_{CM}$  differentiation (Fig. 2*D*). Importantly, LCMV-Armstrong is cleared within 8 days (12), and memory CD8 T cells in Armstrong immune mice after day 30 display a resting phenotype, CD69<sup>low</sup> and CD25<sup>low</sup> (data not shown). Thus, upon re-exposure to Ag, resting memory CD8 T cells can very rapidly acquire lytic activity and kill target cells in vivo.

To compare directly the lytic activity of  $T_{CM}$  and  $T_{EM}$  cells, we measured in vivo killing by normalized numbers of CD62L<sup>high</sup> and CD62L<sup>low</sup> memory CD8 T cells. We adoptively transferred equal numbers of either CD62L<sup>high</sup> or CD62L<sup>low</sup> GP33-specific transgenic memory CD8 T cells (P14 T cells) into naive mice and allowed them to equilibrate for 2 h. An equal number of target cells was then transferred, creating an in vivo E:T ratio of 1. At 4 h post-transfer of targets, CD62L<sup>high</sup> memory cells had killed 63% of the targets and CD62L<sup>low</sup> memory cells had killed 37% (Fig. 2*E*). We verified that equal numbers of T cells were transferred by staining with class I tetramers at the end of the assay. These data indicate that both memory T cell subsets can kill in vivo, and when compared on a per cell basis  $T_{CM}$  are equally efficient, if not slightly more efficient killers than  $T_{EM}$  cells.

However, it is unclear from these experiments how the lytic activity of memory CD8 T cells directly compares with that of day 8 effector CD8 T cells, because there are 10- to 20-fold more Ag-specific T cells present in day 8 mice than in immune mice (Fig. 2*A*). By comparing the number of Ag-specific T cells and the number of unpulsed targets, we were able to calculate



**FIGURE 2.** Resting memory CD8 T cells display rapid cytolytic activity in vivo. *A*, NP396- and GP276-specific CD8 T cells were enumerated with class I tetramers at different days postinfection. *B* and *C*, Memory CD8 T cell cytotoxicity was measured in the spleen (*B*) of day 45 mice at the indicated time points and in the liver and lung of day 37 mice (*C*) 4 h post-transfer of targets. Histograms are gated on PKH26<sup>+</sup> target cells. Numbers represent the percentage of target cells killed. *D*, NP396-specific in vivo killing in the spleen (*top*) and CD62L expression (*bottom*) on NP396-specific memory cells from the blood of the same animal 1 day before the killing experiment was performed. In vivo E:T ratios were as follows for NP396 and GP276, respectively: spleen, 2:1 and 0.5:1; liver, 10:1 and 2.5:1; lung, 35:1 and 9:1. *E*, A total of  $1.5 \times 10^6$  CD62L<sup>high</sup> (97% pure) or CD62L<sup>low</sup> (98% pure) memory CD8 T cells purified from the spleens of day 70 mice were transferred into naive recipients. Two hours later,  $1.5 \times 10^6$  GP33-pulsed target cells were transferred into T cell recipients, and spleens were harvested 4 h later. CD62L and tetramer stains shown pre- and posttransfer are gated on CD8<sup>+</sup> cells, and target cells shown are from the same spleen. Numbers next to gated events indicate the percentage of CD8 T cells that are tetramer<sup>+</sup>. Numbers next to target cell histograms indicate the percent killed.

the in vivo E:T ratio for each experiment. Indeed, the in vivo E:T ratio correlated with the level of killing (Fig. 3*A*), indicating that the higher levels of killing observed in day 8 mice may be due to differences in the number of Ag-specific T cells. In the next series of experiments, we normalized the in vivo E:T ratios and directly compared the lytic activity of effector and memory CD8 T cells.

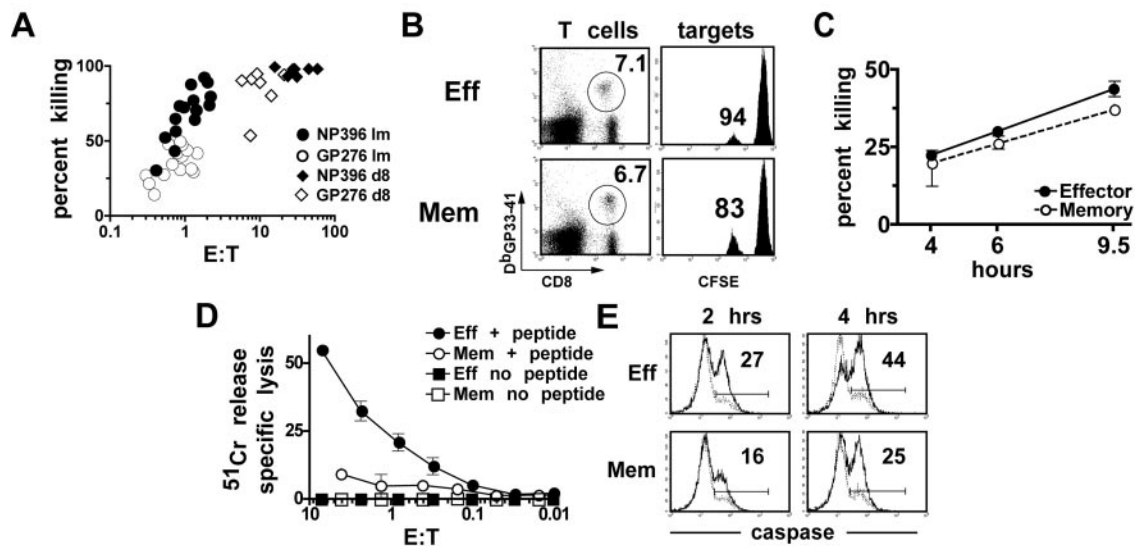
Equal numbers of either effector or memory P14 cells were adoptively transferred into naive mice and allowed to equilibrate for 2–3 h. GP33-pulsed and unpulsed target cells were then transferred into the mice that had received the P14 effector or memory CD8 T cells. We verified that the recipients contained equal numbers of effector or memory cells by staining with class I tetramers at the end of the assay (Fig. 3*B*). At an in vivo E:T ratio of 2:1, effector cells killed 94% of the targets, whereas memory cells killed 83% of the targets. These data indicate that memory cells killed only slightly less efficiently than effector cells at a normalized E:T ratio. This may be explained by slower rates of killing, a delay in the onset of killing by memory CTL, or both. To address these possibilities, we used the P14 adoptive transfer system (13) to measure the kinetics of effector and memory CTL killing at the relatively low E:T ratio of 0.2:1. This low E:T ratio was chosen to enhance the detection of any differences in the ability to serially kill targets. From 4 to 9.5 h, effector and memory CTL killed at similar rates (Fig. 3*C*). However, the memory cells had killed fewer targets at each time point ( $p = 0.02$ ). These data may indicate that memory CD8 T cells have a brief lag time before they begin to kill, but once they have acquired cytotoxic function upon re-exposure to Ag, they kill as rapidly as effector CD8 T cells.

This strikingly rapid in vivo killing by memory CD8 T cells contrasts what is observed by  $^{51}\text{Cr}$  release assays, which fail to

detect appreciable direct ex vivo lytic activity by memory CD8 T cells (Fig. 3*D*). To investigate further the ability of memory CD8 T cells to kill in vitro, we used a flow cytometric based in vitro killing assay that detects early biochemical changes in apoptotic target cells (i.e., caspase activation) (7). At 2 h, memory cells had induced caspase activation in 16% of the targets compared with 27% of the targets for effector cells. By 4 h, this had increased to 25 and 44% for memory and effector cells, respectively (Fig. 3*E*). These data indicate that memory CD8 T cells can induce target cell apoptosis in only 2 h in vitro, further supporting our in vivo detection of memory cell cytotoxicity.

In this study, we have shown that CD8 T cells kill target cells extremely rapidly in vivo. In fact, we detected between 10 and 40% killing of NP396-pulsed targets in only 5 min after transfer into day 8 mice (data not shown). This is consistent with an elegant microscopic study that demonstrated synapse formation in 3 min and characteristically apoptotic morphological changes in target cells only 5 min after contact with the CTL (14). These results indicate that CD8 T cells could control viral infections by killing infected cells within minutes of their detection. It has been shown that when splenocytes from day 8 LCMV-infected mice are adoptively transferred into recipients that had been infected 1 day earlier, the transferred cells can clear the otherwise rapidly replicating virus in <6 h (15). Our results further illustrate how CTL are capable of mediating such impressive control of viral infections.

Effector CD8 T cells contain very high levels of perforin and granzyme B, two key toxic molecules contained within CTL granules. In contrast, memory CD8 T cells contain very little or none (4, 5). It is possible, however, that memory cells rapidly up-regulate these molecules upon Ag encounter. This possibility is consistent with our observation that memory CD8 T cells



**FIGURE 3.** In vivo and in vitro comparisons of effector (Eff) and memory (Mem) CD8 T cell cytotoxicity. *A*, Ag-specific CD8 T cells and unpulsed target cells in the spleen were enumerated to determine an in vivo E:T ratio which is plotted against the percent killing observed in the same spleen. *B*,  $1 \times 10^7$  GP33 specific effector (day 7) or memory (day 40–330) splenic CD8 T cells were transferred into naive mice. Targets ( $5 \times 10^6$ ) were transferred 2 h later, and killing was measured in the spleen 4 h later. Tetramer stains and target cell plots are shown from the same mouse. Numbers on tetramer stains indicate the percentage of CD8 T cells that are tetramer<sup>+</sup>. Target cell histograms are gated on PKH26<sup>+</sup> cells, and numbers indicate the percent of target cells killed. *C*, Effector (day 8) and memory (day 60) CD8 T cells were compared as described in *B* at an E:T ratio of 0.2:1 by transferring  $1 \times 10^6$  T cells and  $5 \times 10^6$  targets. *D*, GP33-specific effector (day 7) or memory (day 50) CD8 T cell lytic activity was measured by  $^{51}\text{Cr}$  release assay. E:T indicates the ratio of tetramer<sup>+</sup> T cells to targets. *E*, Measurement of cytotoxicity by detecting the induction of caspase activity in target cells. NP396-specific effector (day 8) or memory (day 30) CD8 T cells were incubated with peptide-pulsed or unpulsed splenocyte targets for the indicated times, and caspase activity was detected with a fluorogenic caspase substrate. Histograms are gated on PKH26<sup>+</sup> target cells. Numbers indicate the percent killed. E:T ratio was 4:1, corrected to Ag-specific T cells.

killed *lpr* and *TNFR<sup>-/-</sup>* targets as well as normal targets (data not shown), and it is likely that memory CTL use the granule exocytosis pathway for this rapid *in vivo* killing. Unlike the <sup>51</sup>Cr release assay, which requires membrane disruption and release of salts into the culture supernatant to detect killing, killing *in vivo* is most likely scored by the phagocytic removal of killed target cells. Indeed, one of the earliest events in the apoptotic cascade is the exposure of surface molecules, such as phosphatidylserine, that mark the dying cell for phagocytosis. Our data argue that, despite their inability to induce sufficient membrane damage to allow <sup>51</sup>Cr release, memory CTL can very rapidly induce alterations in the target cell that mark it for phagocytic removal. Therefore, it is possible that memory CTL deliver low doses of perforin and granzyme that are sufficient to mark a splenocyte target to be scavenged and score a kill *in vivo*, but not enough to induce rapid <sup>51</sup>Cr release from cell lines *in vitro*.

Our results showing rapid *in vivo* killing by memory CD8 T cells are not limited to the LCMV system because we also observed rapid *in vivo* killing in vesicular stomatitis virus-immune mice >200 days postinfection (data not shown). In addition, Byers et al. (16) have demonstrated rapid *in vivo* killing by polyoma virus-specific memory CD8 T cells. The speed at which memory CD8 T cells acquire lytic competence and kill infected cells upon secondary infection is a critical aspect of memory CD8 T cell-mediated protective immunity, because a long lag time between reinfection and CTL-mediated removal of infected cells could result in the release and spread of substantial viral progeny. Our data indicate that memory CD8 T cells can acquire cytotoxic activity very rapidly and that they may contribute significantly to even the earliest control of viral replication by killing infected cells.

## Acknowledgments

We thank Anthony M. Byers and Dr. Aron E. Lukacher for useful discussions and sharing unpublished data. We thank Drs. Shane Crotty, Dave Masopust, Thandi Onami, and John Shires for their careful review of the manuscript and

Bogna Konieczny, Yelena Blinder, and John Glidewell for their excellent technical assistance.

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