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# Differentiation of Naive CTL to Effector and Memory CTL: Correlation of Effector Function with Phenotype and Cell Division<sup>1</sup>

Stephan Oehen<sup>2</sup> and Karin Brduscha-Riem

Phenotypically and functionally, the early steps of T cell differentiation are not well characterized. In addition, the effector T cell stage shares several phenotypic characteristics with memory T cells, which has made the analysis of T cell memory difficult. In this study, we have investigated *in vitro* and *in vivo* the differentiation of naive CTL into effector and memory CTL as a function of cell division using lymphocytic choriomeningitis virus-specific TCR-transgenic spleen cells labeled with the vital dye carboxyfluorescein diacetate, succinimidyl ester. The following major points emerged. 1) During the first nine cell divisions, the investigated cell surface markers were strongly modulated. 2) The TCR was stepwise down-regulated during viral infection. 3) Cytotoxic effector function was acquired within one cell division and was retained during the next four to five divisions. 4) *In vitro*, CTL reached a CD44<sup>high</sup>CD62L<sup>+</sup> memory phenotype after 6–10 cell divisions and required restimulation to exert effector function. 5) Lymphocytic choriomeningitis virus memory mice contained two distinct memory populations: a CD44<sup>high</sup>CD62L<sup>−</sup> population, predominately located in the spleen and exerting rapid effector function, and a CD44<sup>high</sup>CD62L<sup>+</sup> population found in the spleen and the lymph nodes, which had lost immediate effector function. This finding suggests that two types of memory CTL exist. The correlation between CD62L expression, effector function, and Ag persistence is discussed. *The Journal of Immunology*, 1998, 161: 5338–5346.

In response to specific Ag, naive T cells undergo a number of highly complex changes resulting in proliferation, acquisition of effector function, differential homing, and the modulation of various cell surface markers associated with cell function and signal transduction. Phenotypically and functionally, the early steps of T cell differentiation are poorly characterized, and it is still unclear whether memory T cells develop along a separate lineage or whether they differentiate via an effector stage (1). Usually, effector and memory T cells are phenotypically characterized by the expression of selected cell surface markers (2–4). Yet, these classifications are not precise because memory T cells have been shown to display some of the same commonly used markers as effector T cells, including CD44 and CD62L.<sup>3</sup> Thus, in many instances, the effector T cell stage and consequently the memory T cell stage are not clearly defined. Furthermore, it is debated whether memory T cells represent cells that are continuously activated through persisting or cross-reactive Ag (5–7), or whether they represent long-lived terminally differentiated Ag-independent T cells (8–10). The emerging data suggest that T cell memory may be based on both types of memory cells, but that the continuously activated memory T cells are functionally more closely related to

effector cells. Because they are activated, they can develop antiviral effector function immediately, and therefore seem to be more important in memory responses to peripherally localized viral infections and in the clearance of viral infections from solid tissue (4, 11–13).

Using 5 (and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE)-labeled spleen cells from 318 TCR transgenic mice (14) specific for the lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP), the present study aimed at analyzing the differentiation of naive CTL to effector and memory CTL *in vitro* and *in vivo* and to monitor the expression of selected activation and memory markers during differentiation as a function of cell division. The distinct differentiation stages were then correlated *in vitro* and *in vivo* with cytotoxic effector function and CTL phenotype.

## Materials and Methods

### Mice

Six- to 10-wk-old C57BL/6 mice and TCR transgenic 318 mice (14) were bred at the Labortierkunde at University of Zürich (Switzerland) and kept under specific pathogen-free conditions, according to institutional guidelines. 318 mice express transgenic V $\alpha$ 2 and V $\beta$ 8.1 TCR chains specific for the LCMV glycoprotein peptide p33 presented on H-2D<sup>b</sup> on 50–60% of the CD8<sup>+</sup> CTL. 318 mice have been bred onto the C57BL/6 background for at least six generations, and 318 spleen cells are not rejected after transfer into sex-matched C57BL/6 recipients.

### Virus

The LCMV WE strain was used in this study (15). Recombinant vaccinia virus expressing the LCMV GP (vacc GP (16)) was a gift from D. H. L. Bishop (Oxford, U.K.).

### Cell lines

EL-4 thymoma cells (H-2<sup>b</sup>) were grown in Iscove's modified Dulbecco's medium (Life Technologies, Gaithersburg, MD) containing 5% FCS and supplemented with glutamine and streptomycin/penicillin.

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<sup>3</sup> Abbreviations used in this paper: CD62L, CD62 ligand; BSS, balanced salt solution; CFSE, 5 (and 6)-carboxyfluorescein diacetate, succinimidyl ester; FasL, Fas ligand; GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; PE, phycoerythrin; PFU, plaque-forming unit.

### CFSE labeling

CFSE was purchased from Molecular Probes (Eugene, OR). Erythrocytes were removed from spleen cell suspensions by water lysis. The cells were then washed with ice-cold PBS and resuspended at  $5 \times 10^6$  cells/ml in ice-cold PBS. CFSE was kept as a 0.5 mM stock in DMSO and stored at  $-20^\circ\text{C}$  in a desiccator box. Cells were labeled by diluting the 0.5 mM CFSE stock 1000-fold into the cell suspension (final concentration 0.5  $\mu\text{M}$ ) and incubating them for 10 min at  $37^\circ\text{C}$ . For high CFSE fluorescence intensities, cells were labeled at a final concentration of 5  $\mu\text{M}$  by diluting a 5 mM stock 1000-fold. After labeling, FCS was added to 5% final concentration and the cells were immediately centrifuged and washed with ice-cold PBS. For i.v. injection into the tail vein, labeled cells were resuspended in BSS.

### In vitro activation of transgenic T cells

A total of  $3\text{--}5 \times 10^6$  spleen cells/well (24-well plate) or  $10^7$  spleen cells/well (6-well plate) from 318 TCR transgenic mice was labeled with CFSE and stimulated by adding  $2 \times 10^{-8}$  M of specific LCMV GP peptide p33 (KAVYNFATM) to the cultures. For technical reasons, the original position 41 cysteine in p33 was replaced by a methionine to prevent dimer formation. Cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% FCS, penicillin/streptomycin, glutamine, and  $5 \times 10^{-5}$  M 2-ME.

### Adoptive transfer and in vivo activation of 318 CTL

318 spleen cells were labeled with CFSE, as described above. A quantity amounting to  $3\text{--}5 \times 10^7$  labeled 318 spleen cells (318-CFSE) was resuspended in 400  $\mu\text{l}$  of BSS and injected into the tail vein of sex-matched C57BL/6 recipients (318-CFSE $\rightarrow$ B6). After 8–16 h, recipients were infected i.v. with the indicated doses of the LCMV WE strain to activate LCMV GP p33-specific transgenic CTL.

### Flow cytometry

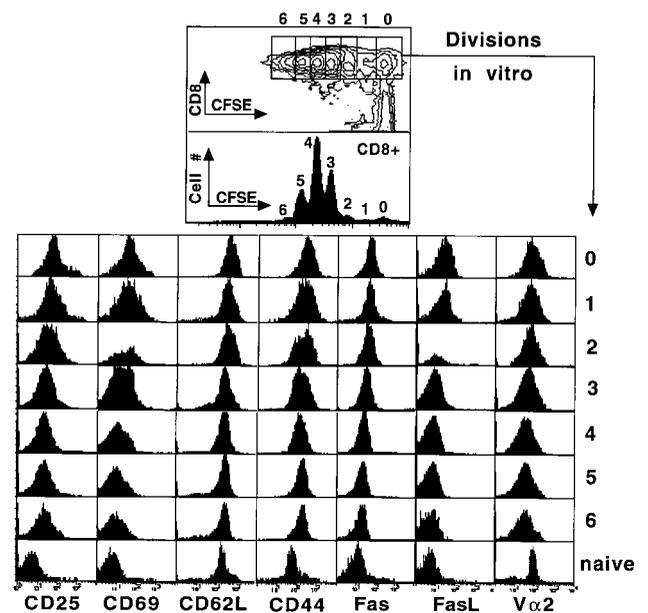
Single cell suspensions were harvested from 318-CFSE CTL cultures or prepared from lymph nodes and spleens of 318-CFSE $\rightarrow$ B6 recipients and incubated with an anti-CD8 PE-coupled Ab (PharMingen, San Diego, CA) to detect the CFSE-positive transgenic CTL. To analyze cell surface expression levels of the various markers, the cell suspensions were incubated with biotinylated mAbs against CD25, CD44, CD69, CD62L, Fas, FasL, or V $\alpha$ 2 (all PharMingen), respectively, followed by a streptavidin-Tricolor (Caltag, South San Francisco, CA) incubation. For cell surface marker analyses,  $5 \times 10^5$  to  $9 \times 10^5$  live cells were acquired in a FACScan and analyzed using CellQuest software (both Becton Dickinson, Mountain View, CA).

### In vitro cytotoxicity assay

After in vitro activation (24-well tissue culture plate) of  $5 \times 10^6$  transgenic T cells/well in a volume of 1 ml (see above), standard cultures were counted where indicated or serially diluted (four steps, threefold dilutions) in MEM supplemented with 2% FCS in 96-well round-bottom plates. Specific cytotoxicity of the diluted cultures was then determined in a standard  $^{51}\text{Cr}$  release assay, as described (16). EL4 cells were coated with peptide p33 at a concentration of  $10^{-6}$  M and were labeled in a total volume of 300  $\mu\text{l}$  with 250  $\mu\text{Ci}$   $^{51}\text{Cr}$  for 2 h at  $37^\circ\text{C}$  on a rocking platform. The labeled target cells were washed three times, and  $10^4$  cells were added to the effector cells in a final volume of 200  $\mu\text{l}$ . After a 5- or 20-h incubation at  $37^\circ\text{C}$ , 60  $\mu\text{l}$  of the supernatants was harvested and counted with a gamma counter.

### In vivo cytotoxicity assay

To prepare target cells for in vivo evaluation of cytotoxic activity, erythrocytes from C57BL/6 spleen cell suspensions were lysed, washed, pulsed with  $10^{-6}$  M p33 peptide for 90 min at  $37^\circ\text{C}$ , washed again, and labeled with a high CFSE fluorescence intensity, as described above. Uncoated control target cells were labeled with a low CFSE fluorescence intensity, as described above. For i.v. injection,  $3 \times 10^7$  cells of each population were mixed in 500  $\mu\text{l}$  BSS. Specific in vivo cytotoxicity was determined by collecting blood from the tail vein at the indicated time points, lysing erythrocytes using FACS lysing solution (Becton Dickinson), and detecting the differentially labeled fluorescent target cell populations by flow cytometry. The ratio  $r$  between the percentages of uncoated versus p33 coated (CFSE<sup>low</sup>/CFSE<sup>high</sup>) was calculated to obtain a numerical value of cytotoxicity.

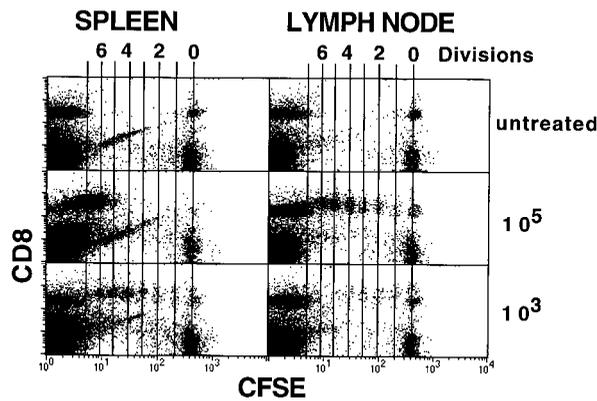


**FIGURE 1.** Surface marker modulation after in vitro activation of 318-CFSE CTL. CFSE-labeled 318 spleen cells were stimulated with  $2 \times 10^{-8}$  M peptide p33 and analyzed after 72 h for cell division in CD8-PE versus CFSE contour plots and in CFSE histograms after gating on CD8<sup>+</sup> CTL. Marker expression levels were determined after gating on the respective populations in the contour plots and displaying the third color in the histograms below. Numbers indicate division cycles. Naive = expression levels on naive 318 CTL. A representative result from at least three independent experiments is shown.

## Results

### Monitoring the differentiation of CTL in vitro

CFSE-labeled 318 TCR transgenic T cells expressing a TCR specific for the LCMV GP epitope p33 on H-2D<sup>b</sup> (318 transgenic mouse line (14)) lose 50% of their fluorescence intensity after each cell division upon stimulation with specific peptide. We have shown previously that labeling of lymphocytes with CFSE does not interfere with their biologic function and homing properties (17) and that adoptively transferred cells can be readily detected in blood and lymphoid organs by flow cytometry for several weeks (17, 18). To monitor the differentiation of a naive CTL population into effector and memory CTL, we followed the modulation of selected cell surface markers associated with the effector and/or memory T cell phenotype as a function of division cycle number. Spleen cells from 318 mice were labeled with CFSE (318-CFSE) and activated in vitro with p33 (Fig. 1) or with LCMV-infected macrophages (data not shown). After 72 h in culture, the cells were harvested and stained for CD8 and CD25, CD44, CD69, CD62L, Fas, FasL, or the transgenic V $\alpha$ 2 TCR chain, respectively. In Fig. 1, the top panel displays a two-dimensional CD8-PE versus CFSE (FITC) fluorescence profile of cultures harvested 72 h after stimulation. The contour plots reveal distinct, prominent peaks in the CD8-positive transgenic CTL population, corresponding to single cell divisions. These peaks are further displayed in the histogram below after gating on the CD8-positive cells. After 48 h (not shown), we observed four peaks, and 72 h after stimulation with p33, six to seven distinct peaks were apparent. Peaks 0 and 1 are more readily identified in the two-dimensional contour plots. Peak 0 represents CTL that have not divided. One hundred percent of the CD8-positive cells were found in the undivided peak when analyzed 24 h after stimulation (data not shown). Thus, taking into account that the cells had not divided within the first 24 h, we can



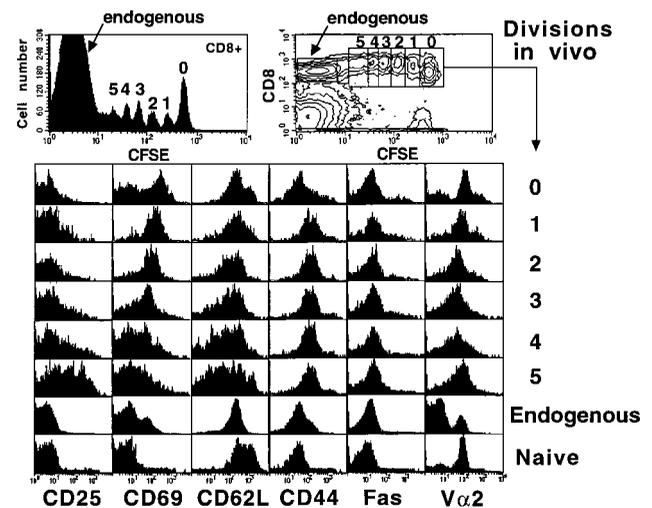
**FIGURE 2.** In vivo detection of cell division after viral infection. 318-CFSE spleen cells were transferred into recipients 1 day before i.v. injection with the indicated doses of LCMV WE. Three days after infection, spleen cells and mesenteric lymph node cells were stained with anti-CD8-PE and analyzed by two-color flow cytometry. Lines represent CFSE fluorescence intensities of CTL that have divided zero to seven times. A representative of at least two experiments is shown.

calculate that the average duplication time of an activated CTL is 8 h.

To follow CTL differentiation in vitro, the expression patterns of selected surface molecules were monitored as a function of cell division. This was achieved by gating on the corresponding populations in the contour plots and displaying the surface marker expression levels in the histograms below (Fig. 1, *bottom panel* of histograms). CD25, CD69, CD44, Fas, and FasL expression levels were increased by a factor of 5–10 even on CTL that had not divided. CD62L and transgenic TCR ( $V\alpha 2$ ) expression levels were unaltered on undivided cells. CD25 and CD44 levels remained elevated during further divisions, while CD69, Fas, and FasL levels declined and reached expression levels comparable with naive cells within six cell divisions. In 72-h cultures, CD62L was not down-regulated below the levels of naive CTL within the first six divisions, which would be characteristic of LCMV effector CTL generated in vivo (19). However, in 48-h cultures, CD62L was down-modulated on dividing cells (see below). TCR levels decreased with increasing numbers of cell divisions.

#### Tracing CTL differentiation in vivo

To follow cell division and CTL activation in vivo, we adoptively transferred  $3\text{--}5 \times 10^7$  318-CFSE spleen cells into nonirradiated recipients and subsequently activated the transfused CTL by i.v. infection of the recipients with LCMV. In the experiments depicted in Fig. 2, recipients were left untreated or were infected with  $10^5$  or  $10^3$  PFU of LCMV, respectively. Three days later, the  $CD8^+CFSE^+$  transgenic CTL in spleen and mesenteric lymph nodes were analyzed for cell division by flow cytometry. CFSE-labeled  $CD8^+$ CTL were readily detected in spleen and lymph nodes. Three days after injection of  $10^3$  PFU of LCMV, the transferred CTL had divided one to six times in the spleen. In the mesenteric lymph nodes, few CTL had divided. Injection of  $10^5$  PFU of LCMV resulted in a more homogenous stimulation and proliferation in the spleen than after injection with a low dose (compare spleen: *middle* and *bottom panels*, Fig. 2): The majority of CTL had divided six or seven times in the spleen after high dose infection, whereas the whole spectrum of CTL that had divided zero to six times was found in spleens after low dose infection. In the lymph nodes, CTL had divided one to six or seven times after high dose infection, but hardly any dividing cells were found in the



**FIGURE 3.** Surface marker analysis of in vivo differentiating CTL. Recipients were transfused with 318-CFSE spleen cells, and mesenteric lymph nodes were analyzed 3 days after i.v. injection of  $5 \times 10^3$  PFU of LCMV WE. Cells were stained for CD8 and for the indicated surface markers. Expression levels in dependence of cell cycle number were determined as described in Fig. 1. Endogenous = endogenous CTL population. Naive = surface marker expression levels on naive 318 CTL. Surface marker expression levels on 318 CTL 3 days after transfer into naive recipients were indistinguishable from fresh naive 318 CTL. A representative result from at least three independent experiments is shown.

lymph nodes of mice receiving a low virus dose. When comparing splenic CTL with mesenteric CTL at a given dose, we found that the majority of the CTL in the spleen had been activated before mesenteric CTL, which is manifested by a more synchronous proliferation of splenic CTL at a high LCMV dose (compare *middle panels* spleen versus lymph node) and the absence of large numbers of dividing CTL in the lymph nodes at low virus doses (compare *bottom panels* spleen versus lymph node), respectively. Thus, after systemic infection, the bulk of CTL was first activated in the spleen, and stimulation was more synchronous in the spleen after infection with high doses of LCMV. Similar as in vitro and given that CTL require approximately 24 h before starting to proliferate, we can calculate  $((72 - 24) \div 6)$  that also in vivo the average duplication time of an activated CTL after viral infection is 8 h.

To follow the differentiation of naive CTL in vivo, recipients that had been transfused with  $3\text{--}5 \times 10^7$  CFSE-labeled 318 spleen cells were challenged i.v. with  $5 \times 10^3$  PFU of LCMV. Three days after infection, mesenteric lymph nodes were removed to monitor cell surface marker expression as a function of cell division in a similar analysis, as described in Fig. 1. Fig. 3 shows that  $CD8^+CFSE^+$ CTL had divided up to five times in the mesenteric lymph nodes (*upper two panels*), and that CD8 was slightly up-regulated on these proliferating CTL. Expression levels of the various markers were then compared with the endogenous CD8 T cells and with naive 318 CTL from controls. In contrast to what was found in vitro (for comparison, see Table I), CD25 was strongly up-regulated only after the fourth cell division. As in cultured CTL, CD69 was immediately up-regulated even on cells that had not yet divided. CD62L was gradually down-modulated with increasing numbers of cell divisions. Compared with in vitro stimulation, CD44 expression increased only after the first cell division in vivo. In vivo, Fas was minimally up-regulated on activated cells. The TCR was down-regulated in a similar fashion in vivo as in vitro. Similar results were obtained with splenic CTL (data not shown).

Table I. Summary of surface marker modulations on CTL activated in vitro and in vivo<sup>a</sup>

Surface Marker	In Vitro Cell Division Number <sup>b</sup>							In Vivo Cell Division Number <sup>c</sup>						
	0	1	2	3	4	5	6	0	1	2	3	4	5	
CD25	↑↑↑	↑↑↑	↑↑	↑	↑	↑	↑	↔	↔	↔	↑	↑↑	↑↑↑	
CD69	↑↑↑	↑↑↑	↑↑	↑	↑	↔	↔	↑↑↑	↑↑↑	↑↑↑	↑↑	↑↑	↑↑↑	
CD62L	↔	↔	↔	↔	↔	↔	↔	↑↑↑	↑↑↑	↑↑↑	↑↑	↑↑	↓↓↓	
CD44	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↔	↑↑	↑↑	↑↑	↑↑	↑↑	
Fas	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↔	↑	↑	↑	↑	↑	↔	
FasL	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↔	ND	ND	ND	ND	ND	ND	
TCR	↔	↓	↓	↓	↓	↓	↓	↔ <sup>d</sup>	↔ <sup>d</sup>	↓	↓	↓	NV <sup>d</sup>	

<sup>a</sup> Marker modulations are compared to naive 318 CTL: ↔, no modulation; ↑, 0- to 5-fold up-regulation; ↑↑, 5- to 10-fold up-regulation; ↑↑↑, ≥10-fold up-regulation; ↓, 0- to 5-fold down-regulation; ↓↓, 5- to 10-fold down-regulation; ↓↓↓, ≥10-fold down-regulation; ND, not done; NV, not visible.

<sup>b</sup> Data taken from Fig. 1.

<sup>c</sup> Data taken from Fig. 3.

<sup>d</sup> Data taken from Fig. 4.

Experiments investigating whether the nature of the priming Ag influenced CTL differentiation were conducted by infecting recipients that had received CFSE-labeled 318 spleen cells with a recombinant vaccinia virus expressing the LCMV GP (vacc GP). In contrast to noncytopathic LCMV (20), cytolytic vaccinia virus abortively replicates in mice and elicits a CTL response that peaks on day 6 after infection (21). In mice, optimal vaccinia virus-induced CTL responses are obtained by i.v. infection of  $2 \times 10^6$  PFU of virus. Two days after infection with vacc GP, LCMV GP-specific CTL had divided in the spleen and followed a differentiation similar to CTL activated by a low dose of LCMV (not shown). Thus, a cytolytic versus noncytopathic priming agent did not influence the overall differentiation pattern of CTL.

For enhanced resolution of TCR modulation (the resolution of detection of TCR expression using Vα2-PE Ab is superior over Vα2-biotin streptavidin-Tricolor staining), we separately stained CFSE-labeled lymph node cells for CD8 or transgenic Vα2, respectively. We found that the TCR was further down-regulated after each cell division on a significant proportion, but not all CTL, resulting in populations of CTL expressing a wide range of TCR levels after each division (reflected by a smear in Fig. 4). After four to five cell divisions, a significant number of CTL that had normal TCR levels were visible (Fig. 4, compare black arrows),

and at the same time a significant number of CTL that expressed low TCR levels were detected (Fig. 4, compare white arrows). Thus, it appears that the TCR was expressed over a broad range, with some of the CTL further decreasing their TCR levels after each cell division.

*Acquisition of the effector and the memory stage and correlation with effector function*

To investigate at which stage differentiating CTL acquired cytotoxic effector function, their potential to lyse target cells was evaluated as a function of cell division. Toward this end, 318-CFSE spleen cells were stimulated in vitro with p33 and assayed after different time points for cell division and cytotoxic effector function. Fig. 5A shows that CTL that had been stimulated for 16 h and had not divided (0 div) did not lyse peptide-coated target cells. In contrast, cultures that had divided once after 30 h (0–1 div) displayed significant specific cytotoxicity. Similarly, CTL that had divided up to four times after 48 h (0–4 div) were cytotoxic in a 5-h <sup>51</sup>Cr release assay (Fig. 5B). Thus, activated CTL gained effector function within the first cell division and continued to express cytotoxic effector function while proliferating.

The next set of experiments aimed at investigating after how many cell divisions a naive CTL reached a CD44<sup>high</sup>CD62L<sup>+</sup> memory phenotype (22, 23). Furthermore, we evaluated whether discrete differentiation stages could be correlated phenotypically and functionally. Fig. 5B (right histogram) shows that 318-CFSE CTL that were stimulated with p33 and were cultured for 7 days had divided 6–10 times. When testing the CTL for effector function, these long-term cultures, unlike effector cultures that had divided 0–4 times, did not display any significant immediate cytotoxicity in a 5-h <sup>51</sup>Cr release assay (Fig. 5B, bottom graph). However, when incubating the CTL for 20 h, they developed maximal cytolysis. Thus, CTL, having divided 6–10 times, displayed delayed effector function. A phenotypic analysis of the differentiating 318 CTL revealed that CTL with immediate effector function were CD44<sup>high</sup> and had down-regulated CD62L, whereas CTL with delayed effector function reexpressed CD62L and displayed a CD44<sup>high</sup>CD62L<sup>+</sup> memory phenotype. (Fig. 5C). Taken together, LCMV-specific CTL differentiated from naive CD44<sup>low</sup>CD62L<sup>+</sup> to CD44<sup>high</sup>CD62L<sup>+</sup> memory CTL via an intermediate CD44<sup>high</sup>CD62L<sup>-</sup> effector stage within 6–10 cell divisions. The intermediate effector stage displayed immediate cytotoxic activity, whereas memory CTL required restimulation to develop cytotoxic activity.

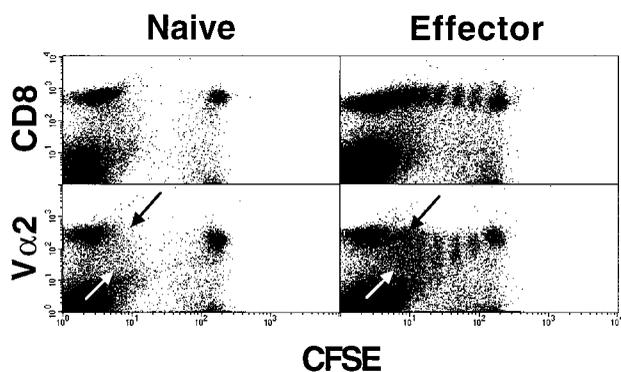
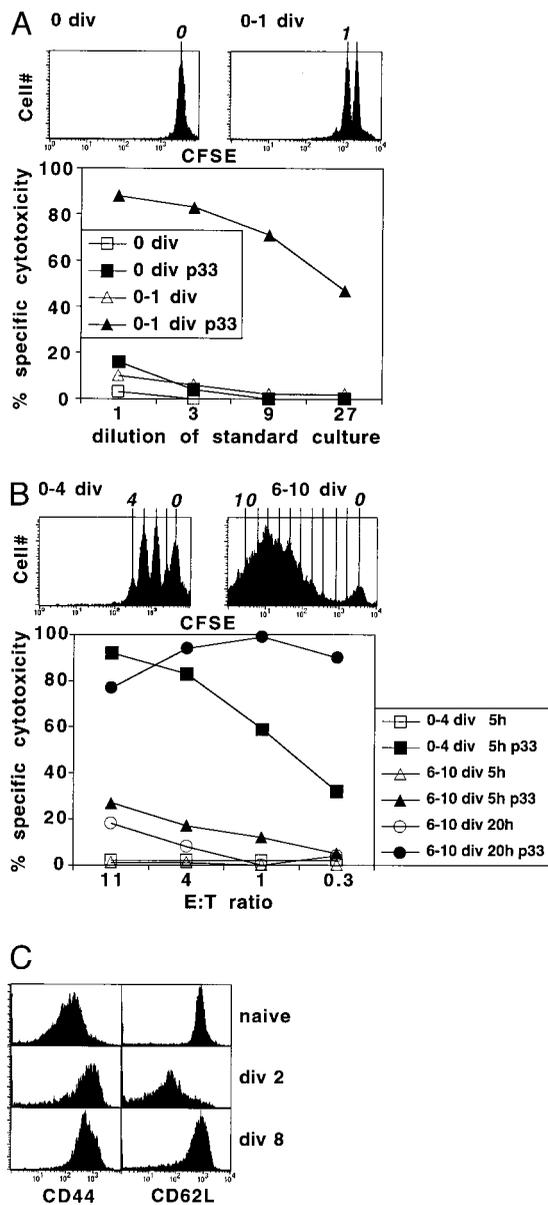


FIGURE 4. TCR modulation after viral infection. 318-CFSE spleen cells were transfused into recipients and activated in vivo by i.v. injection of 5000 PFU of LCMV WE (Effector). Uninfected recipients (Naive) served as a comparison. After 3 days, mesenteric lymph nodes were stained with anti-CD8-PE or anti-Vα2-PE, to directly compare transgenic TCR modulation on proliferating CD8<sup>+</sup> CTL (upper versus lower panels). Black and white arrows point at TCR<sup>high</sup> and TCR<sup>low</sup> CTL, respectively, which are absent in the noninfected controls (left plot), but are visible in the infected recipients (right plot). A representative result from at least four mice is shown.

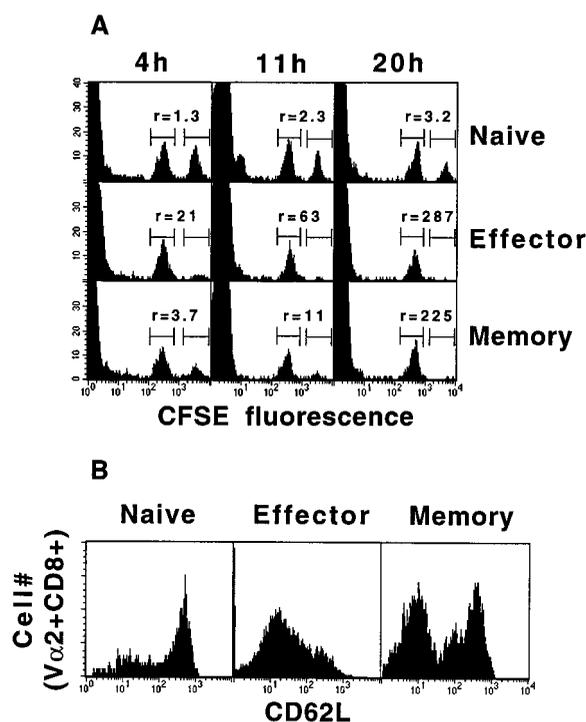


**FIGURE 5.** A, CTL acquire cytotoxic effector function within one cell division. 318-CFSE spleen cells were activated *in vitro* with p33, as described in Fig. 1. After 16 h (0 div) or 30 h (0-1 div), standard cultures were harvested and an aliquot was analyzed for cell division by gating on CD8-PE<sup>+</sup> CTL and displaying CFSE intensities in a histogram (*upper panels*). Cultures were then tested in a 5-h <sup>51</sup>Cr release assay (*lower graph*). Cultures that had not divided yet (boxes) or that had divided once (triangles) were analyzed on unpulsed (open symbols) or p33-pulsed (filled symbols) EL-4 target cells. Spontaneous release was <12% for all targets. B, Cytotoxic effector function of acutely activated and memory CTL. 318-CFSE spleen cell cultures were stimulated with p33 for 48 h (div 0-4) to obtain effector CTL. For long-term cultures, 318-CFSE spleen cells were stimulated by a single addition of p33 (final concentration: 10<sup>-8</sup> M), washed after 3 days, split at a ratio of 1:4, and recultured for an additional 4 days (div 6-10). Cell cycle numbers were determined as described above (*top two panels*). Cytotoxic activity (*bottom panel*) was determined in a <sup>51</sup>Cr release assay at the indicated E:T ratios on p33-pulsed (closed symbols) and unpulsed (open symbols) EL-4 target cells. 0-4 div cultures were incubated for 5 h (squares); 6-10 div cultures for 5 h (triangles) and 20 h (circles). After 5 h, spontaneous releases were <10%; after 20 h, <25%. C, Phenotype of short-term (0-4 div) and long-term (6-10 div) cultures. Cultures from B were stained for CD8 and CD44 or CD62L. After gating on the most prominent division peak (div2 and div8, respectively), surface marker expression levels were displayed in a histogram and compared with naive 318 CTL. A representative of three independent cultures is shown.

#### Acquisition of effector function *in vivo*

To examine the dynamics of effector function during the differentiation of naive CTL to memory CTL *in vivo*, the rejection of p33-presenting target cells was monitored. Since LCMV was already administered to activate naive CTL, a challenge infection with virus early after priming could not be used to assay for cytotoxic activity in a viral plaque assay. To circumvent this problem, we developed an *in vivo* cytotoxicity assay: Normal syngeneic spleen cells serving as target cells were pulsed with the p33 and were labeled with a high CFSE (CFSE<sup>high</sup>) intensity. To control for the elimination of target cells by noncontact-dependent or unspecific lymphokine-mediated mechanism, unpulsed spleen cells were labeled with a low CFSE fluorescence (CFSE<sup>low</sup>) intensity. CFSE<sup>high</sup> and CFSE<sup>low</sup> target cells were mixed at a ratio of 1:1 and transferred into the different types of recipients, and the dynamics of CTL effector function were measured by monitoring the rejection of the CFSE<sup>high</sup> target cells by flow cytometry 4, 11, and 20 h after transfer (Fig. 6A). Effector mice were prepared by adoptively transferring 3 × 10<sup>7</sup> 318 spleen cells into C57BL/6 recipients (318→B6) and infecting the recipients with 1000 PFU of LCMV 3 days before transfer of the target cell mixture. Memory mice were generated by infecting 318→B6 recipients with LCMV 60 days before target cell injection. Untreated 318 transgenic mice (instead of normal mice) served as naive controls to compensate for elevated LCMV CTL precursor frequencies. Fig. 6A shows that naive 318 mice required overnight activation before developing any significant CTL activity, which is evidenced by a reduction of the p33-coated CFSE<sup>high</sup> indicator cells. In contrast, early differentiating CTL in effector mice immediately eliminated p33 CFSE<sup>high</sup> target cells within 4 h. Memory mice showed significant cytotoxicity at 4 and 11 h, and completely eliminated p33-coated target cells by 20 h. This contrasted with the *in vitro* generated memory CTL that required reactivation and suggested that *in vivo* the memory population was more rapidly reactivated and/or contained effector CTL. To resolve this question, the LCMV-specific 318 CTL populations in naive, effector, and memory mice were analyzed for the expression of CD62L. Fig. 6B shows that in naive 318 mice, 90% of the transgenic CTL were CD62L<sup>+</sup> (and CD44<sup>low</sup>, not shown). In effector mice, more than 90% of the LCMV-specific transgenic CTL were CD62L<sup>-</sup> (and CD44<sup>high</sup>, not shown). Interestingly, in memory mice, 40-50% of the transgenic CTL were CD62L<sup>-</sup> (and CD44<sup>high</sup>, not shown), a phenotype typical for LCMV effector CTL.

To further support the idea that also *in vivo* the CD44<sup>high</sup>CD62L<sup>+</sup> CTL conferred delayed effector function, we sought to separately test *in vivo* generated CD44<sup>high</sup>CD62L<sup>+</sup> memory CTL. Since CD62L is a lymph node homing receptor, we reasoned that in memory mice CD44<sup>high</sup>CD62L<sup>+</sup> CTL should be found in lymph nodes and in spleen, but that CD44<sup>high</sup>CD62L<sup>-</sup> CTL should be predominantly found in the spleen due to the lack of CD62L expression. We therefore analyzed CD62L expression on 318 transgenic CTL in spleen and lymph nodes of 318→B6 memory mice and found that 50% of the 318 CTL in the spleen were CD62L<sup>-</sup>, while only 20% of the lymph node 318 CTL were CD62L<sup>-</sup> (Fig. 7). Both populations were CD44<sup>high</sup> (not shown). When spleen and lymph node cells from these mice were tested for immediate effector function in a 6-h cytotoxicity assay, we observed that in contrast to lymph node CTL, splenic CTL significantly lysed p33-coated target cells (Fig. 7). After prolonged incubation for 19 h, lymph node CTL had also gained effector function, yet it was not possible to analyze whether this cytotoxicity was mediated by the residual 19% CD62L<sup>-</sup> CTL population. Taken together, after LCMV infection in

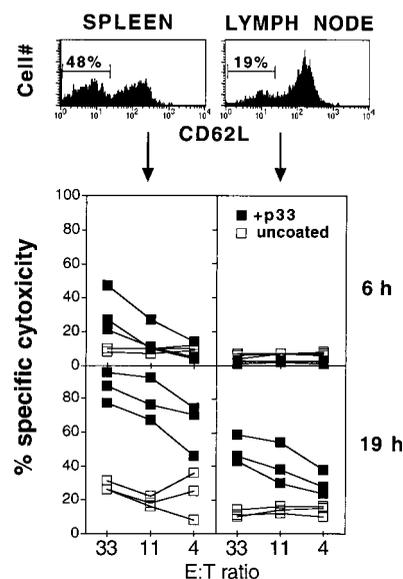


**FIGURE 6.** *A*, In vivo effector function of naive, effector, and memory CTL. Day 3 effector mice (Effector) were generated by transfusing  $3 \times 10^7$  318 spleen cells into syngeneic hosts and infecting them with 1000 PFU of LCMV. Memory mice were obtained by adoptively transferring  $1 \times 10^6$  318 spleen cells into naive recipients and infecting them i.v. with 200 PFU of LCMV WE 30 or 60 days (17) before determination of CTL effector function. Untreated 318 mice served as naive control mice. To analyze CTL effector function in vivo, target cells were prepared by pulsing syngeneic spleen cells with p33 and labeling them with a high CFSE intensity. To control for Ag specificity, unpulsed syngeneic spleen cells were labeled with a low CFSE intensity. A 1:1 mixture of  $3 \times 10^7$  cells of each of the target cell populations was injected i.v. into the naive 318 controls, the effector, or the memory mice. After 4, 11, and 20 h, blood was collected from the tail vein and analyzed in a single-parameter flow-cytometric analysis for the presence of the CFSE<sup>high</sup> (p33-pulsed) and CFSE<sup>low</sup> (unpulsed) target cell populations. To test for contact-dependent, specific cytotoxic effector function, the elimination of p33-pulsed CFSE<sup>high</sup> spleen cells was monitored and the ratio ( $r$ ) between percentage of nonpulsed and percentage of p33-pulsed indicator cells was calculated. Data show a representative of three mice. *B*, CD62L expression on naive, effector, and memory LCMV-specific CTL. Blood was collected from the mice described in *A* before transfer of the target cells and triple stained for transgenic  $V\alpha 2$ , CD8, and CD62L or CD44 (not shown). CD62L expression is shown after gating on CD8- and  $V\alpha 2$ -positive lymphocytes. A representative result from three independent day 30 memory mice is shown.

vivo, CTL differentiated into a CD44<sup>high</sup>CD62L<sup>-</sup> activation stage exerting rapid effector function. In memory mice, a CD44<sup>high</sup>CD62L<sup>-</sup> population with rapid effector function coexisted with a CD44<sup>high</sup>CD62L<sup>+</sup> population exerting delayed effector function. Thus, based on the expression of CD44 and CD62L, two types of phenotypically and functionally distinct memory CTL were found in LCMV memory mice.

## Discussion

This study aimed at analyzing the differentiation of naive CTL to effector and memory CTL in vitro and in vivo in dependence of cell division and to correlate the different stages of differentiation with effector function. Using the vital dye CFSE, we followed an



**FIGURE 7.** CD62L-negative effector memory CTL are predominately located in the spleen and exert rapid effector function. Day 30 memory mice were generated as described in Fig. 6. An aliquot of spleen and pooled mesenteric and inguinal lymph node cells was triple stained for CD8,  $V\alpha 2$ , and CD62L. CD62L expression levels in spleen and lymph node are displayed in the histograms after gating on the CD8<sup>+</sup> $V\alpha 2$ <sup>+</sup> transgenic CTL. To assay for immediate and delayed effector function, spleen cells and lymph node cells were tested in a 6- and 19-h cytotoxicity assay, respectively, on p33-coated (filled boxes) and uncoated (open boxes) EL-4 target cells at the indicated E:T ratios (*bottom panels*). A representative histogram from three mice is shown for spleen and lymph nodes. The results from three memory mice are depicted in the cytotoxicity assay.

anti-LCMV-specific CTL population in vitro upon stimulation with specific peptide and in vivo after LCMV infection.

As previously described for in vitro polyclonally activated B cells (24), we were able to monitor the modulation of selected T cell surface molecules on differentiating CTL in dependence of the division cycle number. The analysis of in vitro and in vivo stimulated CTL revealed that the investigated surface markers were strongly modulated within the first six cell divisions.

## CD25

The activation of CTL in vitro is strictly dependent on IL-2 (25). Consistent with this, we found that in vitro CD25 was up-regulated before the first cell division. In vivo, however, CD25 up-regulation was observed only after the third to fourth cell division, confirming recent studies showing that in IL-2-deficient mice, CTL proliferated in response to superantigen (26) and antigenic peptide (27). Thus, it appears that in vivo other cytokines or, as shown with CD4 T cells, costimulation through B7.2 (28) may substitute for IL-2.

## CD69, Fas, and FasL

As expected, the early activation marker CD69 was rapidly up-regulated and returned to naive levels within six cell divisions. Interestingly, Fas and FasL also followed an expression pattern similar to that of an early activation marker. This suggests that activated T cells may only be susceptible to Fas-induced apoptosis at an early differentiation stage. Experiments conducted to test for this showed that the addition of anti-Fas Ab to CTL cultures (10  $\mu\text{g/ml}$ ) marginally inhibited proliferation (by a factor of 2) when added within the first 48 h (data not shown). Thus, although recent reports have suggested that expression of FasL may generate immune privileged sites by Fas-mediated elimination of autoimmune

T cells (29, 30), our *in vitro* data imply that only a minority of Fas-expressing early effector cells are susceptible to Fas-FasL-induced apoptosis and that late effector CTL may be refractory to Fas-mediated apoptosis. Consistent with this, it was found that the expression of Bcl- $x_L$  in activated T cells can protect them from Fas-mediated apoptosis (31).

#### *CD44 and CD62L*

CD44 is required for extravasation of activated T cells into inflammatory sites by interacting with the ligand hyaluronate (32). Since CD44 was immediately up-regulated within the first cell division, this suggests that activated T cells immediately gain the potential to change their extravasation properties. On the other hand, the lymph node homing receptor CD62L was substantially decreased only after the fourth cell division *in vivo*, suggesting that early during differentiation T lymphocytes maintain the capacity to remain in or home back into lymph nodes. Based on our *in vitro* data, naive T cells started to proliferate after an initial activation lag time of 24 h, and thereafter duplicated every 8 h. Thus, we could calculate that approximately 56 h after encountering specific Ag, activated T cells may lose the potential to home to lymph nodes. From this, one could conclude that early during an immune response, activated T cells are retained in the secondary lymphoid organ, perhaps to eliminate draining Ag and/or to provide helper function for the establishment of a B cell response. T cells that have left the lymph node despite expressing the lymph node homing receptor CD62L, as well as late effector cells that have down-regulated CD62L may be recruited to inflammatory sites by CD44-mediated extravasation.

Even though CD62L is well known as a lymph node homing receptor (33), it was striking to see how accurately the migration patterns of activated CTL could be deduced from the CD62L expression patterns, e.g., we showed that CD62L<sup>-</sup> effector and memory CTL were predominately found in the spleen and were reduced in the lymph nodes. Similarly, experiments in which CTL were primed with a high dose of LCMV down-regulated CD62L on undivided cells within 18 h (not shown) and would mean that, after a strong stimulus, extravasation should be accelerated. Consistent with this, a recent study reported that V $\beta$ 8<sup>+</sup> T cells appeared in the inflammatory site 16 h after priming with the strong stimulus staphylococcal enterotoxin B (32). Thus, it appears that deducing the homing properties of CTL from the expression of CD44 and CD62L matches well with *in vivo* observations.

#### *T cell receptor*

Recent studies have shown that the TCR is down-regulated upon encountering Ag (34, 35). Our data show that a similar phenomenon is observed after stimulation of CTL with peptide *in vitro* or during viral infection *in vivo*. Down-modulation was not a consequence of cell division since nondividing CTL activated by a high Ag dose down-regulated the TCR (not shown). Thus, our data show that TCR modulation is observed during the course of a natural viral infection; however, the biologic role of TCR down-regulation is unclear. Since many intermediate mediators may be shared by different signaling pathways (36), excessive triggering of one signaling cascade may result in nonspecific induction of alternate genes. Thus, one can speculate that, generally, down-regulation of surface molecules coupled to downstream regulatory pathways may serve to desensitize the responding cell toward the original signal and prevent unspecific activation of additional genes. Experiments performed in our adoptive transfer system and showing that a high virus dose capable of immediately delivering a strong stimulatory signal resulted in nonspecific cytotoxic effector function, support this view (not shown).

Our data reveal that within a given cell division cycle, TCR down-regulation was not homogenous: A proportion of the CTL expressed normal, while other CTL had reduced TCR levels. With increasing numbers of cell divisions, the degree of TCR down-modulation increased, resulting in an overall stepwise TCR down-regulation. After four to five divisions, we found significant numbers of CTL expressing normal and cells expressing greater than 10-fold reduced TCR levels. It is possible that TCR<sup>high</sup>CTL emerged from TCR<sup>low</sup> cells that have reexpressed the TCR because they have not reencountered Ag, while TCR<sup>low</sup>CTL are the result of continuous reexposure to viral Ag. Another possibility is that a proportion of the CTL was activated, but divided without any significant TCR modulation, as could be seen in experiments recently published by Iezzi et al. (37). It is also not clear whether the TCR<sup>high</sup> or TCR<sup>low</sup> CTL populations represent separate CTL lineages, either one of which may differentiate into memory CTL. Studies to investigate the fate of these two distinct populations are underway.

#### *Cytotoxic effector function*

It is an open question after how many cell divisions CTL acquire effector function and whether effector function correlates with the expression of a distinct (set of) surface markers. For CTL, one of the primary effector functions is cytotoxic activity. Our results show that undivided CTL that had been stimulated for 16 h *in vitro* failed to develop any cytotoxic activity, whereas cells that had divided once within 30 h specifically lysed target cells. Similarly, differentiating, proliferating CTL developed cytotoxic activity *in vitro* and *in vivo* during a phase in which the investigated surface markers were strongly modulated. Since in this assay system cytotoxicity is mediated by perforin (38), our data suggest that sufficient perforin may have to accumulate in cytotoxic granules within 30 h to promote cytotoxic function. Consistent with this, cultures that were treated with mitomycin C to inhibit proliferation (data not shown) still developed cytotoxic activity, supporting the notion that the acquisition of effector function does not seem to require a certain number of cell divisions, but may rather parallel perforin synthesis. In this context, it should be noted, however, that depending on the T cell lineage and the effector function investigated, the apparent acquisition of an effector stage may vary significantly.

The absence of CD62L has been linked to the effector stage (19, 23, 39–41). In our assays, CD62L was not down-regulated *in vitro* when the cultures were analyzed after 72 h. However, these cultures usually also displayed reduced cytotoxic effector function. In contrast, CD62L was consistently down-modulated in cultures tested 48 h after stimulation or after repetitive administration of p33 (not shown) or on CTL activated *in vivo*. Thus, effector function correlated with low CD62L levels, but also seemed to require persisting Ag. After a single administration of p33, our culture conditions simulate a situation in which the stimulating peptide is rapidly degraded or turned over since it is known that the average  $t_{1/2}$  of peptides in serum-complemented medium is 10–100 min (42). The observation that CD62L was not (anymore) reduced 3 days after stimulation *in vitro* may be explained by the possibility that peptide was degraded too rapidly to result in sustained CD62L down-regulation.

#### *CTL memory*

The nature of T cell memory is debated about. Two opposing but not mutually exclusive views have emerged, one postulating that long-lived memory T cells persist in the absence of Ag (8–10), the other suggesting that protective T cell memory against peripheral infections depends on persisting Ag (6, 7, 11, 12). Long-lived

memory T cells may confer protection against a systemic infection because this allows early restimulation and development of effector function. On the other hand, antivirally protective T cells may rather represent continuously activated, Ag-dependent effector T cells that can promote immediate effector function, migrate through solid tissue, interfere with replication and spreading of a sequestered pathogen, and thus promote protection to a peripheral infection. Thus, in order for long-lived memory T cells to intervene with a local peripheral infection, they may require a period of restimulation before they can exert effector function. This lag period may give a rapidly replicating pathogen enough time to replicate locally and cause direct tissue damage in the case of cytopathic viruses or to infect a sufficient number of target cells, rendering them susceptible to immunopathologic destruction in the case of noncytopathic viruses. In such a situation, long-lived memory T cells would not appear to be antivirally protective to a peripheral challenge (11, 12, 43).

During the acute phase, 100% of the LCMV-specific effector CTL expressed a CD44<sup>high</sup>CD62L<sup>-</sup> phenotype. CTL from 6–9-day-old cultures acquired a previously described CD44<sup>high</sup>CD62L<sup>+</sup> memory stage (22) after 6–10 divisions and, in contrast to acute phase effector CTL, required prolonged incubation periods to develop cytotoxic activity. In LCMV memory mice, we and others (19) detected a CD44<sup>high</sup>CD62L<sup>-</sup> and a CD44<sup>high</sup>CD62L<sup>+</sup> transgenic LCMV-specific CTL population after 30 to 60 days. Such mixed CTL populations were capable of rapidly eliminating Ag in vivo (our data) and in vitro (19). Together with the observation that acute phase effector CTL are CD62L<sup>-</sup> in vivo, we may conclude that the CD62L<sup>-</sup> effector memory CTL population accounts for rapid effector function found in LCMV memory mice. Conversely, the CD62L<sup>+</sup> memory CTL representing the major population in the lymph nodes did not display rapid effector function. A similar correlation has been proposed for human CD4 T cells, in which CD45R<sup>low</sup> T cells represent Ag-primed memory T cells with rapid response kinetics and CD45R<sup>high</sup> memory T cells are long lived and quiescent (44). Reviewing of several studies suggests that the CD62L-negative effector memory phenotype correlates with persisting Ag, whereas the CD62L<sup>+</sup> memory stage appears when Ag is lost: Pihlgren et al. showed that peptide injection without adjuvant resulted in a transient down-regulation of CD62L on day 3, followed by the appearance of CD44<sup>high</sup>CD62L<sup>+</sup> memory CTL after 6 wk (22). At this late time point, one can expect that the soluble priming peptide has been cleared from the host. On the other hand, Rogers et al. analyzed OVA-specific CD62L-negative memory T cells 36 days after s.c. immunization of OVA peptide in CFA (41). Similarly, Bradley et al. (39, 40) showed that memory T cells were confined to a CD62L<sup>-</sup> population in mice primed with keyhole limpet hemocyanin precipitated on alum or emulsified in CFA or IFA. Thus, in general, under conditions in which Ag can persist on an alum depot, in an emulsified form, or in adjuvant-induced granulomas (45), at least a fraction of memory T cells acquired a CD62L-negative phenotype, while CD44<sup>high</sup>CD62L<sup>+</sup> memory CTL developed when Ag had been cleared. CD62L may therefore represent an activation marker that is down-regulated on effector T cells only in the presence of sufficient Ag. The effector memory CTL population in LCMV memory mice may be driven by persisting Ag, which is consistent with the findings that LCMV or retrotranscribed genomic components thereof can be recovered long after the infection has been cleared (46–48). Yet, while it seems clear that persisting Ag can maintain effector memory CTL, it is not resolved whether the presence of effector memory CTL always correlates with persisting Ag. It is also possible that the detection of elevated frequencies of effector memory CTL over

prolonged time periods correlates with the initial clonal burst size (9). Collectively, our interpretation of the data is that the CD44<sup>high</sup>CD62L<sup>-</sup> memory CTL population represents effector memory CTL capable of immediately eliminating specific Ag, whereas CD44<sup>high</sup>CD62L<sup>+</sup> CTL may represent memory cells that require restimulation for cytotoxic effector function.

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