Direct quantitation of rapid elimination of viral antigen-positive lymphocytes by antiviral CD8$^+$ T cells *in vivo*

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Lysis of infected cells by CD8$^+$ T cells is an important mechanism for the control of virus infections, but remains difficult to quantify *in vivo*. Here, we study the elimination kinetics of viral antigen-positive lymphocytes by antiviral CD8$^+$ T cells using flow cytometry and mathematical analysis. In mice acutely infected with lymphocytic choriomeningitis virus, more than 99.99 % of target cells were eliminated each day, corresponding to a half-life of 1.4 h. Even in mice exposed to virus 300 days previously, and with no *ex vivo* killing activity, 84 % of the target cells were eliminated per day. Unexpectedly, the elimination kinetics of antigen-positive lymphocytes was not significantly impaired in mice deficient in either perforin-, CD95 ligand- or TNF-mediated cytotoxicity. For viruses with a particular tropism for lymphocytes, such as Epstein-Barr virus or HIV, our results illustrate how effectively CD8$^+$ T cell-mediated elimination of target cells can potentially contribute to virus control and immunosuppression.

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1 Introduction

Quantitative parameters are important for understanding the dynamics of immune responses to replicating pathogens. The quantitative study of antiviral cytotoxic T cells has recently been significantly advanced by the introduction of MHC-peptide tetramers [1]. While this tool has provided a new perspective on the numbers of virus-specific CD8$^+$ T cells [2–4], it does not allow direct study of T cell function. Antiviral cytotoxic T cell function is usually quantified *in vitro* using ^{51}Cr-release assays [5], which mainly assess contact-dependent perforin-mediated cytotoxicity [6]. It is unclear, however, how the characteristics of tumor cell target lysis *in vitro* relate to the elimination kinetics of epithelial, mesenchymal or lymphohemopoietic cells presenting viral antigens *in vivo*. Although the efficacy in target cell elimination *in vivo* is a key issue for CD8$^+$ T cell-mediated virus control, this has never been directly quantified.

Infection of mice with LCMV is an excellent and well-characterized experimental system to study CD8$^+$ T cell responses under controlled conditions *in vivo* [7, 8]. In this study we adoptively transfused fluorescence-labeled spleen cells expressing a viral CD8$^+$ T cell epitope derived from LCMV [9] into immunized recipient mice. The kinetics of donor cell elimination was then monitored by flow cytometry [10] to quantify the efficacy of the anti-viral CD8$^+$ T cell response in target cell elimination. This experimental approach allowed a clear definition of effector cells, the target cell population, as well as the target antigen under *in vivo* conditions.

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Abbreviations: gp33: LCMV glycoprotein peptide 33–41 **CFSE:** Carboxyfluorescein diacetate succinimidyl ester **Vacc GP:** Vaccinia virus expressing LCMV glycoprotein **Vacc NP:** Vaccinia virus expressing LCMV nucleoprotein **PKO:** Perforin knockout

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2 Results

2.1 Antigen specificity of CD8$^+$ T cell-mediated target cell elimination *in vivo*

Spleen cells from transgenic mice ubiquitously expressing the LCMV glycoprotein peptide gp33–41 (H8 mice [9]) or from C57BL/6 mice were labeled with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) [11]. Cells (7×10^7) were transfused into C57BL/6 recipient mice immunized with recombinant vaccinia virus expressing either the LCMV glycoprotein (vacc GP) or, as a specificity control, the LCMV nucleoprotein (vacc NP). H8 cells were eliminated below the detection limit of 0.02 % within 3 days in vacc GP-primed mice, while they persisted at levels around 4–5 % in mice immunized with vacc NP (Fig. 1 a–c). Viral antigen-negative control cells from C57BL/6 mice were not eliminated in unprimed or vacc GP-primed mice (Fig. 1 d). Among the transfused spleen cells the rate of elimination was similar for CD4$^+$ T cells, CD8$^+$ T cells and B cells as revealed by co-staining with the respective antibodies (not shown). Furthermore, donor cell elimination occurred with similar kinetics in the lung, the liver, the peritoneal cavity, several lymph nodes including Peyer's patches and the spleen (Fig. 1 e, f).

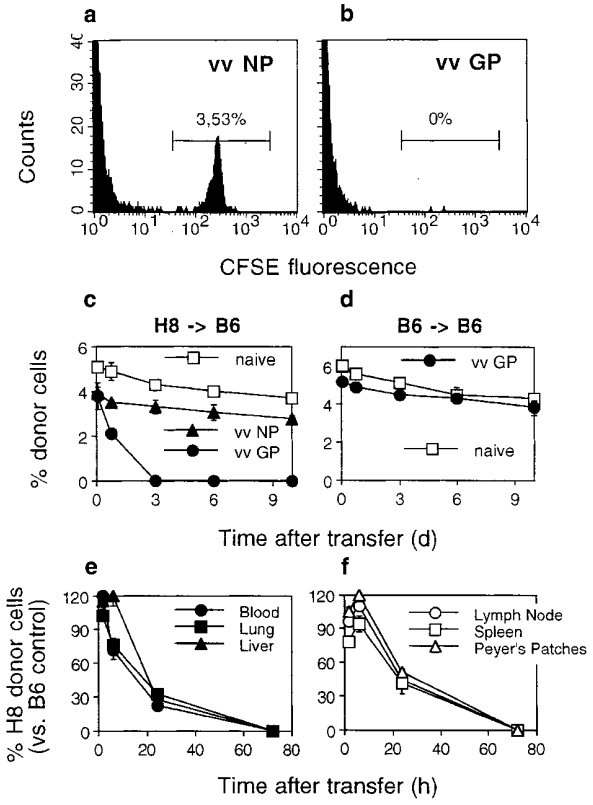

Fig. 1. Antigen specificity of lymphocyte elimination *in vivo*. Spleen cells from gp33-transgenic H8 mice or from C57BL/6 mice were labeled with the fluorescent dye CFSE and transfused (7×10^7 cells) into C57BL/6 recipients. The percentage of CFSE$^+$ cells among total lymphocytes was determined by flow cytometry at different time points after transfer. The recipient mice were either naive (open squares) or had been immunized with 2×10^6 PFU vacc GP (b, c, d circles, e–f) or vacc NP (a, c triangles) 14 days earlier. (a, b) Flow cytometric pictures obtained 3 days after transfer. (c, d) Kinetics of H8 cell (c) or C57BL/6 cell (d) elimination from peripheral blood. (e, f) H8 cell elimination from blood, lung and liver (e), mesenteric lymph nodes, spleen and Peyer's patches (f) expressed as percentage of C57BL/6 donor cells detected in control mice treated with the same protocol.

The mechanism responsible for donor cell elimination was studied in vacc GP-immunized mice deficient in particular effector cell populations. IgM knockout mice [12] and C57BL/6 mice depleted of NK cells [13] eliminated H8 cells with similar kinetics as control mice (Table 1). In contrast, CD8$^+$ T cell-deficient β2-microglobulin knockout mice [14] and mice depleted of CD8$^+$ T cells [15] did not eliminate H8 cells (Table 1), demonstrating that the observed lymphocyte elimination was mediated by CD8$^+$ T cells.

2.2 Lymphocyte elimination *in vivo* in the absence of perforin-, CD95 ligand- or TNF-mediated cytotoxicity

To investigate the effector mechanism of lymphocyte elimination, we transfused H8 cells into vacc GP-immunized mice deficient in perforin [perforin knockout

Table 1. Lymphocyte elimination is mediated by CD8$^+$ T cells

Recipient mice[a)]	% donor cells		
	2 h	18–22 h	3 days
C57BL/6	3.5	2.1	0.03
B6 anti-NK 1.1	3.1	2.5	0.01
B6 Ig M$^{-/-}$	2.6	1.3	0
β2-microglobulin$^{-/-}$	2.9	3	2.5
B6 anti-CD8	5.4	5.5	4.6

a) Mice rendered deficient in NK, B or CD8$^+$ T cells by gene targeting or antibody depletion were immunized with 2×10^6 PFU vacc GP. Fourteen days after infection, they were transfused with 7×10^7 H8 spleen cells and the percentage of CFSE$^+$ donor cells among total lymphocytes was determined by flow cytometry at different time points after transfer. The mean of three mice per group is shown.

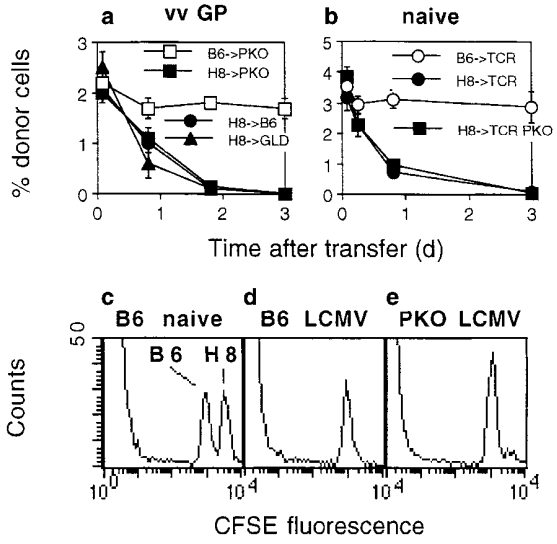

Fig. 2. CD8⁺ T cell-mediated lymphocyte elimination *in vivo* does not require perforin-mediated cytotoxicity. Spleen cells from H8 (closed symbols) or C57BL/6 mice (open symbols) were labeled with CFSE and transfused (7×10^7 cells) into C57BL/6, PKO, gld/gld, TCR318 or TCR318xPKO recipient mice (as indicated). In experiments (c–e), a mixture of 3×10^7 spleen cells from both H8 and C57BL/6 mice labeled with different concentrations of CFSE was transferred. The percentage of CFSE⁺ donor cells among total PBL was determined at different times after transfer. The recipient mice were either naive (b, c) or had been immunized i. v. with 2×10^6 PFU vacc GP 11 days earlier (a) or with 200 PFU LCMV 8 days earlier (d, e).

(PKO) mice] [16] or CD95 ligand. Surprisingly, the kinetics of donor cell elimination was as rapid as in C57BL/6 control mice (Fig. 2 a). Similar results were obtained with perforin-deficient mice expressing a transgenic TCR specific for gp33 (Fig. 2 b). In an additional set of experiments perforin-competent and -deficient mice immunized with LCMV 8 days earlier were transfused with a 1 : 1 mixture of spleen cells from C57BL/6 and H8 mice labeled with different concentrations of CFSE. Fig. 2 d shows selective elimination of the antigen-expressing cells within 5 h without effect on the co-transfused control cells. In the absence of perforin, the elimination was slightly retarded – about 90 % elimination by 5 h (Fig. 2 e), but also complete within 24 h after transfer.

Furthermore, donor cells deficient in CD95 or TNFR p55 [17] and exogenously loaded with gp33 peptide were eliminated from LCMV-immunized mice as efficiently as control cells (Fig. 3 a). Finally, peptide-loaded spleen cells deficient in the CD95 or TNFR p55 were also eliminated in vacc GP-immunized perforin-deficient mice, albeit with slower kinetics than peptide-loaded spleen

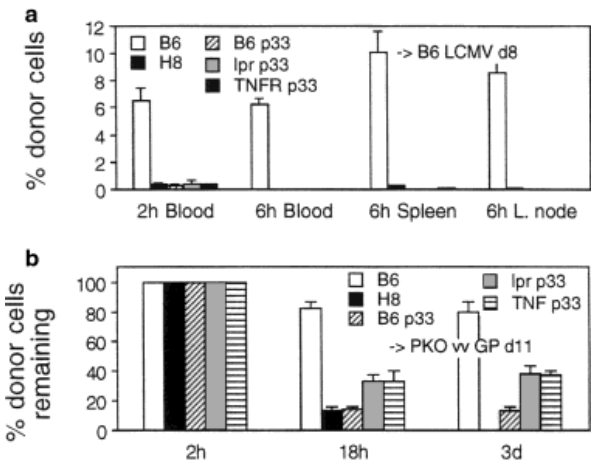

Fig. 3. Role of TNF and Fas in lymphocyte elimination by normal and perforin-deficient mice. Spleen cells from C57BL/6, lpr/lpr or TNFR $p55^{-/-}$ mice were incubated with gp33 peptide and CFSE. Cells (7×10^7) were then transfused either into C57BL/6 recipients that had been infected with LCMV 8 days earlier (a) or into perforin-deficient recipients 8 days after immunization with vacc GP (b). The percentage of CFSE⁺ donor cells among total PBL, spleen or lymph node cells was determined at different times after transfer.

cells from C57BL/6 mice (Fig. 3 b, compare columns 3–5). With peptide-loaded spleen cells, target cell elimination could only be studied during the first 18 h; by then the peptide had apparently been washed off the target cells such that further elimination did not occur (Fig. 3 b, compare time points 18 h and 3 days).

2.3 CD8⁺ T cell-mediated lymphocyte lysis *in vitro* in the absence of perforin and CD95

The unexpected elimination of lymphocytes in the absence of one or two of the three main cytolytic mechanisms raised the question whether the transfused cells were really eliminated or rather redistributed to other compartments. The evidence in Fig. 1, showing comparable kinetics of target cell elimination in various organs, rendered this possibility unlikely. Furthermore, demonstration of similar characteristics of lymphocyte lysis *in vitro*, where redistribution is not an issue, should offer additional evidence. We therefore set up *in vitro* CTL assays using CFSE-labeled lymphocytes as target cells and perforin-competent or perforin-deficient gp33-specific effector cells. Fig. 4 a shows typical results obtained by FACS analysis of unlabeled (upper panel) or gp33-pulsed (lower panel) CFSE-labeled C57BL/6 lymph node target cells after incubation with different numbers of perforin-competent effector cells. The

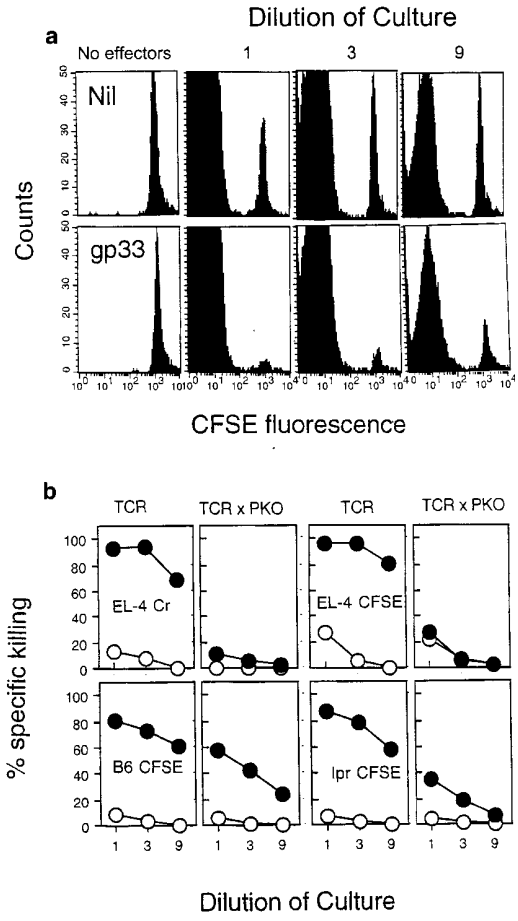

Fig. 4. Mechanisms of $CD8^+$ T cell-mediated elimination of fresh lymphocytes *in vitro*. CFSE-labeled target cells pulsed with gp33 peptide (a, lower panel; b, closed symbols) or left unlabeled (a, upper panel; b, open symbols) were incubated for 12 h with titrated numbers of peptide-stimulated effector cells from perforin-competent (a) or perforin-deficient TCR-transgenic mice (TCR *vs.* TCRxPKO). The absolute number of remaining $CFSE^+$ live lymphocytes was determined 12 h later by FACS analysis. (a) Typical flow cytometric pictures obtained using C57BL/6 lymph node target cells. (b) Lysis of ^{51}Cr-labeled or CFSE-labeld EL4 cells (upper panel) and of CFSE-labeled lymph node cells from C57BL/6 or lpr/lpr mice.

results obtained using this method were comparable to that of a conventional ^{51}Cr-release assay as lysis of EL4 target cells could be observed to a similar extent and was strictly dependent on perforin (Fig. 4 b, upper panel). Interestingly, however, lysis of CFSE-labeled C57BL/6 lymphocytes was only slightly impaired in the absence of perforin (Fig. 4 b, lower panel). Also lymphocytes from mice deficient in CD95 were lysed efficiently by perforin-competent effector cells. In the absence of both CD95 and perforin, target cell elimination was still possible, but more than tenfold less efficient in this 12-h assay (Fig. 4 b, lower panel). From these experiments we conclude that elimination of lymphocyte target cells both *ex vivo* and *in vivo* may occur via several, mutually compensating effector pathways.

2.4 Lymphocyte elimination *in vivo* versus cytolytic activity *in vitro* in acutely infected and long-term memory mice

Groups of female C57BL/6 mice were immunized against the LCMV glycoprotein epitope gp33–41 either with LCMV, vacc GP or with a transfusion of 3×10^5 male H8 spleen cells [18]. At different time points after immunization, mice were either transfused with 7×10^7 CFSE-labeled spleen cells from female H8 mice or killed for analysis of gp33-specific CTL activity *in vitro*. Eight days after LCMV infection, when brisk *ex vivo* CTL responses could be documented, H8 cells were eliminated below detection limit within 6 h (Table 2). Even 300 days after LCMV immunization, the donor cells persisted no more than 40 h. Immunization with male H8 cells did not induce significant *ex vivo* gp33-specific cytotoxicity and 300 days after immunization also sensitive *in vitro* restimulation assays remained negative. Even in these weakly immunized memory mice, however, 7×10^7 gp33-expressing donor cells were eliminated within 4 days after transfer (Table 2). We quantified the efficacy of donor cell elimination by calculating the time until 50 % elimination had been achieved and the percentage of cells eliminated per day. While approximately 3.1 % of the injected cells disappeared each day in non-immunized mice, more than 99.99 % were eliminated per day in mice 8 days after LCMV immunization. The efficacy of donor cell elimination was about 99.6 % per day 30 days and 84 % per day 300 days after LCMV infection (Table 2).

3 Discussion

In this study we have for the first time directly quantified the high efficacy of lymphocyte target cell elimination *in vivo* by antiviral $CD8^+$ T cells. During acute infection, a population of 50–70 million transfused viral antigen-expressing lymphocytes was eliminated within a few hours. Weeks after immunization, nearly the entire transfused lymphocyte target population was eliminated within a day. Even using weak forms of immunization we could detect high rates of target cell elimination when *ex vivo* and sensitive *in vitro* assays of cytotoxicity were largely negative.

What does the lymphocyte elimination assay measure? On the side of the target cells we quantified the specific disappearance of viral epitope-expressing donor spleen cells from the circulation and several organs. The cells

Table 2. Elimination kinetics of lymphocytes *in vivo* correlates with anti-viral CD8$^+$ T cell activity *in vitro*

Immunization[a)]	Days after immunization	% CTL lysis (*ex vivo*)	% CTL lysis (restimulated)	Rejection half-life (h)	% cells eliminated/ day
None	/	2	9	>500	3.1
LCMV	8	89	n.d.	1.4	99.99
	30	42	92	2.9	99.6
	300	6	85	8.9	84.6
Vacc GP	6	32	83	5.8	87.7
	15	5	79	19.7	62.3
H8 male cells	8	14	89	3.8	91.2
	300	0	11	33.2	39.2

a) Groups of two to three C57BL/6 mice were immunized i.v. either with 200 PFU LCMV, 2×10^6 PFU vacc GP or with 3×10^5 male H8 cells. At the indicated time points, mice were either transfused with 7×10^7 CFSE-labeled female H8 spleen cells or killed for determination of LCMV-specific cytotoxicity directly *ex vivo* or after restimulation *in vitro*. Data represent the mean % specific lysis achieved at an E:T ratio of 70:1 (*ex vivo*) or dilution of culture of 1:1 (restimulated). The time until 50 % of the target cell population had been eliminated was determined using the exponential decay equation $y_t = y_0\, e^{-at}$. The percentage of cells eliminated per day is given by $100(1-e^{-a})$ where a is the rate of decline of the donor cell population.

were most likely eliminated and not simply redistributed for the following reasons: (1) they disappeared with similar kinetics from the blood, the lung, the liver, the peritoneal cavity and several lymphoid organs. (2) This process was highly selective for antigen-expressing lymphocytes since the level of co-transfused control lymphocytes remained constant in the same animal during specific target cell disappearance. (3) The characteristics of lymphocyte elimination were similar when tested *in vivo* and *in vitro*, where redistribution could not occur. Compared to target cells presenting viral antigen during a natural virus infection two limitations of our assay should be stated. Thus, the amount of antigen expressed by infected cells may differ from that of cells labeled with peptide or expressing a viral epitope transgene. Also, transfusion of viral antigen-expressing lymphocytes assesses short-term effector pathways and can not model the many viral strategies of immune evasion. On the side of the effector cells viral epitope-specific CD8$^+$ T cells were required and they were directly responsible for the selective elimination of gp33-expressing lymphocytes. The transfused donor cells did not express a Th cell epitope and antibodies or NK cells were not involved. Although it is difficult to conceive how the rapid elimination of CFSE$^+$ target cells could be caused by a non-lytic mechanism, to our surprise lymphocyte elimination occurred with only slightly impaired kinetics in the absence of perforin, CD95 or TNFR.

These observations are in striking contrast to the strict perforin dependence of CD8$^+$ T cell-mediated control of acute LCMV infection *in vivo* and lysis of most tumor cell targets *in vitro*. Differences between the mechanisms required for elimination of LCMV and that of antigen-expressing lymphocytes may exist for the following reasons. First, while LCMV is a rapidly replicating pathogen, the transfused cells do not significantly increase in number after transfer. For replicating agents, the kinetic requirements for control may be much more demanding [19], such that compensation by different, potentially less effective effector mechanisms in the absence of perforin may not be sufficient. Second, LCMV predominantly infects macrophages, dendritic cells and some parenchymal cells, but infects lymphocytes only to a negligible extent. CD8$^+$ T cell-mediated killing of different cell types may have different requirements. Our experiments using CFSE-labeled target cells *in vitro* support these considerations. While conventional tumor cell line targets were killed in a perforin-dependent fashion, lysis of fresh lymphocytes was little impaired in the absence of perforin. In line with previous studies we have presented some evidence that for killing of lymphocytes several effector mechanisms may compensate for one another [6, 20]. Additional not investigated mechanisms of cytotoxicity could also play a role in this system. If they were receptor mediated, they would have to be executed via a receptor expressed on both T and B cells, since both cell types were eliminated with the same kinetics. Overall, our findings are in good keeping with previous observations in a model of GVH disease, showing that also in this setting CD8$^+$ T cell-mediated lysis of splenic lymphocytes can occur in the absence of perforin [9].

How does the lymphocyte elimination assay relate to other tests for antiviral CD8$^+$ T cells? The introduction of MHC-peptide tetramers has allowed to determine the frequency of virus-specific CTL directly *ex vivo* [1–4]. However, this method fails to give information about functional CTL activity and uncertainties as to binding strength of tetramer versus target cell binding remain unresolved. Assays quantifying the amount of cells secreting certain cytokines upon stimulation with viral antigens such as ELISpot or flow cytometric anlysis of intracellular cytokines also allow *ex vivo* analysis of antiviral CD8$^+$ T cells and show good correlation with tetramer studies [2]. However, it has been shown that detection of virus-specific CD8$^+$ T cells by this method does not necessarily correlate with antiviral effector function *in vivo* [21]. The classical functional test to quantify target cell elimination by antiviral CD8$^+$ T cells is the ^{51}Cr-release assay [5]. A major limitation of this assay is the difficulty to adequately label fresh cells, such that tumor cell lines or *in vitro* activated cells are used as a substitute. In contrast, the CFSE-based assays used in this study permit the use of fresh target cells, opening new possibilities to study lytic CD8$^+$ T cell activity both in mice and in humans. Our data show that lysis of these *ex vivo* targets may follow different rules than those established with cultured tumor cells.

To what extent does the quantification of CD8$^+$ T cell-mediated lymphocyte elimination give information about virus control *in vivo*? For LCMV, the different molecular mechanisms required for the elimination of the natural (mostly non-lymphocytic) target cells and the transfused lymphocytes render a direct comparison difficult. Nevertheless, the observed kinetics of CD8$^+$ T cell-mediated target cell elimination *in vivo* correlates well with published observations on the rates of CD8$^+$ T cell-mediated viral clearance during the acute phase of LCMV infection ([22]; data not shown). Furthermore, the still surprisingly rapid elimination *in vivo* of target cells during the memory phase – extending recent *in vitro* observations [23–26] – provides a quantitative explanation for the fact that even in the absence of antibodies it is difficult to establish an infection in primed mice.

In summary, our results give a good quantitative illustration of the surprisingly rapid CD8$^+$ T cell-mediated elimination of antigen-expressing cells *in vivo*. We have used a transgenic model to develop the assays, but since donor cells can also be labeled with peptide ([27], Fig. 3) the same method can be applied to other viral infections. It is a new and unexpected finding that this rapid elimination of lymphocytes *in vivo* may be highly efficient in the absence of perforin or CD95. For viruses with a particular tropism for lymphocytes, such as EBV or HIV, our results illustrate how effectively CD8$^+$ T cell-mediated elimination of target cells can potentially contribute to virus control and immunosuppression.

4 Materials and methods

4.1 Mice and viruses

H8 mice [9], mice expressing a transgenic TCR specific for gp33 (TCR 318 [10]) and PKO [16] mice have been described previously. C57BL/6 lpr/lpr and gld/gld mice were obtained from the Jackson Laboratory (Bar Harbor, ME). TNFR p55-deficient [17], IgM-deficient [12] and β2-microglobulin-deficient [14] mice were obtained from the referenced sources and bred locally. Mice were depleted of NK cells using a single i.p. injection of anti-NK 1.1 [13] and of CD8$^+$ T cells by two i.p. injections of anti-CD8 mAb [15] 3 and 1 days before cell transfer. All viruses and immunization protocols have been described earlier [18, 19].

4.2 Cell labeling and transfer studies

Single-cell suspensions were prepared from donor spleens and incubated at a concentration of 5×10^6 cells/ml in balanced salt solution (BSS) containing 0.5 μM CFSE (Molecular Probes, Eugene, OR) for 10 min at 37 °C. They were washed twice in BSS/1 % FCS and injected i.v. in a volume of 500 μl BSS [11]. For some experiments, donor cells were incubated with LCMV gp33 (10^{-6} M) for 2 h before CFSE labeling. At the indicated time points, recipient mice were bled from the tail vein and/or killed and single-cell suspensions were prepared from spleen, mesenteric lymph nodes, inguinal lymph nodes, Peyer's patches, peritoneal lavage, lung and liver. The cells were either used directly or after Ficoll separation (liver and lung) for flow cytometric analysis (Becton Dickinson, CellQuest software). For some experiments, the cells were additionally labeled with PE-conjugated anti-CD8, -anti-CD4 or -anti-B220 (Pharmingen).

4.3 CTL assays

The ^{51}Cr-release assays for determination of LCMV-specific cytotoxicity *ex vivo* or after restimulation in *in vitro* were performed on MC57G and EL4 target cells as described earlier [19]. Spontaneous ^{51}Cr-release was below 25 % in all assays shown. CFSE-labeled target cells were generated by labeling EL4 target cells or fresh spleen or lymph node cells from C57BL/6 or lpr/lpr mice with gp33 peptide and CFSE as outlined above. Spleen cells from perforin-competent or perforin-deficient mice transgenic for an LCMV gp33-specific TCR (TCR *vs.* TCRxPKO mice) were cultured with 10^{-6} M gp33 peptide for 3 days and used as effector cells at different dilutions of the standard culture. Effector and target cells were incubated for 12 h in 96-well plates. The wells were then harvested into individual FACS tubes and the absolute number of remaining CFSE$^+$ cells per well was

determined by flow cytometry using a gate for live lymphocytes. The percentage specific lysis was calculated as follows: ($CFSE^+$ cells remaining after incubation of target cells alone – $CSFE^+$ cells remaining after incubation of a given amount of effector cells and target cells)/$CFSE^+$ cells remaining after incubation of target cells alone × 100.

4.4 Mathematical analysis

The efficacy of the $CD8^+$ T cell response *in vivo* was quantified as the percentage of cells lysed per day. The exponential decay equation $y_t = y_0 e^{-at}$ was used to describe the kinetics of target cell elimination. y_t denotes the number of donor cells at time *t*, y_0 the initial number of cells injected, and *a* the rate of donor cell decay. The percentage of cells eliminated each day is given by $100(1-e^{-a})$. The time until 50 % of the target cell population had been eliminated was denoted as "elimination half-life". This term refers to the elimination of a population of cells rather than the lysis of individual target cells, which is characterized by a distribution of elimination times.

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