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# Killing of targets by effector CD8 T cells in the mouse spleen follows the law of mass action

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## Abstract

In contrast with antibody-based vaccines, it has been difficult to measure the efficacy of T cell-based vaccines and to correlate the efficacy of CD8 T cell responses with protection against viral infections. In part, this difficulty is due to poor understanding of the *in vivo* efficacy of CD8 T cells produced by vaccination. Using a recently developed experimental method of *in vivo* cytotoxicity we have investigated quantitative aspects of killing of peptide-pulsed targets by effector and memory CD8 T cells, specific to three epitopes of lymphocytic choriomeningitis virus (LCMV), in the mouse spleen. By analyzing data on killing of targets with varying number of epitope-specific effector and memory CD8 T cells, we find that killing of targets by effectors follows the law of mass-action, that is the death rate of peptide-pulsed targets is proportional to the frequency of CTLs in the spleen. In contrast, killing of targets by memory CD8 T cells does not follow the mass action law because the death rate of targets saturates at high frequencies of memory CD8 T cells. For both effector and memory cells, we also find little support for the killing term that includes the decrease of the death rate of targets with target cell density. Interestingly, our analysis suggests that at low CD8 T cell frequencies, memory CD8 T cells on the per capita basis are more efficient at killing peptide-pulsed targets than effectors, but at high frequencies, effectors are more efficient killers than memory T cells. Comparison of the estimated killing efficacy of effector T cells with the value that is predicted from theoretical physics and based on motility of T cells in lymphoid tissues, suggests that limiting step in the killing of peptide-pulsed targets is delivering the lethal hit and not finding the target. Our results thus form a basis for quantitative understanding of the process of killing of virus-infected cells by T cell responses in tissues and can be used to correlate the phenotype of vaccine-induced memory CD8 T cells with their killing efficacy *in vivo*.

Short running title: Killing by CD8 T cells in tissues

Abbreviations: LCMV, lymphocytic choriomeningitis virus, CTLs, cytotoxic T lymphocytes, TDLs, thoracic duct lymphocytes, CIs, confidence intervals, RSS, residual sum of squares, AIC, Akaike's An Information Criterion.

# 1 Introduction

Vaccination is often considered as one of the greatest medical achievements of the last century. Despite widespread use of vaccination, we still have a very limited understanding of how to generate a good vaccine. In particular, no successful vaccine has been yet produced to protect against AIDS, malaria or tuberculosis despite decades of extensive research.

It is generally believed that most of currently used effective vaccines provide protection by inducing high titers of pathogen-neutralizing antibodies (e.g., (1)). In such cases, the efficacy of an antibody-inducing vaccine can be evaluated after vaccination as an inverse of the titer of neutralizing antibody (refs?). In contrast, some vaccines that are currently being developed against chronic infections such as HIV and malaria, are aimed to stimulate T cell responses. It is unclear, however, what parameters of T cell memory, induced by vaccination, best correlate with protection (1). It has been suggested that polyfunctional memory CD4 T cells may be superior in providing protection following infection with *Leishmania* (2), but evidence is still lacking for many other important infections of humans (3, 4).

In part, our limited understanding of how memory T cells provide protection comes from the fact that most of the effector functions of effector and memory T cells are generally measured *in vitro* (after short- or long-term restimulation), and we know very few quantitative details of how T cells may control pathogen growth in tissues (e.g., (5)). Recently, a new experimental technique to measure cytotoxic efficacy of CD8 T cells *in vivo* has been introduced (6–10). In this assay, peptide-pulsed and unpulsed target cells are transferred into mice harboring peptide-specific effector or memory CD8 T cells, and elimination of pulsed targets is used as indication of Ag-specific killing *in vivo* (11–15). We use the data from recently published experiments (12) and our recently developed mathematical model (16) to investigate quantitative details how effector and memory CD8 T cells, specific to three epitopes of lymphocytic choriomeningitis virus (LCMV), kill peptide-pulsed targets in the mouse spleen.

Unexpectedly, our results suggest that killing in the spleen by effector CD8 T cells (present at the peak of the immune response) follows the law of mass action: rate of killing is simply proportional to the density of targets and the frequency of effector CD8 T cells in the spleen. Such linear dependence of the death rate of targets on the frequency of effectors in the spleen was observed with the latter changing about 100 fold. In contrast, killing of targets by LCMV-specific memory CD8 T cells does not follow the law of mass action: the death rate of peptide-pulsed targets saturates at high frequencies of memory CD8 T cells. This suggests that there might be an upper bound level of efficacy of the total memory T cell response, and this potentially may limit the efficacy of T-cell based vaccines.

This analysis may form a basis for quantitative understanding of efficacy of T cell-based vaccines. By correlating expression of various cell surface and intracellular markers with the in

vivo killing efficacy of memory T cells of different specificities in mice, we may better understand which qualities of memory cells provide best protection. Such information can potentially be further used to predict efficacy of T cell-based vaccines in humans.

## 2 Material and Methods

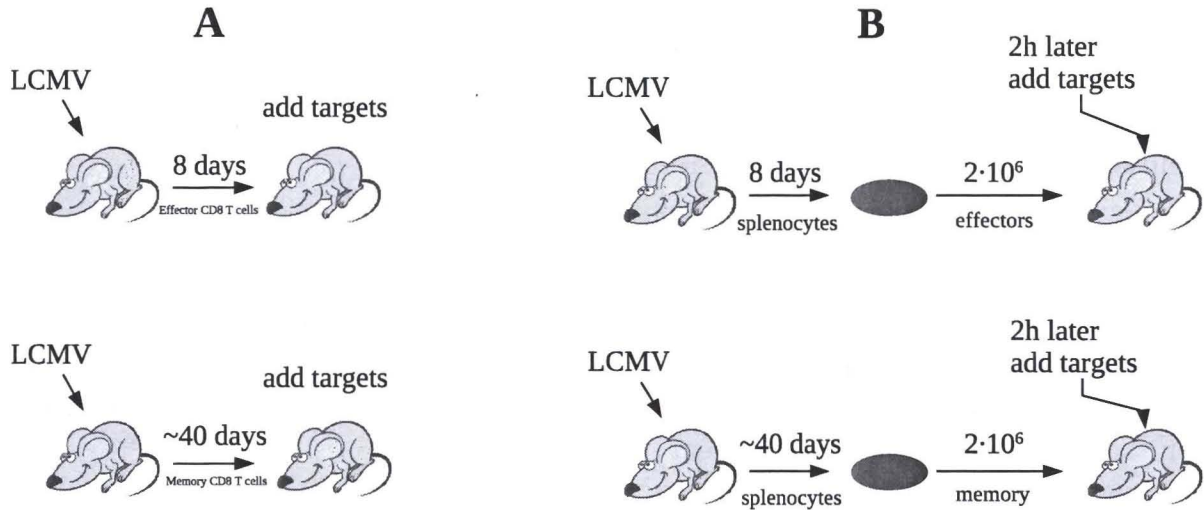
### 2.1 Cytotoxicity in vivo

The method of measuring cytotoxicity of CD8<sup>+</sup> T cells in vivo has been described in great detail elsewhere (e.g., (15)). In this report, we analyze recently published data on killing of peptide-pulsed splenocytes by LCMV-specific effector and memory CD8<sup>+</sup> T cells (12, Figure 1). The reader is referred to the original publication for more detail. In the first set of experiments, target splenocytes were pulsed with NP396 or GP276 peptides of LCMV (10  $\mu$ M) or left unpulsed. Targets were transferred into syngenic mice either infected with LCMV 8 days previously (“acutely infected” mice) or recovered from LCMV infection (LCMV-immune or “memory” mice). At different times after the transfer, spleens were harvested, and the number of pulsed and unpulsed targets, splenocytes, and peptide-specific CD8 T cells was calculated.

In the adoptive transfer experiments,  $10^6$  of P14 CD8 T cells, expressing TCR specific for the GP33 epitope of LCMV, were transferred into recipient B6 mice and infected i.p. with LCMV-Arm (17). Eight (effectors) or 40 (memory) days later different numbers of P14 CD8 T cells were transferred into new naive recipients (Figure 1B). The number of effector CD8 T cells transferred into different recipients was  $10^6$ ,  $2 \times 10^6$ ,  $10^7$ , and  $2 \times 10^7$ . The number of memory CD8 T cells transferred into different recipients was  $10^6$ ,  $2 \times 10^6$ , and  $10^7$ . Two hours later, two populations of CFSE labeled splenocytes, one of which was pulsed with GP33 peptide of LCMV (1  $\mu$ M), were transferred into recipient mice, harboring GP33-specific effector or memory CD8 T cells. Percent targets killed was calculated at different times after target cell transfer as described earlier (12, 15). The ratio of the frequency of pulsed to unpulsed targets, used in fitting of the data, was calculated as  $R = 1 - L/100$  where  $L$  is the percent of peptide-pulsed targets killed (15, 16).

### 2.2 Mathematical model for the cytotoxicity in vivo assay

Details of the mathematical model proposed to describe migration of injected targets from the blood to the spleen and killing of peptide-pulsed targets in the spleen is given in great detail elsewhere (16, see also Supplementary Information). In short, target cells injected i.v. migrate from the blood to the spleen at a rate  $\sigma$ , die at a rate  $\epsilon$  due to preparation techniques (independent of CD8 T cell mediated killing), or migrate to other tissues and/or die elsewhere



**Figure 1:** Schematic representation of the in vivo cytotoxicity assays undertaken to investigate the quantitative details of CD8 T cell mediated killing of peptide-pulsed targets in the mouse spleen. In the first set of experiments (panel A), B6 mice were infected with LCMV-Arm and 8 or 37-100 days later, three populations of  $5 \times 10^6$  target cells (pulsed with either NP296 or GP276 peptides of LCMV and unpulsed) were transferred into these mice. In the second set of experiments, P14 TCR Tg CD8 T cells, specific to the GP33 epitope of LCMV, were transferred into B6 mice and then infected with LCMV-Arm. Eight or 40 days later, different number of effector (day 8) or memory (day 40) P14 CD8 T cells were transferred into naive B6 mice. Two hours later, two populations of  $5 \times 10^6$  targets (pulsed with the GP33 peptide of LCMV and unpulsed) were transferred into mice harboring epitope-specific CD8 T cells. In both sets of experiments, killing of peptide-pulsed targets was measured in spleens of mice at different times after cell transfer (12).

at a rate  $\delta$ . In the spleen, targets die due to preparation-induced death rate  $\epsilon$ , and peptide-pulsed targets also die due to CD8 T cell mediated killing, described by the rate  $K$ . The dynamics of unpulsed targets  $S(t)$  and the ratio of the frequency of peptide-pulsed to unpulsed targets  $R(t)$  in the spleen is given by equations (16)

$$S(t) = \frac{S_B(0)\sigma}{d - \epsilon} [1 - e^{-(d-\epsilon)t}] e^{-\epsilon t}, \quad (1)$$

$$R(t) = \frac{(d - \epsilon)}{(K - (d - \epsilon))} \left[ \frac{e^{-(d-\epsilon)t} - e^{-Kt}}{1 - e^{-(d-\epsilon)t}} \right] e^{-\epsilon t}, \quad (2)$$

where  $d = \sigma + \epsilon + \delta$  is the rate of removal of cells from the blood and  $S_B(0) = 5 \times 10^6$  is the initial number of unpulsed targets in the blood (12).

### 2.3 Recruitment of targets to the spleen

We have shown previously that the rate of recruitment of target cells from the blood to the spleen depends on the spleen size (16). Therefore, to describe recruitment of targets into the spleen we let the rate of recruitment to be  $\sigma = \alpha \times (N_s)_i$  where  $(N_s)_i$  is the number of splenocytes in the  $i^{\text{th}}$  mouse and  $\alpha$  is a coefficient (16).

### 2.4 Killing of peptide-pulsed targets in the spleen

In our previous study we have estimated the death rate of targets  $K$ , pulsed with NP396 or GP275 peptides of LCMV, due to killing by effector or memory CD8 T cell response (16). To estimate the per capita killing efficacy of CD8 T cells, we have to relate the death rate of peptide pulsed targets  $K$  to the density of epitope-specific CD8 T cells in the mouse spleen. It is generally assumed that killing of targets follows the law of mass action (18, 19), that is the death rate of peptide-pulsed targets is proportional to the frequency of peptide-specific CD8 T cells. However, spleen tissue has a complicated structure and the assumption of mass-action like encounter of targets and CD8 T cells need not hold. Therefore, here we test several different killing terms in how well they describe the data from the in vivo cytotoxicity assay. In a mass-action, killing occurs at the rate that is proportional to the number of targets and the frequency of epitope-specific CD8 T cells  $E_i$  in the spleen of the  $i^{\text{th}}$  mouse. Then the death rate of peptide-pulsed targets in the  $i^{\text{th}}$  mouse due to CD8 T cell mediated killing is  $K = kE_i$ . Alternatively, it is possible that the encounter rate between targets and killers does not follow the law of mass-action and may be affected by the frequency or the number of CD8 T cells or targets. A simple analysis shows that at high number of CD8 T cells, the death rate of peptide-pulsed targets will saturate with the frequency of killers in the spleen (20, see Supplementary Information); then

$$K = \frac{kE_i}{1 + c_E E_i}, \quad (3)$$

where  $c_E$  is the inverse frequency of CD8 T cells at which killing is half maximal. On the other hand, at large numbers of pulsed targets, the death rate of pulsed targets may decrease with increasing target cell frequency  $\mathcal{T}_i = T_i/(N_s)_i$  in the  $i^{\text{th}}$  mouse (21, see Supplementary Information); then

$$K = \frac{kE_i}{1 + c_T \mathcal{T}_i}, \quad (4)$$

where  $c_T$  is the inverse frequency of pulsed targets at which killing is half maximal. Under some circumstances, both processes may become important (20, results not shown). Finally, the death rate of a single target may depend on the effector to target ratio,  $E_i/\mathcal{T}_i$  (22). Then assuming saturation in the death rate with the ratio of effectors to targets, we obtain

$$K = \frac{k(E_i/\tau_i)}{c_T + (E_i/\mathcal{T}_i)} = \frac{kE_i}{E_i + c_T \mathcal{T}_i}, \quad (5)$$

Note that in those cases, when the death rate of targets due to CD8 T cell mediated killing  $K$  depends on the target cell frequency ( $K = K(\mathcal{T})$ , see eqn. (4) and (5)), eqn. (2) cannot be used. Instead, we numerically solve the model given by differential equations (see Supplementary Information) and fit the numerical solution of the model to data.

## 2.5 Statistics

To fit the data on recruitment of targets into the spleen and on killing of peptide-pulsed targets in the spleen at the same time we log-transform the data and the model predictions. To access lack of fit of the data with repeated measurements we use the F-test (23, p. 29). To compare nested models we also use the F-test (23, p. 104). Fittings were done in Mathematica 5.2 using the routine FindMinimum.

## 3 Results

To investigate quantitative aspects of how effector and memory CD8 T cells kill their targets in the mouse spleen we analyze data from recently published experiments on killing of targets pulsed with either NP396, GP276, or GP33 peptides of LCMV by LCMV-specific effector or memory CD8 T cells (12). Mice, infected with LCMV-Armstrong develop a vigorous CD8 T cell response, specific for LCMV, that peaks 8 days after the infection (24, 25). By 15-30 days after the infection, most of effectors die and a small population of LCMV-specific memory CD8 T



cells persist for the life of the animal (25). To measure the efficacy of effector and memory CD8 T cells, target cells pulsed with LCMV-specific peptides (NP396 or GP276) were transferred into mice infected 8 or 37 – 100 days previously (12), and the percent of targets killed by effectors or memory CD8 T cells was calculated (see Materials and Methods and Figure 1).

We have previously developed a mathematical model to estimate the killing efficacy of LCMV-specific T cell responses from the data obtained in such in vivo cytotoxicity assay (16). The model describes several important processes: recruitment of target cells from the blood to the spleen, death of targets due to preparation, and killing of peptide-pulsed targets by the peptide-specific CD8 T cell response in the spleen (16, see Materials and Methods). From these data we could estimate the death rate of peptide-pulsed targets due to killing by effector or memory CD8 T cell response (16).

We have now extended this model by allowing different terms describing killing of targets by epitope-specific CD8 T cells (see Materials and Methods). Using the approach of the previous study (18), we fitted the data on killing of peptide-pulsed targets by LCMV-specific CD8 T cells in acutely infected and LCMV-immune mice assuming that killing of targets is proportional to the frequency of epitope-specific CD8 T cells in the spleen of a given mouse. Although this extended model could fit the data, to our surprise, the quality of the model fit to data was rather poor (lack of fit test:  $F_{30,158} = 3.1$ ,  $p = 2.6 \times 10^{-6}$ ). Furthermore, assuming that the death rate of peptide-pulsed targets  $K$  is dependent on the total number of epitope-specific CD8 T cells led to even worse fits of the data (lack of fit test:  $F_{30,158} = 5.54$ ,  $p = 3.4 \times 10^{-13}$ ). The reason for the poor fit in both cases is because the frequency (or the total number) of epitope-specific CD8 T cells measured in individual mice did not predict well the killing of targets in the same mice (Figure 5 in Supplementary Information).

We have therefore explored modifications of the model that could lead to an improved fit of the model to data. Including a decrease in the death rate of targets with the frequency of targets (see eqn. (4)) failed to improve the quality of the model fit to data (F-test for nested models,  $F_{1,187} = 0.001$ ,  $p = 0.98$ ). This was not surprising since this change in the killing term predicts an increased rate of loss of peptide-pulsed targets with time as more targets are killed, but the opposite trend is observed in the data (e.g., Figure 2C).

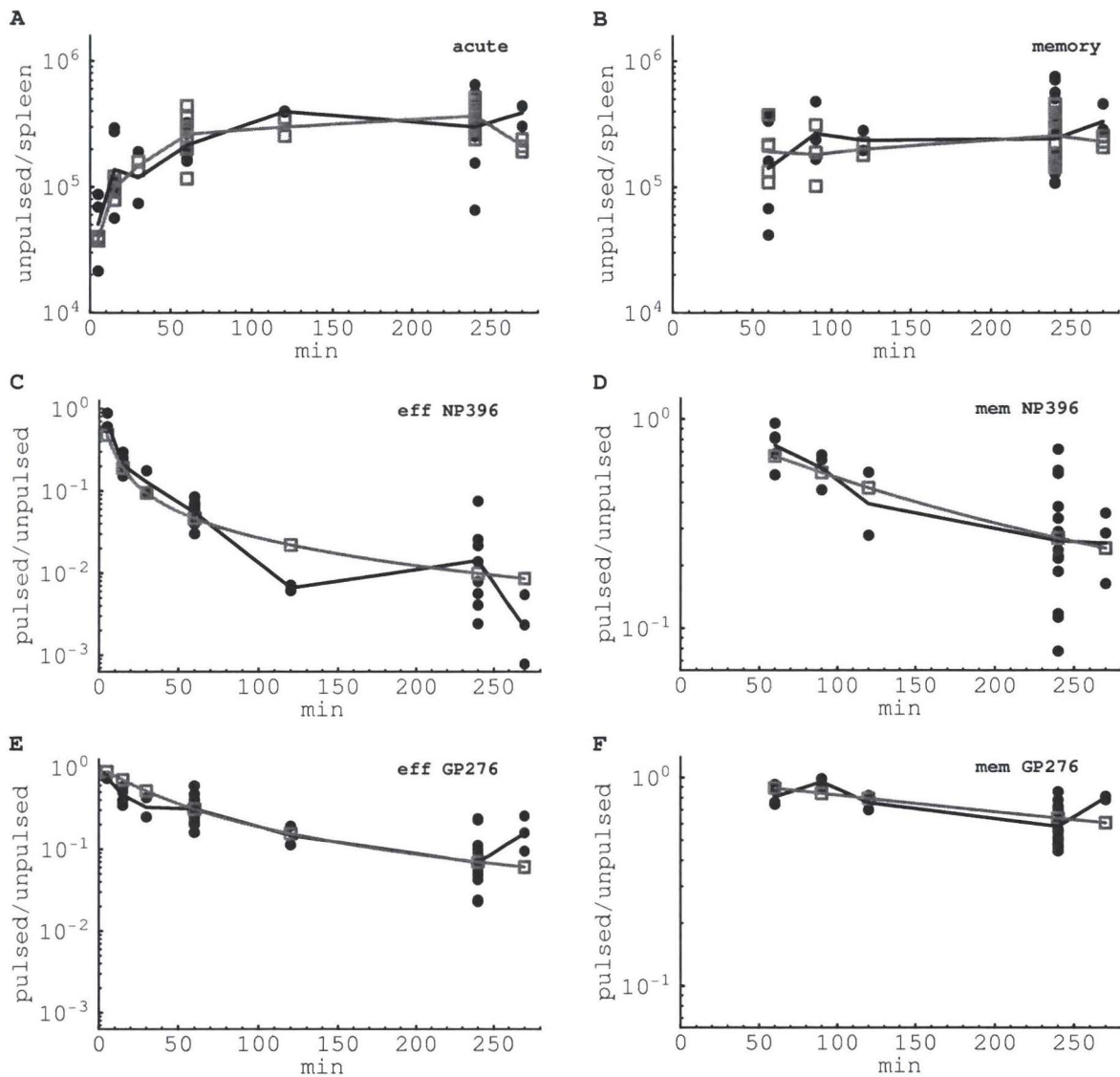
Three modifications, however, did lead to a dramatic improvement of the model fit to data. First, we assumed that the average frequency of epitope-specific CD8 T cells rather than values measured in individual mice is predictive of the rate of killing of peptide-pulsed targets. Therefore, we fitted the data on killing of peptide-pulsed targets using the mass-action type term  $K = kE$  where  $E$  is the average frequency of epitope-specific CD8 T cells in acutely infected or LCMV-immune mice. This resulted in a significantly better model fit with reasonably small confidence intervals for the estimates of the model parameters (lack of fit test:  $F_{30,162} = 0.79$ ,  $p = 0.77$ ; see also Figure 2 and Table 1). The good fit of this model to data suggests that

variation in the frequency of epitope-specific CD8 T cells measured in different mice represents noise and is not translated into variation in the killing of peptide-pulsed targets, since the average CTL frequency predicted the number of target cells killed significantly better. The absence of a strong correlation between the number of targets killed and the CTL frequency in a given mouse further supports this conclusion (see Figure 6 in Supplementary Information). Interestingly, this model fit predicted that both NP396- and GP276-specific memory CD8 T cells are only half as efficient as effectors of the same specificity (Table 1), that is in contrast with a previously obtained result (18).

Second, we allowed the death rate of peptide-pulsed targets to saturate with the frequency of epitope-specific CD8 T cells (see eqn. (3)). This also led to a significantly improved fit of the data (F-test for nested models:  $F_{1,186} = 50.3$ ,  $p = 2.7 \times 10^{-11}$ ), but predicted a low per capita killing efficacy of effector and memory CD8 T cells ( $k_{NP396}/(1 + c_E) = 0.36 \text{ min}^{-1}$ ,  $k_{GP276}/(1 + c_E) = 0.06 \text{ min}^{-1}$ , and  $c_E = 400.4$ ; compare these estimates to values given in Table 1). Moreover, this model predicted that memory CD8 T cells are only 6% (NP396) or 14% (GP276) as efficient as effectors. Such improvement of the fit with including saturation of the death rate of peptide-pulsed targets with CTL frequency is expected if measurements of the frequency of epitope-specific CD8 T cells are noisy. Saturation in the death rate with CD8 T cell frequency was also important in LCMV-immune mice that had low frequencies of LCMV-specific CD8 T cells, since allowing for saturation led to an improved fit of the model to the subset of data ( $F_{1,78} = 15.3$ ,  $p = 2.0 \times 10^{-4}$ ).

Finally, by allowing the death rate of peptide-pulsed targets to depend on the ratio of the frequency of killers to targets (see eqn. (5)) we could also obtain an improved fit of the model to data (lack of fit test:  $F_{28,158} = 1.38$ ,  $p = 0.11$ ). Interestingly, we found a relatively small estimate for the parameter  $c_T$  ( $c_T = 1.37$ ). Because effector to target ratios are rather high in most mice (e.g.,  $E/\tau \approx 10^3$  for NP396- and  $E/\tau \approx 10^2$  for GP276-specific effectors and epitope-expressing targets, see Table 1), a small estimate for the constant  $c_T$  suggests that the death rate of peptide-pulsed targets saturates with the frequency of CD8 T cells (compare eqn. (5) for  $E \gg c_T \tau$  and eqn. (3) for  $c_E E \gg 1$ ). Therefore, the last two models appear to be similar with respect to these in vivo data since both models require saturation in the death rate of peptide-pulsed targets with the frequency of epitope-specific CD8 T cells for a satisfactory description of the data.

Thus, we have generated two alternative models that provide good description of the data but generate highly distinct predictions on the efficiency and nature of killing of targets in the mouse spleen. To discriminate between these alternative models we have analyzed data from additional experiments involving transfer of different numbers of effector or memory CD8 T cells specific to the GP33 epitope of LCMV (12, see Figure 1B). Two hours after transfer of CD8 T cells, GP33-pulsed and unpulsed target cells were transferred into mice harboring



**Figure 2:** Fits of the model given in eqn. (1)–(2) that assumes that the rate of recruitment of targets into the spleen depends on the spleen size and that killing of peptide-pulsed targets depends on the average frequency of epitope-specific CD8 T cells, to data. Panels A-B show the recruitment of unpulsed targets into the spleen, and panels C-F show the decline in the the ratio of the frequency of peptide-pulsed to unpulsed targets over time. Panels A, C, and E are for acutely infected mice, and panels B, D, F are for LCMV-immune (memory) mice. Panels C and D are for NP396-pulsed targets and panels E and F are the GP276-pulsed targets. Black dots ( $\bullet$ ) denote measurements from individual mice, and black lines denote the log average value per time point. Red boxes ( $\blacksquare$ ) show the number of recruited cells predicted by the model for individual mice (panels A and B) or the predicted average ratio  $R$  (panels C-F). Red lines show the log average between individually predicted values. Note the different scale for killing of target cells in acutely infected (panels C and E) and memory (panels D and F) mice. Parameters providing the best fit of the model are shown in Table 1. The lack of fit test confirms good quality fits of the data (after removing two outliers,  $F_{30,162} = 0.79$ ,  $p = 0.77$ ). The residual sum of squares of the fit was  $RSS=65.56$ .

Parameter	Mean	95% CIs	$E/T$	$E, \%$	$E, 10^6$ cells
$\alpha_E, 10^{-12} \text{ min}^{-1}$	7.17	5.63 – 9.25			
$\alpha_M, 10^{-11} \text{ min}^{-1}$	1.30	0.90 – 1.88			
$\epsilon, 10^{-3} \text{ min}^{-1}$	4.71	3.37 – 6.12			
$k_{NP396}, \text{ min}^{-1}$	5.50	4.30-7.27	2471	6.3	10.4
$k_{GP276}, \text{ min}^{-1}$	2.35	1.85-2.89	130	2.1	3.6
$\gamma_{NP396}$	0.49	0.35-0.63	4.9	0.54	0.32
$\gamma_{GP276}$	0.49	0.35-0.63	1.5	0.35	0.21

**Table 1:** Parameters providing the best fit of the mathematical model that assumes that the rate of recruitment of targets into the spleen depends on the spleen size and that killing of peptide-pulsed targets depends on the average frequency of epitope-specific CD8 T cells in the spleen. Here  $\alpha_A$  and  $\alpha_M$  are coefficients relating the recruitment rate of cells into the spleen  $\sigma = \alpha N_s$  and  $N_s$  is the number of splenocytes in individual mice,  $\gamma$  is the ratio of the killing efficacy of epitope-specific memory CD8 T cells to that of effector CD8 T cells. In the fits the rate of migration of labeled splenocytes to other organs  $\delta$  was fixed to 0 since this did not affect the quality of the model fit to data ( $F_{1,191} = 0.15$ ,  $p = 0.70$ ). Data and model fits are shown in Figure 2. CIs were calculated by bootstrapping the data with 1000 simulations (26). Note that the fits predict that epitope-specific memory CD8 T cells on the per capita basis are half as efficient as effectors ( $\gamma = \gamma_{NP396} = \gamma_{GP276} \approx 0.5$ ). For different experiments, we also show the average effector to target ration ( $E/T$ ), the average percentage and the total number of epitope-specific CD8 T in the spleen.

GP33-specific CD8 T cells, and killing of pulsed targets was measured longitudinally (12, Figure 3). Approximately 2 to 10% of adoptively transferred CD8 T cells accumulated in the mouse spleen (Table 1). Since the transfer of different numbers of epitope-specific CD8 T cells led to different frequencies of these cells in the spleen as well as to different effector to target ratios (Table 2), these data allowed for a unique opportunity to investigate whether the per capita killing efficacy of LCMV-specific effector and memory CD8 T cells is independent of these two quantities.

Therefore, we fitted the model given in eqn. (1)-(2) to these data assuming that the death rate of peptide-pulsed targets  $K$  depends on the frequency of GP33-specific CD8 T cells in the spleen (i.e., follows the law of mass action). The model described the data very well with the exception of one time point with very few unpulsed targets being recruited into the spleen (Figure 3A at  $2 \times 10^7$  effector CD8 T cell transferred; lack of fit test with two outliers removed:  $F_{20,50} = 0.92$ ,  $p = 0.56$ ). By fitting the model, we estimated parameters determining the rate of migration of targets from the blood to the spleen as well as the per capita killing efficacy of GP33-specific effector (given by  $k_i$ ) and memory ( $\gamma_i \times k_i$ ) CD8 T cells at different frequencies of effectors of memory CD8 T cells in the spleen (Table 2 and Figure 4). Surprisingly, the model fits predicted that the per capita killing efficacy of effector CD8 T cells was largely independent of the frequency of effectors in the spleen equaling on average  $k_{GP33} = 2.1 \pm 0.65 \text{ min}^{-1}$  per frequency of CD8 T cells. Most importantly, this estimate is almost identical to the estimate of the killing efficacy of GP276-specific effectors obtained by fitting the in vivo data using the average frequency of epitope-specific CD8 T cells in the spleen (see Table 1). Thus, this analysis suggests that changing the frequency of epitope-specific CD8 T cells in the mouse spleen from  $6 \times 10^{-4}$  (transfer of  $10^6$  effectors, see Table 2) to  $2 \times 10^{-2}$  (GP276-) or  $6 \times 10^{-2}$  (NP396-specific effectors, see Table 1) does not dramatically affect the per capita killing efficacy of LCMV-specific effector CD8 T cells. In other words, this implies that killing of targets in the mouse spleen by effector CD8 T cells follows the law of mass action where the rate of killing is simply proportional to the frequency of epitope-specific CD8 T cells (ref on mass action?).

In contrast with effectors, we estimated that the killing efficacy of memory CD8 T cells declines with an increasing number of memory T cell transferred (Table 2 and Figure 4). The killing efficacy of memory CD8 T cells was higher than that of effectors at low number of epitope-specific CD8 T cell transferred (Figure 4). The ratio of the killing efficacy of memory CD8 T cells to that of effectors was declining with increasing frequency of epitope-specific CD8 T cells in the spleen (Figure 4). This suggests that the death rate of peptide-pulsed targets due to killing by memory CD8 T cells saturates with the frequency of CD8 T cells and/or the effector to target ratio in the mouse spleen.

It is important to compare the killing efficacy of memory CD8 T cells obtained from the adoptive transfer experiments with that found during the in vivo infection (Table 1 and 2).

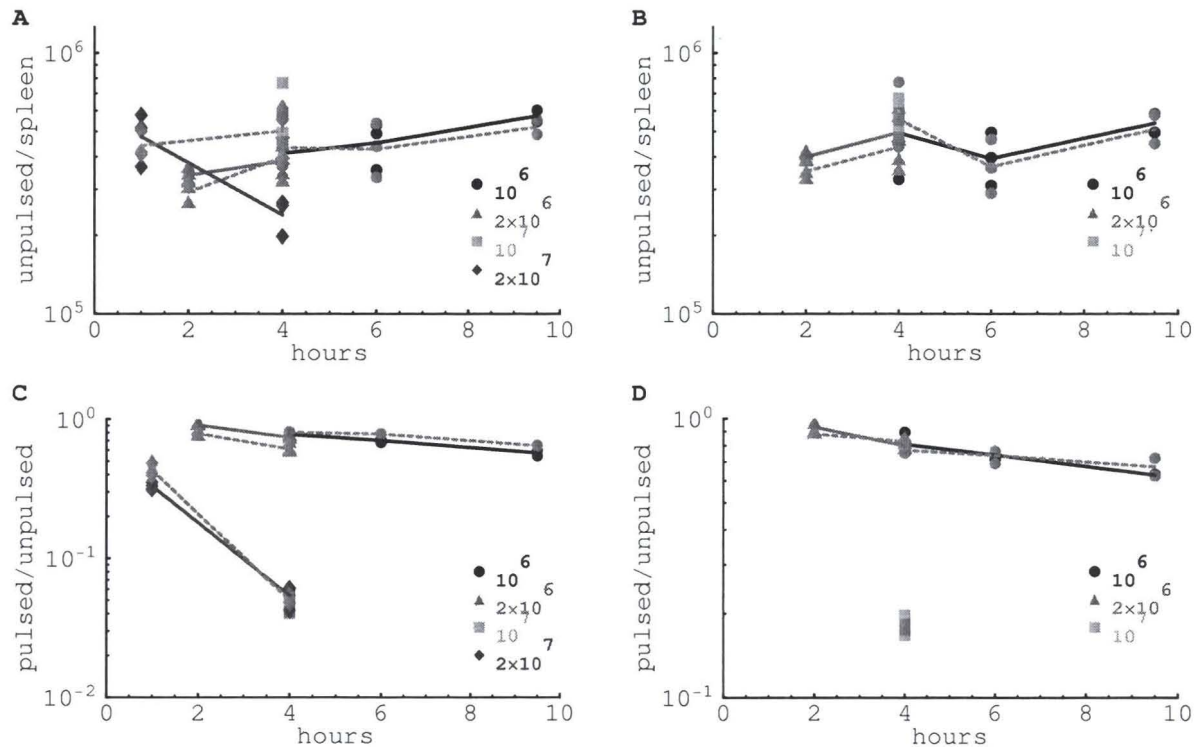
After clearance of LCMV infection, there are about  $2 - 3 \times 10^5$  of NP396- or GP276-specific memory CD8 T cells in the mouse spleen, that constitutes 0.3-0.5% of all splenocytes (Table 1). Following transfer of  $2 \times 10^6$  GP33-specific memory CD8 T cells, after 2 hours, transferred memory cells constitute 0.15-0.18% of all splenocytes (Table 2). At these frequencies in vivo, both in adoptive transfer experiments and following LCMV infection, memory CD8 T cells are only half as efficient at killing peptide-pulsed targets as are effector CD8 T cells of the same specificity. The remarkable match of the killing efficacy of effector and memory CD8 T cells following the in vivo LCMV infection and after adoptive transfer argues in favor of the first modified model that assumed that the death rate of peptide-pulsed targets is proportional to the average frequency of effector or memory CD8 T cells in the spleen (Figure 2). The lack of a large decrease of the killing efficacy of effector or memory CD8 T cells at high CD8 T cell frequencies ( $E \approx 1.25 - 1.54\%$ , see Table 2) or effector to target ratios ( $E/T \approx 10 - 40$ ) as would be predicted in the model with saturation of the death rate of targets with CD8 T cell frequency or  $E/T$  (e.g.,  $k_{GP276}/(1 + c_E) = 0.06 \text{ min}^{-1}$ , see eqn. (3) and text above), provides little support for the alternative models.

Assuming that the death rate of peptide-pulsed targets is proportional to the total number of epitope-specific CD8 T cells in the mouse spleen, we have found that following acute LCMV infection effector and memory CD8 T cells have a similar per capita killing efficacy (results not shown). This is contrast to the result obtained from the adoptive transfer experiments where at similar numbers of epitope-specific effector and memory CD8 T cells, memory T cells are only 30% to 50% as efficient as effectors. This argues that killing targets in the spleen is proportional to the frequency and not the total number of epitope-specific CD8 T cells confirming a previously made assumption (18).

## 4 Discussion

Recent interest in T cell based vaccines against several chronic infections of humans requires the development of experimental and theoretical tools to access the efficacy of such vaccines (27). It is generally believed that memory CD8 T cells induced by vaccination are not able to provide sterilizing immunity, because T cells react only to infected cells, i.e., after the infection has been established. However, a recent study has shown an example where generation of a large population of memory CD8 T cells by vaccination did provide sterilizing immunity against malaria (28).

Quantitative approaches aimed at estimating the in vivo efficacy of effector and memory CD8 T cells and at quantitative details of how CD8 T cells control growth of pathogens are necessary to understand how protection induced by T cell-based vaccines is achieved. This study utilized a recently developed experimental technique of in vivo cytotoxicity to investigate

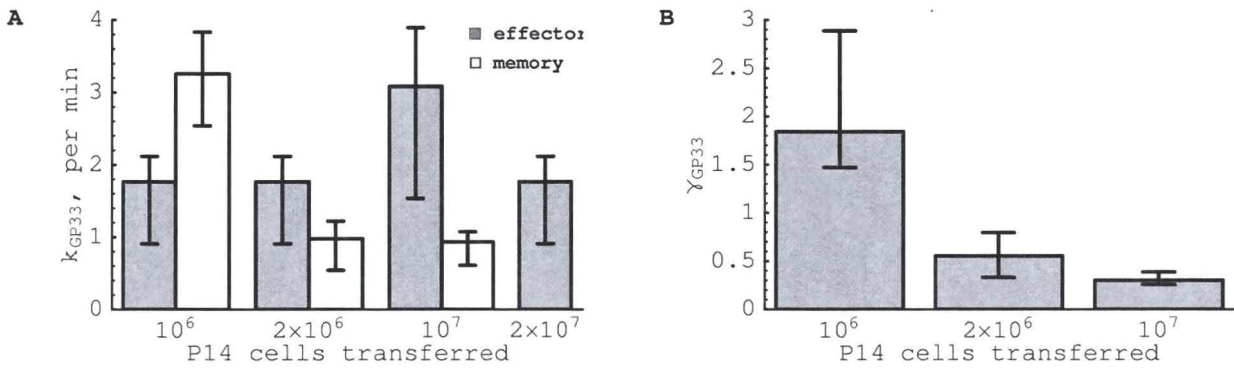


**Figure 3:** Fits of the mathematical model to data from experiments involving adoptive transfer of different numbers of epitope-specific effector (panels A&C) or memory (panels B&D) CD8 T cells. Panels A and B show the number of unpulsed targets at different times after cell transfer. Panels C and D show the change in the ratio of the frequency of peptide-pulsed to unpulsed targets with time. Different symbols denote data from different adoptive transfer experiments with  $10^6$ ,  $2 \times 10^6$ ,  $10^7$ , or  $2 \times 10^7$  T cells transferred. Symbols denote individual measurements with averages per time point being connected by solid lines. Light brown symbols are the model predictions with averages being connected by dashed lines. Parameters providing the best fits of the model are shown in Table 2. Note that in panel A, the model does not predict the decline in the number of unpulsed targets with time in experiments with transfer of  $2 \times 10^7$  GP33-specific effectors. Such decline in the number of unpulsed targets in the spleen is unexpected and most likely is due to a measurement error.

Parameter	Mean	95% CIs	$E/T$	$E$ , %	$E$ , $10^6$ cells	Cells transferred
$\alpha$ , $10^{-11} \text{ min}^{-1} \text{ cell}^{-1}$	2.14	1.88–6.16				
$\epsilon$ , $10^{-3} \text{ min}^{-1}$	1.1	0.4–1.6				
$\delta$ , $10^{-2} \text{ min}^{-1}$	1.0	0.7–4.3				
$k_1$ , $\text{min}^{-1}$	1.77	0.91–2.12	0.14	0.06	0.05	$10^6$
$\gamma_1$	1.84	1.47–2.78	0.09	0.04	0.03	
$k_2$ , $\text{min}^{-1}$	1.77	0.91–2.12	0.34	0.18	0.10	$2 \times 10^6$
$\gamma_2$	0.55	0.36–0.81	0.25	0.15	0.09	
$k_3$ , $\text{min}^{-1}$	3.08	1.6–3.9	23.7	0.87	0.68	$10^7$
$\gamma_3$	0.3	0.26–0.39	9.48	1.25	1.05	
$k_4$ , $\text{min}^{-1}$	1.77	0.91–2.12	44.4	1.54	1.43	$2 \times 10^7$

**Table 2:** Estimates of parameters of the mathematical model fitted to the data from the adoptive transfer experiments. In different experiments,  $10^6$ ,  $2 \times 10^6$ ,  $10^7$  or  $2 \times 10^7$  effector or memory CD8 T cells were transferred resulting in the shown average effector to target ratio  $E/T$ , percentage  $E$  or the total number of transferred cells in the recipient mice. We estimated killing efficacy of effectors ( $k_i$ ) or the ratio of the killing efficacy of an effector to that of a memory cell ( $\gamma_i$ ) by assuming that the death rate of peptide pulsed targets is proportional to the frequency of epitope-specific CD8 T cells in the spleen,  $K = kE$ . Killing efficacies  $k_1$ ,  $k_2$  and  $k_4$  were fitted as one parameter since this did not significantly affect the quality of the model fit to data ( $F_{2,68} = 2.84$ ,  $p = 0.07$ ). Further reduction of the number of model parameters resulted in the significantly worse description of the data (results not shown). Interestingly, this data also lead to a non-zero estimate of the preparation-induced cell death rate that we have previously postulated to exist (16), although in these experiments this rate was smaller than during acute LCMV infection (see Table 1).





**Figure 4:** Estimated per capita killing efficacy of GP33-specific effector CD8 T cells (A) and the ratio of killing efficacy of GP33-specific memory to that of effector CD8 T cells (B) as the function of the number of transferred effectors or memory cells. Error bars show the 95% confidence intervals for estimated parameters. These results suggest that there is a minimal change in the killing efficacy of GP33-specific effector CD8 T cells (with an average of  $2.1 \pm 0.66 \text{ min}^{-1}$ ) and the efficacy of memory CD8 T cells declines at high numbers of transferred cells. This implies that at high densities, memory CD8 T cells are on per capita basis less efficient than effector T cells. This is quantitatively consistent with the conclusion reached from the analysis of data following LCMV infection by assuming that killing of targets in the spleen is proportional to the average frequency of epitope-specific CD8 T cells in the spleen.

how effector and memory CD8 T cells, specific to LCMV, kill peptide-pulsed targets in the mouse spleen. Using a novel mathematical model we have analyzed data from experiments on in vivo killing of targets following LCMV infection of mice and experiments involving adoptive transfer of different numbers of LCMV-specific effector or memory CD8 T cells. Results of our analysis suggest that death rate of targets due to LCMV-specific effectors is simply proportional to the average frequency of epitope-specific CD8 T cells in the mouse spleen. This is rather surprising result on several grounds. First, this result suggests that variation in the frequency of epitope-specific CD8 T cells in the spleen is not reflected in the variation in the percent of target cells killed and rather represents measurement noise. Second, even for frequencies of LCMV-specific effectors in the spleen ranging over 100 fold from 0.06% to 6%, we find no evidence in saturation in the death rate of peptide-pulsed targets with CD8 T cell frequency. This in turn suggests that killing of targets by CTLs follows the law of mass action (29) and that CTLs do not compete for access to targets. Finally, killing in the spleen is dependent on the frequency of epitope-specific CD8 T cells and not their total number. This has important implications for vaccination since this suggests that inducing high numbers of virus-specific CD8 T cells may not be highly advantageous if their frequency in tissues is low. Notably, a similar discussion on whether transmission of pathogens is density- or frequency dependent still exists in epidemiology (30).

We have found that following LCMV infection, LCMV-specific memory CD8 T cells are half as efficient as are effector CD8 T cells of the same specificity. However, at low frequencies of CD8 T cells, LCMV-specific memory CD8 T cells are even more efficient at killing peptide-pulsed targets than effectors. The reduction in the per capita killing efficacy of memory CD8 T cells with their frequency may limit the overall efficacy of T cell-based vaccines, since boosting the frequency of memory T cells may not lead to a proportional increase in the efficacy of the memory T cell response.

Different efficacies of effector and memory CD8 T cells may be due to different localization of these two subsets in the spleen. A recent study suggests that LCMV-specific effectors localize mainly in the red pulp, while memory CD8 T cells reside in the T cell zones of the mouse spleen (31). Additional studies addressing the question of localization of target cells in the mouse spleen are necessary to investigate this issue further.

We have found that NP396-specific effector CD8 T cells present at the peak of the immune response have a per capita killing efficacy of  $k_{NP396} = 5.5 \text{ min}^{-1}$ . This value suggests that if most of splenocytes are NP396-specific CD8 T cells, NP396-pulsed targets will have a half-life of  $\ln 2/5.5 = 7.5$  seconds in the spleen. This time is a sum of the time required by a CTL to find its target and the time to kill the target.

By assuming that the rate of killing of peptide-pulsed targets is limited mainly by the time required by an effector T cell to find its target, we can calculate an approximate upper

bound estimate for the killing efficacy of T cells. Given the estimated motility of activated T cells in lymph nodes and the size of targets, the maximum killing efficacy of CD8 T cells is  $k'_D \approx 72 \text{ min}^{-1}$  (see Supplementary Information). Given that the actual killing efficacy of effector T cells is much lower than this value, our result suggests that the main limiting step of killing of peptide-pulsed targets is the actual process of killing, and not finding the target. This is consistent with a recent study on imaging of killing of B cells by effector CD8 T cells where the process of killing of targets on average took tens of minutes (5).

Effector CD8 T cells, specific to two other epitopes of LCMV, are estimated to have a lower killing efficacy ( $k_{GP276} = 2.4 \text{ min}^{-1}$ ,  $k_{GP33} = 2.1 \text{ min}^{-1}$ ). Since we do not expect these effectors to behave differently from NP396-specific effectors, a lower killing efficacy of GP276- and GP33-specific CD8 T cells is likely because of longer times required by effectors to kill their targets. This in turn may arise because of the reduced affinity of T cell receptors, specific to these peptides, or shorter half-life time of peptide-MHC complexes on target cells.

Reduced killing efficacy of memory CD8 T cells as compared to effectors of the same specificity is expected as memory T cells generally express low levels of perforin, granzymes, and FasL required for mediating cytotoxicity (12). Even though memory CD8 T cells may have a reduced motility in lymphoid tissues as compared to activated, effector T cells, this is an unlikely reason for their reduced killing efficacy. We have found that GP33-specific memory CD8 T cells are more efficient killers than effectors at low T cell frequencies. Whether this holds for T cells of other specificities requires additional investigation.

Our results have important implication for the loss of protection by memory CD8 T cells. It has been suggested that infection with new pathogens leads to attrition (loss) of memory specific to previously encountered pathogens (32–35). A recent study has challenged this conclusion by showing that infection of mice with Vesicular stomatitis virus (VSV) and Vaccinia virus (VV) can also lead to an increase in the total number of memory T cells, and as the result, to a very moderate loss of the total number of memory CD8 T cells, specific to previously encountered virus (LCMV), in the spleen (36). However, this study has also shown a dramatic reduction in the frequency of LCMV-specific memory CD8 T cells in the spleen and in peripheral tissues following infection with VSV and VV. Our results suggest that reduction in the frequency of virus-specific memory CD8 T cells may have a dramatic affect on the efficacy of the memory response even if the total number of virus-specific CD8 T cells is not dramatically reduced. Reduction in the cytotoxic efficacy of Vaccinia virus- or Pichindie virus-specific memory CD8 T cell responses has indeed been observed after exposure to LCMV (35, 37).

Our study may provide practical guidelines for estimating the efficacy of T cell based vaccines. It has been recently shown that cytotoxic potential of HIV-specific CD8 T cells may be one of the most important components of effective control of viral growth (38). Because of practical difficulties in performing in vivo cytotoxicity assay in humans, it would be interesting

to correlate the in vivo killing efficacy of murine CD8 T cells with their phenotype measured ex vivo by flow cytometry (e.g., cell surface and intra cellular markers). This may allow for quantitative understanding of what constitutes an effective memory T cell; this understanding can be further applied to compare efficacy of T cell-based vaccines in humans.

We have investigated quantitative aspects of killing of peptide-pulsed targets in the spleen. It would be important to investigate if killing of virally infected cells and/or targets in other organs such as lung or gut follows the same principle. Including these processes may require the use of more sophisticated mathematical models, and as such will hopefully lead to more collaborations between experimentalists and theoreticians.

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## 5 Supplementary Information

### 5.1 Mathematical model for estimating the killing efficacy of CD8 T cell responses

The dynamics of unpulsed and peptide-pulsed targets in the blood and in the spleen are given by equations

$$\frac{dS_B(t)}{dt} = -(\delta + \sigma + \epsilon)S_B(t), \quad (6)$$

$$\frac{dS(t)}{dt} = \sigma S_B(t) - \epsilon S(t), \quad (7)$$

$$\frac{dT_B(t)}{dt} = -(\delta + \sigma + \epsilon)T_B(t), \quad (8)$$

$$\frac{dT(t)}{dt} = \sigma T_B(t) - \epsilon T(t) - KT(t), \quad (9)$$

where  $S_B(t)$  and  $T_B(t)$  are the numbers of unpulsed and peptide-pulsed target cells in the blood, respectively, and  $S(t)$  and  $T(t)$  is the number of unpulsed and pulsed targets in the spleen, respectively,  $\sigma$  is the rate of migration of target cells from the blood into the spleen, and  $\delta$  is the rate of cell migration/death from blood to other organs,  $\epsilon$  is the extra death rate of transferred splenocytes due to preparation (independent of epitope-specific CD8 T cells), and  $K$  is the death rate of peptide-pulsed targets due to CD8 T cell mediated killing in the spleen. The initial conditions for the model are  $S_B(0) = T_B(0) = 5 \times 10^6$  cells and  $S(0) = T(0) = 0$



(12). In the case when all model parameters, including the death rate of targets due to CD8 T cell mediated killing, are independent of time, the model can be solved analytically (16); some solutions are shown in eqn. (1) and (2).

## 5.2 Deriving the general killing term

In tissues, CD8 T cells scan many cells to find virus-infected targets, and many of the cells scanned are uninfected (5). Scanning uninfected cells also takes some time (5), and if the majority of cells in a tissue is uninfected, a CD8 T cell can spend a substantial amount of time “looking” for the infected targets. The process of scanning of uninfected targets and killing peptide-expressing targets can be described mathematically to enzyme kinetics (e.g., (20)). We let  $E$ ,  $S$ , and  $T$  be the number of killer CD8 T cells, uninfected (bystander) and peptide-expressing targets, respectively. Effector CD8 T cells by scanning uninfected targets form a complex  $C_1$ , and form a complex  $C_2$  when they scan infected cells. Both complexes can dissociate. The kinetic diagram of cell interactions is then



where  $k_1$  and  $k_{-1}$  are the rates for binding and dissociation of a killer T cell and peptide-expressing cell;  $k_{-2}$  is the dissociation rate of a complex of a killer T cell and a bystander (uninfected) cell;  $k_2$  is the dissociation rate of the complex of killer T cell and an infected cell resulting in the death of the infected cell (denoted as  $D$ ). The rate of removal of pulsed targets is then simply  $k_2 C_1$ . Making a quasi-steady state assumption for  $C_1$  and  $C_2$ , we obtain

$$C_1 = \frac{k_1}{k_2 + k_{-1}} TE = K_1 TE, \quad (12)$$

$$C_2 = \frac{k_1}{k_{-2}} SE = K_2 SE. \quad (13)$$

where  $K_1 = k_1/(k_{-1} + k_2)$  and  $K_2 = k_1/k_{-2}$ . If the number of killers  $E$  is much larger than the number of target  $T$ , then rewriting eqn. (12) and (13) in terms of the total number of unpulsed and pulsed targets,  $\hat{S} = S + C_2$ , and  $\hat{T} = T + C_1$ , respectively, after simple algebra for the complex  $C_1$  we find

$$C_1 = \frac{K_1 E}{1 + K_1 E} \hat{T}, \quad (14)$$

This suggests that the death rate of peptide-pulsed targets,  $k_2 C_1 / \hat{T}$ , saturates at high numbers of killer CD8 T cells approaching the rate of dissociation of the complex  $k_2$ .

Similarly, if the number of pulsed targets  $T$  is much larger than the number of killers  $E$ , then rewriting eqn. (12) and (13) in terms of the total number of killer CD8 T cells,  $\hat{E} = E + C_1 + C_2$ , we find

$$C_1 = \frac{K_1 \hat{E}}{1 + K_1 T + K_2 S} T, \quad (15)$$

where  $K_1$  and  $K_2$  are defined above. This expression shows that 1) the death rate of peptide-pulsed targets,  $k_2 C_1 / T$ , may decrease at high numbers of targets, and 2) if the number of bystander (unpulsed) targets is high,  $K_2 S \gg K_1 T$ , the killing of targets depends on the frequency of effectors in the spleen,  $\hat{E}/S$ , and not on their absolute number.

### 5.3 Deriving the killing rate constant

Based on the assumption that the speed of a chemical reaction may be limited by rate at which chemicals are colliding, it has been derived from basic physical principles how the rate of reaction depends on the properties of interacting chemicals (29). Similar approaches have been applied in biology to model infection of target cells by a virus (39, 40). Bearing on these studies, killing of peptide-pulsed target cells  $T$  by peptide-specific CD8 T cells  $E$  can be described by a simple diagram



with the kinetics of target cells  $T$  given simply as

$$\frac{dT(t)}{dt} = -k_D T E. \quad (17)$$

Note that in eqn. (17),  $T$  and  $E$  are given as cell concentrations in the spleen (i.e., number of cells per unit of volume). From a fundamental result of Smoluchowski (ref?), the rate of the reaction  $k_D$  is given by

$$k_D = 4\pi(D_E + D_T)(R_E + R_T), \quad (18)$$

where  $D_E$  and  $D_T$  are the diffusion (or motility) coefficients of effectors and targets, respectively, and  $R_E$  and  $R_T$  are radii of the cells. The rate  $k_D$  needs to be converted to be comparable to the killing efficacy  $k$  that we have estimated from the data (see Table 1 and 2).

Because in eqn. (17), concentration of effectors  $E$  is given as cells/volume, converting the cell concentration to the frequency of cells in the spleen yields

$$k'_D = \frac{k_D N_s}{V}, \quad (19)$$

where  $N_s$  is the number of splenocytes and  $V$  is the volume of the spleen. Since spleen is packed with lymphocytes, the simplest assumption is that the spleen volume can be calculated as  $vN_s$  where  $v$  is the average volume of a splenocyte given by a sphere with the radius  $R_S$ . Then

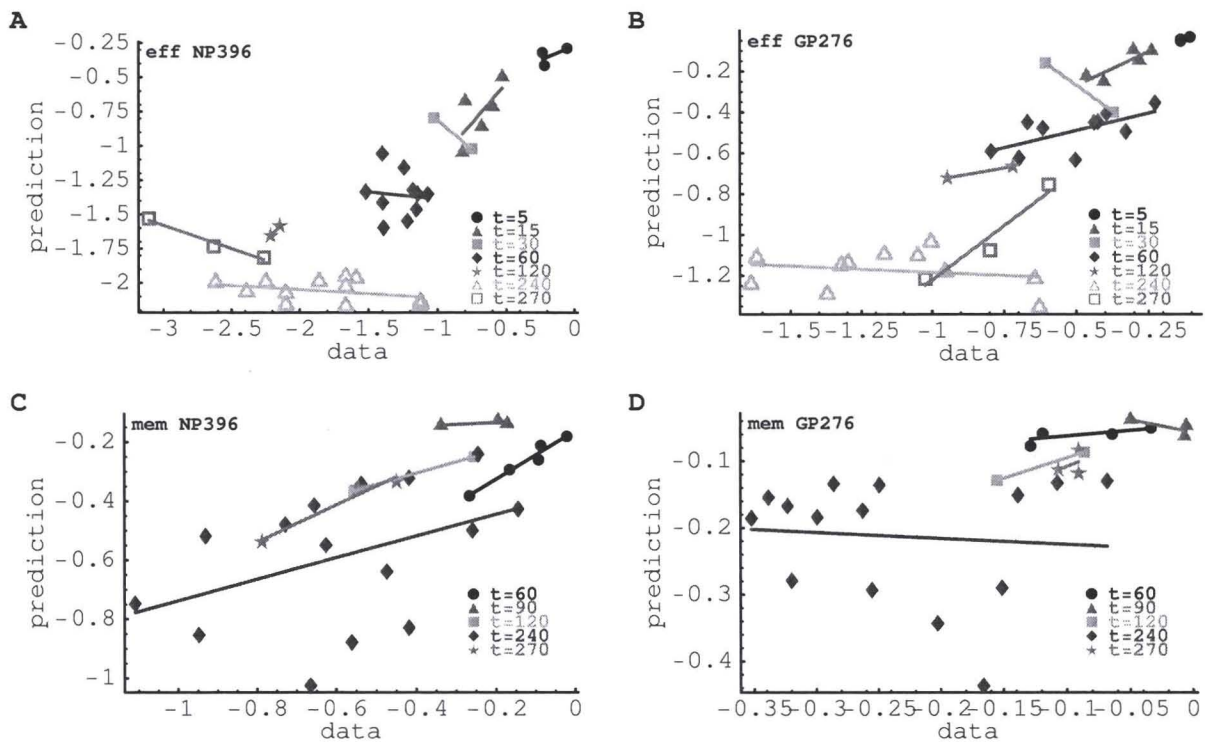
$$k'_D = \frac{4\pi(D_E + D_T)(R_E + R_T)}{v} = \frac{3(D_E + D_T)(R_E + R_T)}{R_S^3}. \quad (20)$$

since volume of a splenocytes is simply  $v = 4/3\pi R_S^3$ . Motility (diffusion) coefficients of T cells in lymph nodes have been estimated in several studies employing in vivo two photon microscopy (41, 42). Depending on type of cells, presence of the antigen and activation status of cells, the motility coefficient has been estimated to range from 10 to 100  $\mu m^2/min$  (42, 43). The average size of a mouse lymphocyte is about 7 – 10  $\mu m$  (44). Assuming that motility of activated effectors is higher than that of targets, we let  $D_E = 100 \mu m^2/min$  and  $D_T = 10 \mu m^2/min$ . Given that targets used in our experiments are splenocytes, we also let  $R_T = R_S = 4 \mu m$ , and for effectors  $R_E = 10 \mu m$ . Then the diffusion limited estimate for the killing efficacy of CD8 T cells in vivo is given by

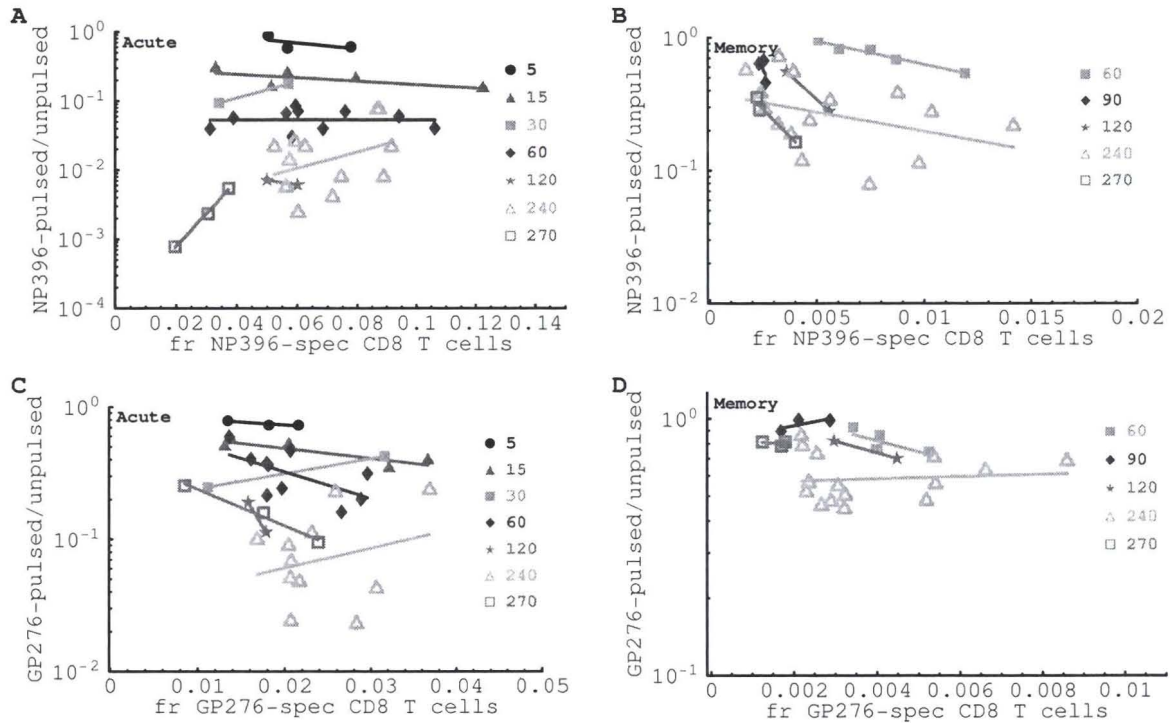
$$k'_D = \frac{3(100 + 10)(10 + 4)}{4^3} = 72.2 \text{ min}^{-1}. \quad (21)$$

This estimate is over an order of magnitude higher than the killing efficacy of NP396-specific effector CD8 T cells suggesting the finding the target is not the limiting step in the process of killing of peptide-pulsed targets in the mouse spleen.

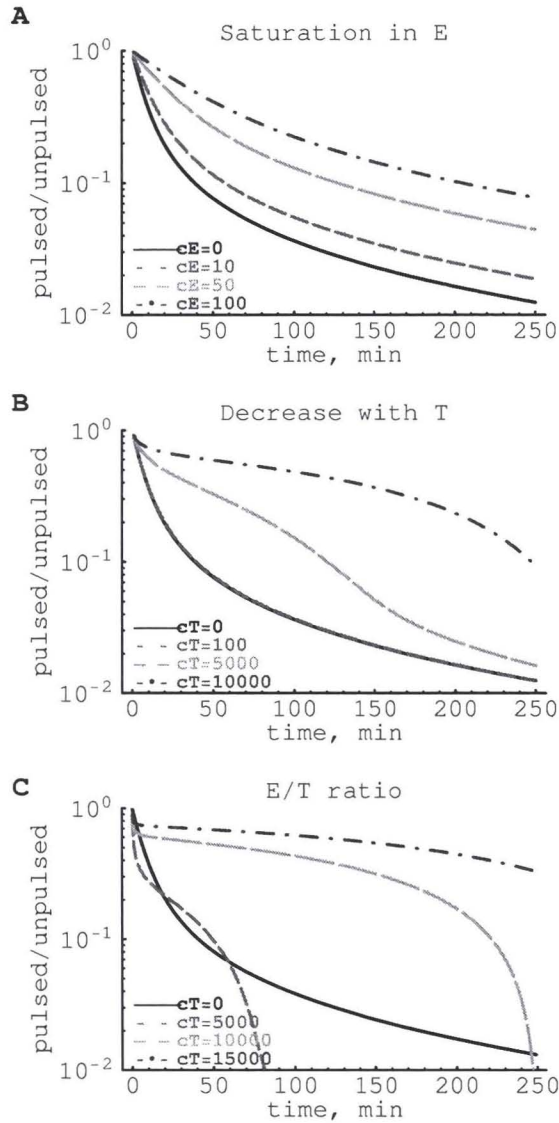
## 5.4 Extra results



**Figure 5:** Poor prediction of the model taking into account individual variation in the measured frequency of epitope-specific effector and memory CD8 T cells. We fit the model, that predicts that mice with more epitope-specific CD8 T cells should lead to higher killing, to the data. Here we plot the log ratio of the frequency of peptide-pulsed to unpulsed target cells that is observed in the data versus the log ratio, that is predicted by the best fit of the model, for different times after transfer of target cells. Lines show linear regressions. If the model were to predict well the data, the points are expected to lie on a line with a positive slope. Instead, we observe a large scatter, and for many data, a negative correlation between the observation and the prediction.



**Figure 6:** No strong correlation between the ratio of the frequency of peptide-pulsed to unpulsed targets at different time points after cell transfer (shown in minutes) and the percent of epitope-specific CD8 T cells in the mouse spleen in acutely infected (panels A and C) and LCMV-immune (panels B and D) mice. To visualize the data, we use different scales on the plots. If CD8 T cells were to affect the frequency of peptide-pulsed targets, a negative correlation between the ratio  $R$  and CD8 T cells would be expected. However, in acutely infected mice (panels A and C), there often are positive correlations between the ratio and the frequency of peptide-specific CD8 T cells.



**Figure 7:** Changes in the ratio of the frequency of peptide-pulsed to unpulsed targets in the mouse spleen as predicted by the mathematical model (given in eqn. (1)-(2)) with different killing terms (given in eqn. (3) – (5)). The death rate of peptide-pulsed targets saturates with the frequency of peptide-specific CD8 T cells (panel A), decreases with the frequency of target cells (panel B) or saturates on the effector to target ratio (panel C). We solve the mathematical model analytically (panel A) or numerically (panels B-C) with the following parameters:  $S_B(0) = T_B(0) = 5 \times 10^6$ ,  $S(0) = T(0) = 0$ ,  $\delta = 0.001 \text{ min}^{-1}$ ,  $\sigma = 0.001 \text{ min}^{-1}$ ,  $\epsilon = 0.005 \text{ min}^{-1}$ ,  $k = 5 \text{ min}^{-1}$ ,  $E = 0.05$  (see also Table 1). The frequency of targets in the spleen is calculated as  $\mathcal{T} = T(t)/N_s$  where  $N_s = 8 \times 10^7$  is the total number of splenocytes. In all panels, solid lines predict changes in the ratio  $R$  if killing follows the law of mass-action, i.e.,  $K = kE$ .