

Immediate Cytotoxicity But Not Degranulation Distinguishes Effector and Memory Subsets of CD8⁺ T Cells

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Abstract

CD8⁺ T cells play a central role in the resolution and containment of viral infections. A key effector function of CD8⁺ T cells is their cytolytic activity toward infected cells. Here, we studied the regulation of cytolytic activity in naive, effector, and central versus effector memory CD8⁺ T cells specific for the same glycoprotein-derived epitope of lymphocytic choriomeningitis virus. Our results show that the kinetics of degranulation, assessed by a novel flow cytometric based assay, were identical in effector and both subsets of memory CD8⁺ T cells, but absent in naive CD8⁺ T cells. However, immediate cytolytic activity was most pronounced in effector T cells, low in effector memory T cells, and absent in central memory T cells, correlating with the respective levels of cytolytic effector molecules present in lytic granules. These results indicate that an inherent program of degranulation is a feature of antigen-experienced cells as opposed to naive CD8⁺ T cells and that the ability of CD8⁺ T cells to induce target cell apoptosis/death is dependent on granule protein content rather than on the act of degranulation itself. Furthermore, these results provide a potential mechanism by which central memory CD8⁺ T cell-mediated death of antigen-presenting cells within the lymph node is avoided.

Key words: cytotoxic T cell • central memory • effector memory • lytic granules • virus

Introduction

CD8⁺ T lymphocytes recognize, via their TCR, processed peptides usually originating from intracellular proteins that are presented on the cell surface bound to MHC class I molecules. Activation of naive CD8⁺ T cells in secondary lymphoid organs induces a developmental program that leads to proliferation and differentiation into effector and memory cells (1, 2). Effector CD8⁺ T cells exhibit direct ex vivo effector function, such as cytotoxicity and cytokine production, upon TCR engagement (3, 4). Memory CD8⁺ T cells exhibit only minimal ex vivo cytolytic activity if compared with effector cells but are able to produce cytokines with equivalent kinetics to effector CD8⁺ T cells (3, 4). Two subsets of memory CD8⁺ T cells have been described according to their recirculation pattern (5, 6) as follows: (a) central memory T cells that express lymph node homing markers, such as CCR7 and CD62L, and preferentially reside in secondary lymphoid organs, and (b) effector memory T cells devoid of LN homing markers and

preferentially localized in peripheral tissue. Both subsets of memory cells can be found in the blood and in the spleen. Central and effector memory CD8⁺ T cells have initially been described to have different kinetics in acquisition of effector functions (5, 7). However, several recent analyses have shown that purified central and effector CD8⁺ T cells exhibit only minimal differences in their ability to exert immediate effector functions, such as cytotoxicity and cytokine production (4, 8, 9).

Cytolytic activity mediated by CD8⁺ T cells is crucial for the resolution/containment of viral infections, in particular for infections with noncytopathic viruses (10, 11). CD8⁺ T cells mediate cytolytic activity against target cells via two major pathways: the granule exocytosis pathway, involving the directed release of granule contents such as perforin and granzymes toward the target cell, and a granule-independent pathway involving the ligation of death receptors on the target cell (e.g., fas/fasL interaction; for review see reference

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Abbreviations used in this paper: APC, allophycocyanin; CFSE, carboxyfluorescein diacetate succinimidyl ester; CHX, cyclohexamide; grB, granzyme B; LAMP, lysosomal associated membrane protein; LCMV, lymphocytic choriomeningitis virus.

12). The granule-dependent pathway does not require de novo synthesis of effector molecules, but relies on the presence of stored effector molecules in lytic granules (for review see reference 13). Lytic granules are membrane-bound secretory lysosomes containing a dense core of various effector molecules (perforin and granzymes). The membrane of lytic granules contains lysosomal-associated glycoproteins (LAMPs) such as CD107a (LAMP-1), CD107b (LAMP-2), and CD63 (LAMP-3; reference 14). These glycoproteins are usually not expressed on the plasma membrane of T lymphocytes, but cell surface expression has been observed after activation of T cells with PHA or ionomycin (15, 16). It has been shown recently that antigen-specific activation of human peripheral CD8⁺ T lymphocytes leads to cell surface expression of CD107a and CD107b, which can be followed by flow cytometric analysis (17). Cell surface expression of CD107a and -b occurred rapidly after antigen encounter, was associated with release of lytic granule proteins (perforin and granzyme B [grB]), and was inhibited by the microtubule inhibitor colchicine, indicating that antigen-induced expression of CD107a and -b was identifying T cells that had degranulated and released effector proteins from their lytic granules (17). Furthermore, it was shown in mice that activation of CD8⁺ T cells leads to up-regulation of CD107 on the cell surface (18).

Based on this novel assay, we analyzed the kinetics of degranulation, release, and production of effector proteins and cytolytic potential in defined populations of murine virus-specific CD8⁺ T cells. These populations included naive TCR transgenic CD8⁺ T cells specific for the lymphocytic choriomeningitis virus (LCMV) GP peptide epitope gp33–41, effector CD8⁺ T cells from acutely LCMV-infected mice, and central and effector memory CD8⁺ T cells with the same antigen specificity.

Materials and Methods

Mice. Transgenic mice expressing a TCR specific for LCMV peptide gp33–41 (P14 tg) presented in association with H2-D^b and transgenic mice expressing a TCR specific for LCMV peptide gp64–80 (Smarta tg) have been described previously (19, 20). C57BL/6 (B6) mice were purchased from Harlan and kept under specific pathogen-free conditions. Animal experiments were performed according to the regulations of the cantonal veterinary office.

Virus and Peptides. The LCMV isolate WE was provided by R.M. Zinkernagel (University Hospital, Zürich, Switzerland) and was propagated on L929 fibroblast cells at low multiplicity of infection. Mice were infected i.v. with 200 PFU LCMV-WE. The LCMV glycoprotein peptides amino acids 33–41 (gp33 peptide, KAVYNFATM) and 64–80 (gp64 peptide, GPDYKGVYQFKSVEFD) were purchased from Neosystem.

Antibodies and Peptide MHC Class I Tetramers. Allophycocyanin (APC)- or PE-conjugated peptide-MHC class I tetrameric complexes were generated as described previously (21). The following anti-mouse monoclonal antibodies and APC-conjugated streptavidin were purchased from BD Biosciences: anti-CD107a (FITC), anti-IFN γ (FITC, PE, or APC), anti-CD8 (peridinin chlorophyll protein), anti-CD4 (peridinin chlorophyll protein), anti-CD49d (biotin), and anti-CD62L (APC). Staining for grB

was performed using PE-labeled anti-human grB (Caltag). The specificity of this reagent for mouse grB has been confirmed previously (4).

Cell Stimulation. Spleen cells were stimulated in vitro with 1 μ g/ml gp33 peptide in 1 ml RPMI 1640 medium containing 10% FCS and penicillin/streptomycin. FITC-conjugated anti-mouse CD107a and Monensin A (Sigma-Aldrich) was added to the cells during the stimulation period. In every experiment, a negative control (no addition of peptide) was included to control for spontaneous production of cytokine and/or CD107a expression. In some experiments, cycloheximide (Sigma-Aldrich) was present at 20 μ g/ml for the duration of stimulation.

Immunofluorescent Staining and Analysis. After the indicated stimulation periods, cells were washed once and surface stained at 4°C with directly labeled antibodies, peptide MHC class I tetramers, or primary biotinylated antibodies, followed by washing and incubation with APC-conjugated streptavidin. Cells were washed once and fixed/permeabilized using 500 μ l of FIX/perm solution (FACSLyse™ [BD Biosciences] diluted to 2 \times concentration with H₂O and 0.05% Tween 20 [Sigma-Aldrich]). Cells were washed once and incubated at room temperature with directly conjugated antibodies specific for intracellular proteins. Cells were washed and resuspended in PBS containing 1% paraformaldehyde (Sigma-Aldrich). Four-color flow cytometric analysis was performed using a FACSCalibur™ flow cytometer (BD Biosciences) with CELLQuest™ software (BD Biosciences). List mode data were analyzed using WinList software (Verity Software House, Inc.). In all cases, at least 100,000 events were collected for analysis.

Isolation of T Cell Subsets. CD62L positive and CD62L negative lymphocytes were purified using anti-CD62L magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions. The purity of samples ranged from 91 to 96% for CD62L negative cells and from 83 to 95% for CD62L positive cells.

Direct Ex Vivo Cytotoxicity Assay. ⁵¹Cr release assays were used for the determination of LCMV-gp33-specific cytotoxicity ex vivo on EL4 target cells as described previously (22). In all cases, the starting E/T ratio was adjusted to obtain identical ratios of D^b-gp33-specific CD8⁺ T cells to target cells. Spontaneous release was <25% in all assays shown.

In Vivo Cytotoxicity Assay. Splenocytes and LN cells from naive B6 mice were costained with PKH26 (Sigma-Aldrich) and either 5 μ M or 0.5 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) according to the manufacturer's instructions. The labeled cells were pulsed with 10⁻⁶ M gp33 peptide for 1 h at 37°C or incubated in medium, washed three times with PBS, mixed at equal amounts, and a total of 10⁷ cells were adoptively transfused i.v. into acutely LCMV-infected mice, into LCMV memory mice, or into naive control recipients. At the indicated time points, lymphocytes were isolated from the blood, spleen, and LN. Target cells were distinguished from recipient cells by gating on PKH26 positive cells. Gp33-loaded and unloaded target cells were distinguished according to the CFSE fluorescence intensity. The percentage of specific target cell elimination was calculated as follows: 100 - [(% peptide pulsed in infected recipients)/(% unpulsed in infected recipients)]/(% peptide pulsed in uninfected recipients)/(% unpulsed in uninfected recipients)] \times 100.

Results

Rapid Degranulation of Effector and Memory CD8⁺ T Cells after Antigen Encounter. To compare the capacity of antigen-specific naive, effector, and memory CD8⁺ T cells to

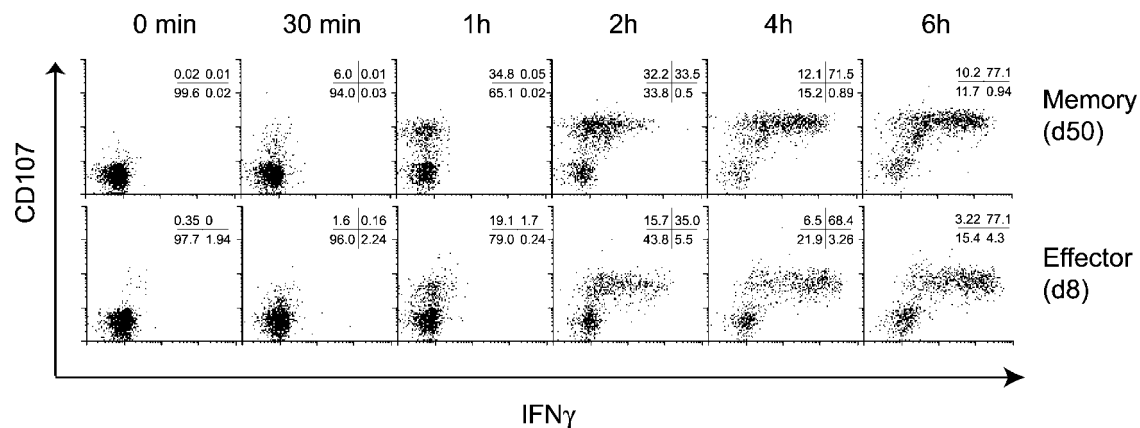


Figure 1. Kinetics of cell surface expression of CD107 and production of intracellular IFN γ . Spleen cells of acutely infected mice (“Effector,” day 8 after i.v. infection with 200 pfu LCMV WE) or memory mice (50 d after i.v. infection with 200 pfu LCMV WE) were incubated with 1 μ g/ml gp33 peptide. At the indicated time points, surface expression of CD107a and the intracellular content of IFN γ was analyzed by flow cytometry. Plots are gated on CD8⁺ gp33 tetramer⁺ T cells. One representative staining of at least six different experiments is shown.

degranulate upon antigen encounter, we used either naive TCR transgenic (P14 tg, specific for LCMV gp33–41) CD8⁺ T cells or CD8⁺ T cells from acutely infected C57BL/6 mice (day 8, 200 PFU of LCMV WE) or from memory mice (day 50, 200 PFU of LCMV WE). To examine the kinetics of activation of LCMV gp33-specific CD8⁺ T cells, we measured cytokine production (IFN γ) and degranulation by cell surface modulation of CD107a (LAMP-1). LCMV gp33-specific CD8⁺ T cells were identified by tetramer staining (Fig. 1). Effector and memory cells exhibited the same degranulation kinetics; degranulation was detectable after 30 min of peptide stimulation and increased up to 4 h after stimulation. IFN γ synthesis was slower than degranulation and required 2 h of peptide stimulation. Similar kinetics of degranulation and IFN γ production have been reported for human T cell responses (17). Naive P14 tg CD8⁺ T cells neither expressed cell surface CD107 nor intracellular IFN γ within 6 h of peptide stimulation (not depicted).

Next, we analyzed whether antigen-induced cell surface modulation of CD107 was exclusively observed in CD8⁺

T cells or also in CD4⁺ T cells. Therefore, we stimulated naive TCR tg CD4⁺ T cells (Smarta tg), CD4⁺ T cells from acutely infected C57BL/6 mice, or from memory mice with the MHC class II-restricted gp64 peptide and analyzed CD107 and IFN γ expression up to 6 h after stimulation. Naive Smarta tg CD4⁺ T cells neither expressed CD107 nor IFN γ and acute and memory gp64-specific CD4⁺ T cells expressed IFN γ but little if any CD107 (not depicted). Hence, for LCMV-specific T cells, antigen-induced abundant surface expression of CD107 is a specific feature of CD8⁺ T cells.

Degranulation of LCMV-specific CD8⁺ T Cells Is Not Necessarily Correlated with Cytotoxic Activity. Because effector and memory cells degranulated with similar kinetics, we compared the cytotoxic potential of effector and memory (day 50 after infection) cells in ⁵¹Cr release assays (Fig. 2). As expected, effector CD8⁺ T cells exhibited stronger cytotoxic potential as compared with memory CD8⁺ T cells. This discrepancy between degranulation and lytic effector function could be related to differences in lytic granule

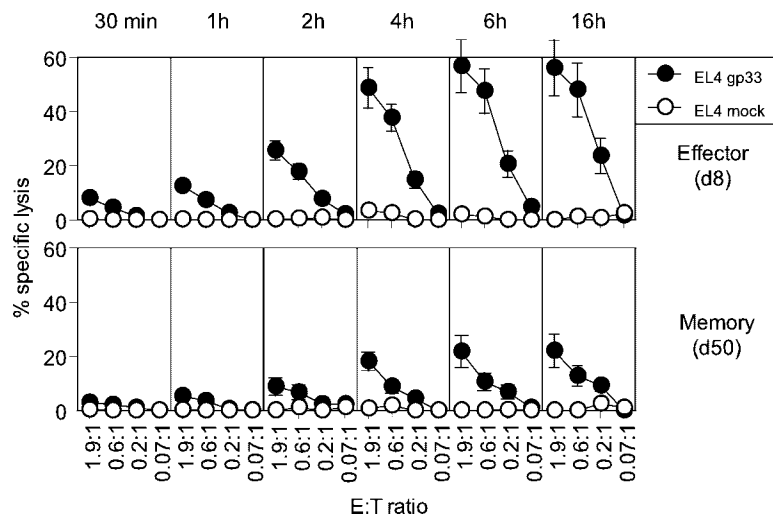


Figure 2. Kinetics of cytotoxic activity of effector and memory gp33-specific T cells. Cytotoxic activity of spleen effector and memory CD8⁺ T cells was analyzed by ⁵¹Cr release assay. EL4 cells, pulsed with gp33 peptide or unpulsed, were used as target cells. Specific lysis was determined at the indicated time points of incubation. E/T ratios indicate the ratio between gp33 tetramer positive T cells and target cells. One of four similar experiments is shown.

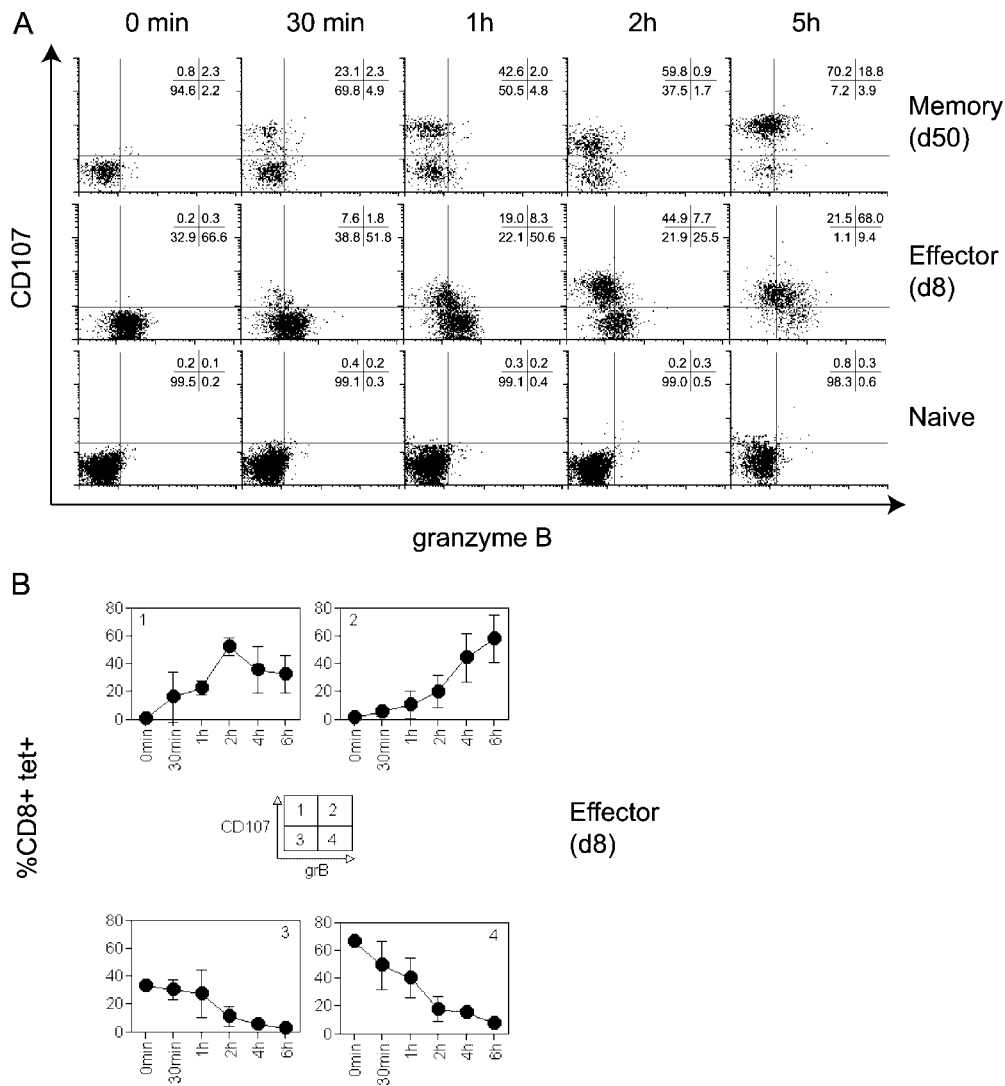


Figure 3. Kinetics degranulation and grB content of naive, effector, and memory gp33-specific T cells. (A) Spleen cells from memory (d50), acutely infected, or naive P14 transgenic mice were stimulated with 1 μ g/ml gp33 peptide. At the indicated time points, cell surface expression of CD107a and intracellular grB content was analyzed by flow cytometry. Plots are gated on CD8⁺ gp33 tetramer⁺ T cells. One of three similar experiments is shown. (B) Acquisition of cell surface CD107 and changes in grB content in stimulated effector CD8⁺ T cells. The plots 1–4 show the percentage of CD8⁺ tet⁺ T cells present in the four quadrants (1, CD107⁺grB⁻; 2, CD107⁺grB⁺; 3, CD107⁻grB⁻; and 4, CD107⁻grB⁺) after different time points of stimulation.

content. Therefore, we analyzed the kinetics of degranulation by staining for CD107 in context with intracellular grB expression in memory, effector, and naive CD8⁺ T cells (Fig. 3). Effector CD8⁺ T cells contained substantial amounts of intracellularly stored grB (66 \pm 5% of CD8⁺tet⁺ cells). Activation of effector CD8⁺ T cells induced degranulation, as identified by surface CD107 staining that coincided with loss of grB staining, indicating that effector CD8⁺ T cells released cytotoxic effector molecules immediately upon antigen encounter (Fig. 3 B). In similar experiments conducted with human CD8⁺ T cells, degranulation could also be correlated with loss of intracellularly stored grB and in addition to loss of perforin (17).

In contrast to effector CD8⁺ T cells, memory CD8⁺ T cells showed only marginal staining for grB, which explained (despite rapid degranulation) the insufficient cytolytic effector function upon antigen encounter. Naive P14 tg CD8⁺ T cells neither degranulated nor contained intracellular grB. Interestingly, after 5 h of stimulation, effector and memory gp33-specific CD8⁺ T cells showed a significant increase in grB content, particularly in cells that co-

stained for CD107. This suggested that both effector and memory CD8⁺ T cells began de novo synthesis of grB after antigen stimulation. To address this hypothesis, effector and memory (d50) CD8⁺ T cells were stimulated in the presence of the protein synthesis inhibitor cyclohexamide (CHX), and the kinetics of degranulation, grB staining, and IFN γ production were analyzed (Fig. 4). Cell surface expression of CD107 was not significantly affected by the presence of CHX, indicating that cell surface expression of CD107 originated from translocated CD107 molecules rather than from newly synthesized molecules. In the presence of CHX, no IFN γ synthesis was observed in stimulated effector and memory CD8⁺ T cells. CD107 positive cells stained negative for grB for the first 2 h after activation, indicating the release of stored grB. However, at 4 and 6 h after stimulation, gp33-specific effector and memory CD8⁺ T cells showed a significant increase in grB staining in the absence of CHX, whereas only a reduced increase in grB staining was observed in the presence of CHX (Fig. 4).

These data show that degranulation initially releases stored effector molecules that differ in quantity between ef-

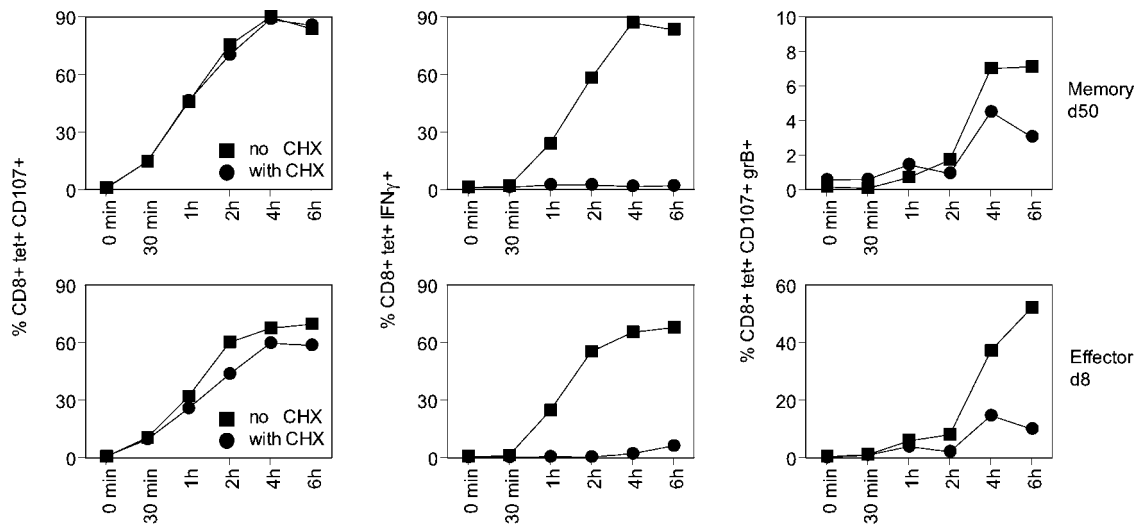


Figure 4. Cell surface expression of CD107a, intracellular IFN γ production, and grB content was analyzed at the indicated time points in effector and memory (d50) gp33-specific CD8⁺ T cells in the presence or absence of CHX. Plots are gated on CD8⁺ gp33 tetramer⁺ T cells. One of two equivalent experiments is shown.

effector and memory CD8⁺ T cells, but that de novo synthesis of effector molecules occurs after 4–6 h of antigen stimulation, allowing lytic granules to refill. These results are in agreement with previous findings that TCR engagement induced refilling of lytic granules in antigen-experienced CD8⁺ T cells (23).

Degranulation Occurs with Similar Kinetics in Effector and Central Memory Cells and Is Temporarily Linked to Down-Regulation of CD62L in Central Memory Cells. We had observed that memory cells expressing CD107 after activation were always CD62L negative (unpublished data). Therefore, we examined whether acquisition of CD107 was linked to down-regulation of the LN homing marker CD62L. CD62L positive and negative cells were sorted from the spleens of LCMV immune memory mice (day 50) and separately stimulated in vitro. Surface expression of CD107 and CD62L was analyzed over an observation period of 6 h (Fig. 5 A). Within 30 min, a substantial proportion of the LCMV gp33-specific CD8⁺ T cells expressed CD107 and this proportion increased over the first 2 h of observation. Acquisition of CD107 (i.e., degranulation) in sorted CD62L positive central memory T cells was associated with down-modulation of cell surface CD62L. This showed that activation leads to degranulation and to down-modulation of LN homing markers on the cell surface of CD8⁺ T cells and, thus, to a transition from central memory to effector memory cells. Similar results were obtained with memory mice up to 250 d after infection.

The ability of effector memory T cells to infiltrate inflamed peripheral tissue is dependent on the expression of adhesion molecules such as integrins and selectins. The integrin $\alpha_4\beta_1$ (VLA-4; CD49d/CD29) binds to fibronectin, VCAM-1, and MadCAM-1 and is one of the principal surface proteins that mediate homing of lymphocytes to endothelium at peripheral sites of inflammation (24, 25). Therefore, we analyzed the expression of CD49d, the integrin α_4

chain, on the surface of d50 unstimulated and stimulated central memory LCMV-specific CD8⁺ T cells (Fig. 5 B). Interestingly, unstimulated as well as stimulated CD62L⁺ gp33 tetramer⁺ CD8⁺ T cells expressed CD49d. Similar results were obtained for central memory cells up to day 250 after infection.

To further address the question whether cell surface up-regulation of CD107 results from relocalization of intracellularly present CD107, we compared cell surface with intracellular CD107 staining in central and effector memory CD8⁺ T cells and in effector CD8⁺ T cells before antigen stimulation (Fig. 5 C). Although no CD107 expression was detected on the cell surface, a strong staining for CD107 was observed intracellularly, supporting the notion that cell surface expression of CD107 after activation occurs by CD107 relocalization from intracellular lytic granules to the plasma membrane.

CD62L Positive Central Memory Cells Are Less Cytolytic than CD62L Negative Effector Memory T Cells Due to the Lack of Cytolytic Effector Molecules. Next, we analyzed the kinetics of degranulation, immediate cytolytic effector function and cytokine production of LCMV-specific central and effector memory CD8⁺ T cells. Our results demonstrate that LCMV-gp33-specific central end effector memory CD8⁺ T cells have similar kinetics of CD107 up-regulation and IFN γ production (Fig. 6 A). However, sorted CD62L negative but not CD62L positive cells were able to exert low level of immediate cytotoxicity in a 6-h ⁵¹Cr release assay when equivalent numbers of gp33 tetramer positive central and effector memory cells were used (Fig. 6 B). Consistent with this result, we observed more intracellularly stored grB in effector memory T cells than in central memory T cells (Fig. 6 B).

Next, we analyzed the grB content in d8 effector CD8⁺ T cells and in memory CD8⁺ T cell subsets at different time points after infection (Fig. 6 C). Although >60% ef-

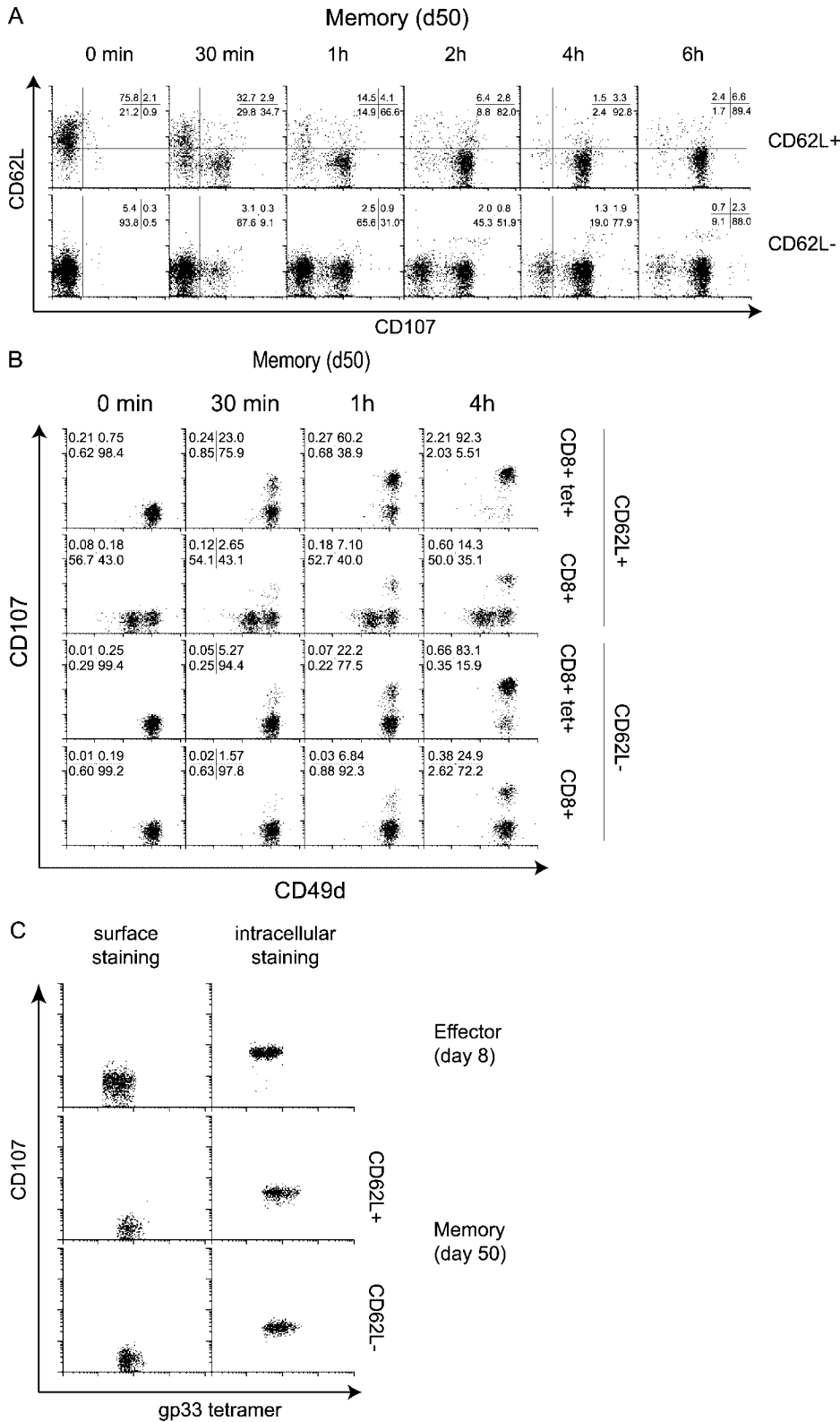


Figure 5. Degranulation and surface expression of CD62L and CD49d of central and effector gp33-specific memory T cells. (A) CD62L positive and CD62L negative cells were sorted from the spleen of LCMV memory mice (day 50) and stimulated with 1 μ g/ml gp33 peptide. After the indicated time points, cell surface expression of CD107a and CD62L was analyzed by flow cytometry. Plots are gated on CD8⁺ gp33 tetramer⁺ T cells. One of three similar experiments is shown. (B) CD62L positive and CD62L negative cells were sorted from the spleen of LCMV memory mice (day 50) and stimulated with 1 μ g/ml gp33 peptide. After the indicated time points, cell surface expression of CD107a and CD49d was analyzed by flow cytometry. Plots are either gated on CD8⁺ gp33 tetramer⁺ T cells or on CD8⁺ T cells. One of three similar experiments is shown. (C) Cell surface versus intracellular staining of CD107a. CD62L positive and CD62L negative cells were sorted from the spleen of LCMV memory mice (day 50). Sorted memory cells and day 8 effector cells were stained extracellularly or intracellularly for CD107a. Plots are gated on CD8⁺ T cells. One of two similar experiments is shown.

effector T cells expressed grB, fewer memory cells expressed grB before stimulation. The overall percentage of grB-expressing memory cells decreased with time after infection; however, at all time points analyzed, more grB-expressing

effector memory CD8⁺ T cells were observed than grB-expressing central memory T cells.

Thus, although central and effector memory T cells produce cytokines and degranulate with identical kinetics,

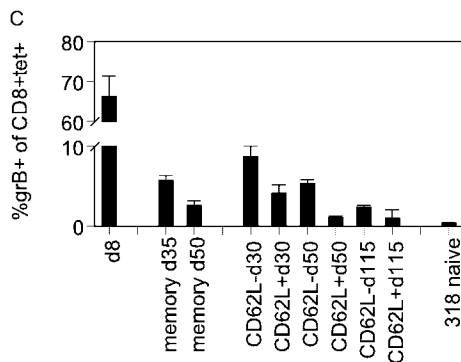
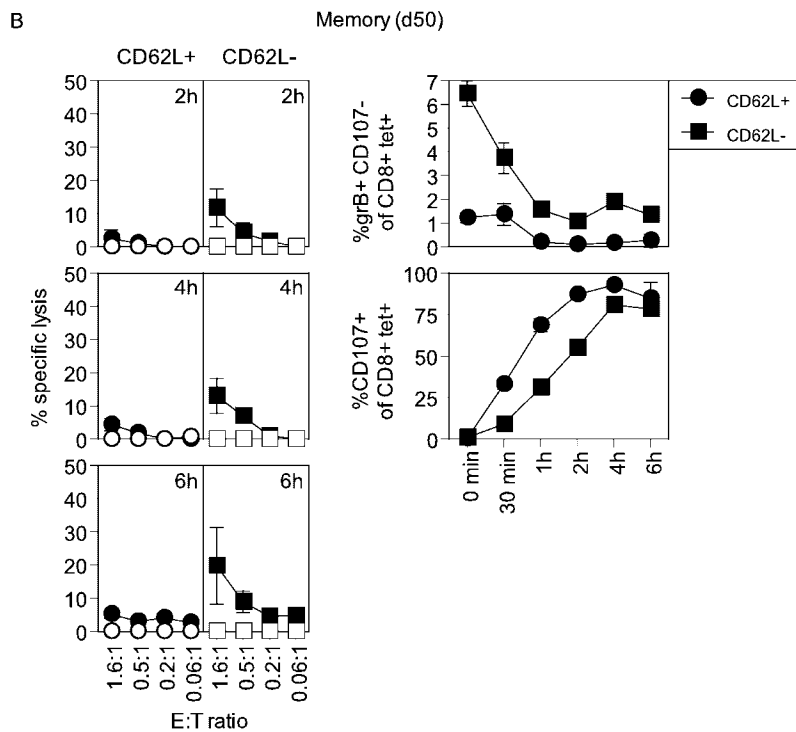
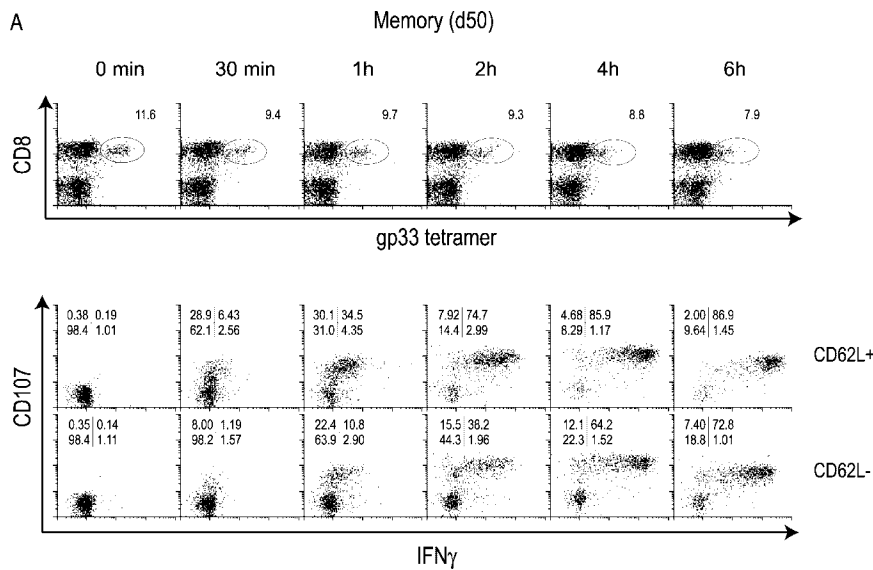


Figure 6. Kinetics of degranulation, IFN γ production, and cytotoxicity of gp33-specific central and effector memory T cells. (A) Tetramer staining, cell surface CD107a expression, and intracellular IFN γ production was analyzed at the indicated time points in sorted CD62L positive and negative cells from LCMV memory mice (day 50). The top row shows staining of CD62L⁺ T cells (day 50) for CD8 and gp33 tetramer after the indicated time points of stimulation. The two bottom rows show staining for cell surface CD107 and intracellular IFN γ . Plots are gated on CD8⁺ gp33 tetramer⁺ T cells. One of three similar experiments is shown. (B) Cytotoxic activity of sorted CD62L positive and CD62L negative spleen cells of memory mice (day 50) was analyzed by ⁵¹Cr release assay. EL4 cells, pulsed with gp33 peptide or unpulsed were used as target cells. Specific lysis was determined at the indicated time points of incubation. E/T ratios indicate the ratio between gp33 tetramer positive T cells and target cells. The graphs on the right side show the percentage of grB⁺CD107⁻ and CD107a⁺ cells of CD8⁺ tet⁺ T cells at the indicated time points after stimulation. One of three similar experiments is shown. (C) Percentage of grB⁺ cells of CD8⁺ tet⁺ cells. grB positive cells were quantified before antigen stimulation amongst CD8⁺ tet⁺ T cells of d8 effector mice, of memory mice (days 35 and 50), of sorted CD62L⁺ and CD62L⁻ memory cells (days 30, 50, and 115), and of naive TCR transgenic mice.

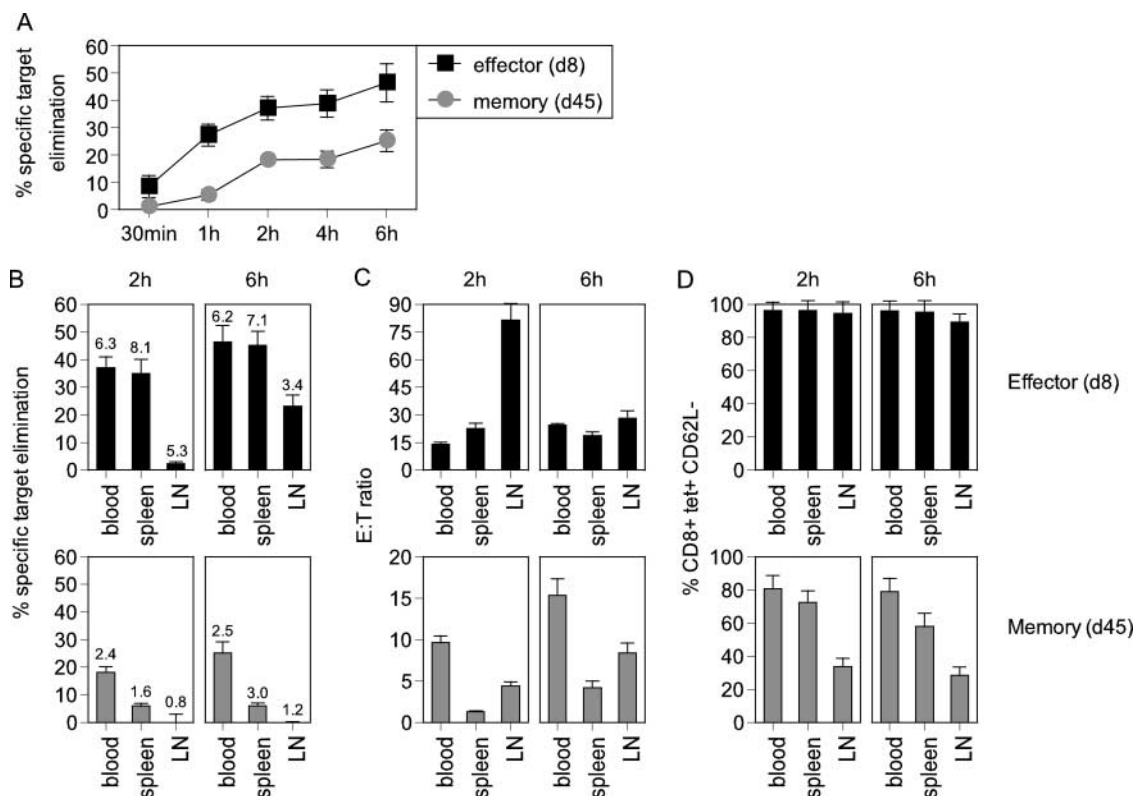


Figure 7. In vivo cytotoxicity of acutely LCMV-infected mice and memory mice. (A) gp33 peptide-loaded and unloaded target cells were adoptively transferred into acutely infected or memory recipient mice. Percentage of specific target cell elimination was analyzed in the blood at the indicated time points. (B) Specific target cell elimination was quantified in blood, spleen, and LN at 2 and 6 h after adoptive transfer of target cells. Numbers indicate the percentage of gp33 tetramer positive lymphocytes in the respective organs. (C) E/T ratio in blood, spleen, and LN at 2 and 6 h after adoptive transfer of target cells. Effector cells were gp33 tetramer positive lymphocytes. (D) Percentage of CD8⁺gp33 tetramer⁺CD62L⁺ cells in blood, spleen, and LN at 2 and 6 h after adoptive transfer of target cells. One of two similar experiments is shown.

only effector memory T cells exhibited immediate cytolytic activity.

In Vivo Cytotoxicity Is Reduced in LN Compared With Blood and Spleen In Memory Mice. Our results showed that activation of CD62L positive central memory CD8⁺ T cells resulted in rapid degranulation with concordant down-modulation of cell surface CD62L. Therefore, activated central memory cells might emigrate from the LN, their preferential localization site, and migrate to peripheral tissues before they acquire cytolytic potential. This would implicate a reduced immediate cytolytic activity against antigen-presenting cells within the LN. To test this hypothesis, we transfused gp33 peptide-loaded and unloaded antigen-presenting cells into acutely LCMV-infected recipients (d8 effector mice) or in LCMV memory mice. The target cell populations were uniformly labeled with PKH-26 and with two different concentrations of CFSE to differentiate between peptide-loaded and unloaded target cells. At several time points after transfer, specific elimination of gp33-loaded target cells was assessed in the blood, in the spleen and the LN. Acutely infected mice were faster and more efficient in specific target cell elimination measured in the blood as compared with memory mice (Fig. 7 A), as has been shown previously (26). For example, by 1 h after

transfer of target cells, acutely infected mice had eliminated almost 30% of gp33-loaded target cells, whereas memory mice had eliminated <5%. Because the frequencies of gp33 tetramer positive CD8⁺ T cells were 2–3 times higher in effector compared with memory mice, this difference represents at least one reason for faster and more efficient in vivo elimination of antigen-presenting cells.

In acutely infected mice, specific target elimination in blood and spleen was comparable at 2 and 6 h after target cell transfer (Fig. 7 B), whereas it was slower and less prominent in the LN. In LCMV memory mice, specific target cell elimination was most prominent in the blood but completely absent in the LN. This reduced killing in the LN of effector mice and in particular the absence of specific killing in the LN of memory mice could partially be accounted for by the relatively lower frequencies of tetramer⁺ CD8⁺ T cells in the LN compared with blood and spleen (Fig. 7 B, numbers above bars). However, this difference was maximally 1.5–3-fold. Alternative explanations for the reduced killing in LN could be a low E/T ratio. We calculated the effector (gp33 tetramer positive lymphocytes)-to-target ratios in the different organs (Fig. 7 C); the E/T ratios in the LN were always higher compared with spleen.

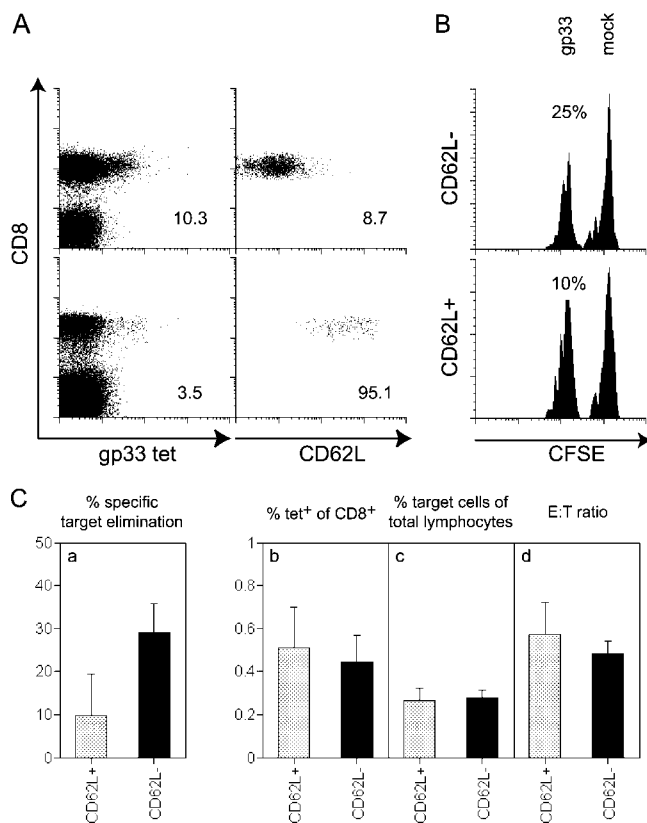


Figure 8. In vivo cytotoxicity of adoptively transfused gp33-specific central and effector memory cells. (A) CD62L positive and negative CD8 T cells were purified from d35 LCMV memory mice. (left) gp33 tetramer staining: numbers indicate the percentage of CD8⁺ tet⁺ T cells. (right) CD62L expression on tetramer-gated CD8⁺ T cells. (B) Target cell numbers in LN 4 h after transfer. 3×10^5 CD62L positive or CD62L negative CD8⁺ tet⁺ T cells were transferred into naive recipient mice followed by transfer of 5×10^6 gp33-pulsed and mock-pulsed CFSE/PKH26 stained target cells 3 h later. CFSE^{low} cells were pulsed with gp33 peptide and CFSE^{high} cells were mock pulsed. 4 h after target cell transfer, the ratio of CFSE^{low/high} target cells was analyzed in the LN. One representative staining of each group is shown. Cells are gated on PKH26 positive cells. Numbers indicate the percentage of specific target cell elimination. (C) Specific target cell elimination (a) 4 h after target cell transfer into recipients having been transfused with equal numbers of CD62L positive or CD62L negative LCMV memory T cells. (b) Percentage of CD8⁺ tet⁺ T cells 4 h after target cell transfer. (c) Percentage of PKH26⁺ target cells 4 h after target cell transfer. (d) E/T ratio (E = percentage of tetramer⁺ lymphocytes; T = percentage of PKH26⁺ lymphocytes) 4 h after target cell transfer.

In memory mice, the percentage of gp33-specific T cells that were CD62L negative (i.e., effector or effector memory T cells) in blood, spleen, and LN (Fig. 7 D) correlated best with the observed pattern of specific killing in vivo, suggesting that the predominantly CD62L positive gp33-specific CD8⁺ T cells in the LN of memory mice were performing worst with respect to immediate cytolytic activity.

To experimentally address this hypothesis, we adoptively transfused equal numbers of gp33 tetramer positive purified central and effector memory cells (Fig. 8 A) into separate naive recipient animals, followed by transfer of equal numbers of gp33-labeled and unlabeled target cells. Specific target cell elimination was analyzed 4 h after target cell trans-

fer (Fig. 8 B). Indeed, we observed that CD62L negative effector memory cells exhibited higher levels of specific killing compared with CD62L positive central memory cells (Fig. 8 C). These in vivo results corroborate the in vitro findings that CD62L negative effector memory cells show higher levels of immediate cytotoxicity as compared with central memory cells (Fig. 6 B).

Discussion

In this paper, we analyze the kinetics of antigen-induced degranulation, cytotoxic activity, and effector protein production in defined populations of LCMV-specific CD8⁺ T cells. We compare naive TCR tg CD8⁺ T cells, effector CD8⁺ T cells, and central and effector memory CD8⁺ T cells originating from LCMV-infected mice. Degranulation was analyzed using a novel flow cytometry-based assay that measures the appearance of lytic granule-associated markers on the plasma membrane (17). LCMV-specific effector and memory CD8⁺ T cells degranulated with identical kinetics upon antigen contact. However, only effector T cells showed strong cytolytic activity measured by ⁵¹Cr release assays that was associated with the release of stored cytotoxic effector molecules. Memory CD8⁺ T cells had only marginal amounts of stored effector molecules, resulting in low level immediate cytotoxic activity. Both effector and memory CD8⁺ T cells initiated de novo synthesis of grB 4–6 h after antigen contact, whereas de novo synthesis of IFN γ was already measurable at 2 h after antigen encounter. It has been shown that IFN γ and perforin mRNA is present at equivalent levels in effector and memory CD8⁺ T cells (3, 27) and that regulated on activation, normal T cell expressed, and secreted (RANTES) mRNA is present in memory CD8⁺ T cells (28), indicating that regulation of cytokine, chemokine, and cytolytic effector molecule expression occurred at the translational level. In contrast, naive LCMV-specific CD8⁺ T cells neither degranulated nor produced cytokines or cytolytic molecules within 20 h of antigen stimulation (unpublished data). These findings indicate that a program for immediate degranulation is a feature of antigen-experienced CD8⁺ T cells that is not yet functional in naive CD8⁺ T cells, and that regulation of cytolytic activity occurs at the effector protein level. Hence, degranulation is necessary but not sufficient for performance of cytolytic activity.

Two subsets of memory CD8⁺ T cells can be distinguished by their recirculation pattern and accordingly by the expression of LN homing markers such as CD62L, termed central and effector memory cells (5, 6). These memory CD8⁺ T cell subsets have been ascribed different levels of immediate effector functions (5, 7). However, more recent studies question this differential effector function (4, 8, 9) and propose that effector memory CD8⁺ T cells are a transitory population between effector cells and central memory cells (4, 27, 29). We observed that central and effector memory CD8⁺ T cells degranulated and produced IFN γ with similar kinetics after antigen contact, in-

dicating that these functions were not restricted to effector CD8⁺ T cells. However, a significant number effector memory CD8⁺ T cells contained stored grB that could be released upon antigen encounter, leading to low levels of immediate cytotoxicity. In contrast, only very low numbers of central effector cells had intracellularly stored grB, and as a result, were incapable of immediate cytolytic activity. However, the overall percentage of grB-expressing effector memory cells decreased with time after LCMV infection, suggesting that grB expression in effector memory CD8⁺ T cells might be related to relatively recent antigen exposure. Thus, central memory and effector memory CD8⁺ T cells differ at the functional level primarily in their expression of cytolytic effector molecules, and not in their inherent ability to degranulate in response to stimulation. These data establish a clear difference in immediate cytolytic effector function of central and effector memory T cells, which is in contrast to some papers that did not find any differences in cytolytic potential of central and effector CTL (4, 8). A potential explanation for this difference could be use of TCR transgenic CD8⁺ T cells in these analyses as opposed to endogenous polyclonal CD8⁺ T cells in the present paper; the adoptive transfer of higher numbers of central and effector memory CD8⁺ T cells for in vivo killing experiments (26); or the discrimination of effector and central memory CD8⁺ T cells by CCR7 expression (8) as opposed to CD62L expression in the present paper.

The difference of immediate in vitro cytolytic activity between central and effector memory CD8⁺ T cells might have important in vivo implications with regards to the efficiency of target cell eliminations in different tissues. These in vitro observations led to the hypothesis that central memory CD8⁺ T cells residing in LNs, when activated by cognate antigen, would degranulate but not exert immediate effector function due to lack of stored cytolytic effector molecules. Concomitant down-regulation of CD62L and expression of tissue homing markers, such as CD49d, combined with delayed de novo synthesis of cytolytic effector molecules might result in the emigration of restimulated CD8⁺ T cells from the LN before acquisition of cytolytic effector function, thus avoiding the immediate destruction of the restimulating antigen-presenting cells in the LNs. Analysis of in vivo cytotoxicity (26, 30, 31) in different organs showed that specific target cell elimination was more pronounced in acutely infected mice than in memory

mice. We observed in acutely infected mice that specific target cell elimination was comparable in blood and spleen (in agreement with reference 26), but delayed in LN. In contrast, we did not observe any specific target cell elimination in the LN of memory mice within 6 h, whereas the same mice eliminated target cells from the spleen and blood. Several potential explanations could account for this observation as follows. First, relatively lower numbers of gp33-specific CD8⁺ T cells in LN as compared with blood and spleen. The frequency of gp33-specific CD8⁺ T cells was ~50% in LN compared with spleen, and this reduced percentage might partly be responsible for the delayed/absent killing in the LN. Second, lower E/T ratios were in LN compared with spleen and blood. This explanation was refuted by the finding that E/T ratios were higher in LN than in spleen and comparable to blood. Third, there was reduced cytotoxic potential of predominantly gp33-specific central memory T cells in the LN. This possibility seemed likely because 70% of gp33-specific CD8⁺ T cells were central memory cells in the LN as opposed to blood and spleen, where 70–80% of gp33-specific CD8⁺ T cells were effector memory cells. Fourth, the LN environment could influence the target cell killing. It has been shown that DC target cell elimination in the LN of memory mice occurs relatively slowly compared with acutely primed mice (32); however, in this work, target cell killing was analyzed only in LN, therefore precluding a comparison between different tissues. Furthermore, it has been shown that DCs are partially resistant to CD8⁺ T cell-induced apoptosis due to expression of serpine protease inhibitor 6 (33, 34); however, we have not analyzed whether upon adoptive transfer the target cells present in the LN contained a higher percentage of DCs as compared with blood and LN. The fact that target cell killing occurred in the LN of acutely infected mice would argue for the ability of effector cells to perform cytotoxicity in the LN environment.

To directly assess the hypothesis that effector and central memory cells differ in their immediate cytolytic potential in vivo, we transfused equal numbers of gp33-specific purified central and effector memory CD8⁺ T cells in naive recipients and measured their ability to eliminate gp33-loaded target cells in vivo. In agreement with our in vitro findings, we observed that effector memory CD8⁺ T cells showed enhanced immediate in vivo cytolytic potential as compared with central memory cells with the same speci-

Table I. Features of Naive, Effector, and Central and Effector Memory CD8⁺ T Cells

	Degranulation program	Effector molecules stored in granules	Cytotoxicity (4–6 h)	De novo synthesis of effector molecules (5 h)	IFN γ synthesis (2–4 h)
Naive	–	–	–	–	–
Effector	+++	+++	+++	++	+++
Effector memory	+++	+	+	+	+++
Central memory	+++	–	–	+	+++

ficity. This is in contrast with a recent work showing no in vivo difference in cytolytic potential between LCMV-specific effector and central memory cells (26). Potential explanations for this discrepancy could be the time point after infection used for the isolation of memory cells, the viral strain and the dose used for infection, the numbers of adoptively transfused memory cells, the compartment in which target cell elimination is assessed, and the use of TCR transgenic CD8⁺ T cells as opposed to endogenous polyclonal CD8⁺ T cells.

In summary (Table I), our data show that immediate degranulation upon antigen encounter is a feature of antigen-experienced but not of naive CD8⁺ T cells. Degranulation of effector and memory CD8⁺ T cells occurred with similar kinetics and is not necessarily correlated with immediate cytolytic activity. Immediate cytotoxic activity is dependent on the presence of stored cytotoxic effector molecules in lytic granules. Central and effector memory CD8⁺ T cells degranulated and produced IFN γ with identical kinetics. Effector memory cells as opposed to central memory cells contained small amounts of stored cytotoxic effectors that mediated low level immediate cytotoxicity. This is reflected in vivo by the observation that immediate target cell killing was not detectable in LNs of memory mice containing predominantly central memory T cells, but apparent in spleen and blood, suggesting that this might be a mechanism preventing rapid elimination of restimulating antigen-presenting cells in the LN.

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