Revisiting Estimates of CTL Killing Rates In Vivo

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Recent experimental advances have allowed the estimation of the in vivo rates of killing of infected target cells by cytototoxic T lymphocytes (CTL). We present several refinements to a method applied previously to quantify killing of targets in the spleen using a dynamical model. We reanalyse data previously used to estimate killing rates of CTL specific for two epitopes of lymphocytic choriomeningitis virus (LCMV) in mice and show that, contrary to previous estimates the “killing rate” of effector CTL is approximately twice that of memory CTL. Further, our method allows the fits to be visualized, and reveals one potentially interesting discrepancy between fits and data. We discuss extensions to the basic CTL killing model to explain this discrepancy and propose experimental tests to distinguish between them.

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INTRODUCTION

A detailed understanding of immune responses requires quantifying the population dynamics of pathogens and immune cells. A key element of these dynamics is the killing of infected cells by cytototoxic T lymphocytes (CTL). We want to be able to accurately measure how fast CTL can find and kill infected target cells in vivo. This information will help answer many basic immunological questions. For example, how do killing rates differ between effector and memory CTL, and do these rates change as CTL become exhausted in chronic infection? Answering these questions will be particularly important for persistent infections such as HIV.

Many studies have quantified cytototoxic T cell activity in vitro [1–8]. More recently, mathematical models in conjunction with advances in experimental techniques have allowed estimates of CTL killing rates in vivo [9–14]. For a summary of the work in both of these areas, we refer the reader to our review [15].

In this paper we present a refined analysis of a dataset relating to CTL killing of LCMV-infected cells in a mouse model, first presented by Barber et al. [16] and subsequently analysed quantitatively by some of us [13]. Barchet et al. [17] were among the first to use this simple but powerful assay. Briefly, effector or memory CTL are generated by infecting mice with LCMV and waiting either 8 or >30 days respectively. To measure the rate of killing of target cells by CTL, a mixture of fluorescently labelled cells containing equal proportions of unpulsed controls and cells pulsed with either of the two immunodominant LCMV epitopes (NP396 and GP276) is injected intravenously into mice. To study effector and memory CTL responses to these targets, the frequencies of CTL, control cells and pulsed targets are measured in the spleen following sacrifice of mice during the first few hours after injection of targets (Figure 1).

To estimate killing rates from this assay, we need to take into account two complications. First cells are flowing into the spleen while killing is taking place. This results in some target cells being missed during CTL surveillance for shorter durations than others. This was addressed in [13] using a simple dynamical model of migration and killing of target cells. They also took into account a second problem, namely that different mice have different numbers of splenocytes and spleen-resident CTL.

There are, however, further problems with this method, which we address here. The most significant of these is the uncertainty in the ‘take’ of injected cells (i.e. the number of injected cells that end up in the spleen). This is variable for a combination of reasons; different numbers of cells in each inoculum and difficulties associated with targeting injections precisely into the tail vein.

We show how this uncertainty can be removed from the calculation by using the pairing of the estimates of unpulsed and pulsed target cell frequencies in each animal. Importantly, this new approach also allows clearer visualisation of the fits and the data. Applying the method to the dataset first presented in [16] more than doubles previous estimates of effector CTL killing rates. Furthermore, the clearer visualization allowed by the new approach reveals a systematic discrepancy between the data and the model in one case. We propose several hypotheses to account for this shortcoming of the current framework, and we describe additional experiments that may allow us to discriminate between these hypotheses.

METHODS

The basic model—the dynamics of target cells in blood and spleen

We begin by describing the original method of estimation, and then explain how it can be improved.

The flux of control or unpulsed cells from the blood to spleen immediately following tail-vein injection is modelled as follows. If N denotes the number of unpulsed cells in blood and U the number of pulsed cells in blood, then:

\[ \frac{dN}{dt} = \lambda N - \nu N \]

\[ \frac{dU}{dt} = \nu N - \lambda U \]

\[ \frac{dY}{dt} = \nu N - \lambda Y \]

\[ \frac{dY}{dt} = \lambda Y - \nu Y \]

where \( \lambda \) is the rate of injection of cells into the blood, \( \nu \) is the rate of movement of cells from blood to spleen, \( \lambda Y \) is the rate of killing of injected targets, \( \lambda Y \) is the rate of killing of injected targets, and \( \lambda Y \) is the rate of killing of injected targets.

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number of unpulsed cells in the spleen, then

$$N(t) = -(\sigma + \delta)N(t)$$  \hspace{1cm} (1)

$$\dot{U}(t) = \sigma N(t)$$  \hspace{1cm} (2)

or

$$U(t) = \frac{\sigma N(0)}{\sigma + \delta} \left(1 - e^{-(\sigma + \delta)t}\right)$$  \hspace{1cm} (3)

where $N(0)$ is the number of control cells injected into the blood at the start of the experiment. We assume that the influx of pulsed and unpulsed targets into the spleen is identical, but that in addition pulsed targets are killed in the spleen. The total rate of killing is assumed to take a mass-action form, proportional to the product of the dimensionless frequencies of target cells and CTL in the spleen. Assuming all mice have identical total splenocyte numbers, then,

$$T(t) = \frac{\dot{U}(t)}{N_s} - kC T(t),$$  \hspace{1cm} (4)

where $T(t)$ and $C$ are the frequencies of target cells and CTL in the spleen, respectively. This equation assumes equal numbers of pulsed and unpulsed target cells in the inoculum.

Ideally, all mice would be identical, with the same inoculum $N(0)$, spleen size $N_s$ and CTL frequency $C$, and would differ only in the time of sacrifice. If this were the case, we would estimate $k$ as follows. First fit the data for unpulsed target cells (Figure 1, green line) to obtain the parameters governing the dynamics of unpulsed cells. Then use these parameters to estimate $k$ by fitting equation (4) to a time series of measured target cell frequencies (Figure 1, red line).

### Biological and Experimental Limitations

The above approach needs modification, because although we would prefer all mice to be identical, in reality they differ substantially in the following:

1. Spleen sizes (splenocyte numbers);
2. Number of CTL specific for GP and NP epitopes—this depends on their response to the LCMV immunization;
3. The inocula for different mice contain slightly different proportions of pulsed and unpulsed target cells.

We took these sources of variation into account in our original study [13] by measuring $N_s$ and $C$ for each animal, as well as the ratio of unpulsed to pulsed targets, $f$ (measured separately for NP- and GP-pulsed targets). Then for animal $i$ sacrificed at time $t_i$,

$$T_i(t) = f_i \frac{U(t)}{N_s} - kC T_i(t),$$  \hspace{1cm} (5)

where $k$ is the rate constant for killing, $N_s$ is the total number of splenocytes, $U(t)$ is the total number of unpulsed targets in the spleen and $C$ is the frequency of CTL in the spleen (numbers/total splenocytes) specific for the target cells of interest.

In our original approach we fitted the following equation to the unpulsed cell data pooled from all animals at all timepoints to obtain averaged estimates of $c = \sigma N(0)$ and $d = \sigma + \delta$:

$$U(t) = \frac{\sigma N(0)}{\sigma + \delta} \left(1 - e^{-(\sigma + \delta)t}\right) = \frac{c}{d} (1 - e^{-dt}).$$  \hspace{1cm} (6)

This follows from equation (3). Using this in equation (5),

$$T_{i,\text{predicted}}(t) = f_i \frac{\sigma N(0)}{N_s} \left(\frac{e^{-(\sigma + \delta)t} - e^{-kC(t)}}{kC(t) - (\sigma + \delta)}\right).$$  \hspace{1cm} (7)

![Figure 1. The CTL killing assay. Peptide-pulsed target cells and control (unpulsed) cells are injected intravenously (A). The control cells allow us to measure the flux of both populations into the spleen, and the differences between numbers of pulsed and unpulsed cells in the spleen at later timepoints (panel B) is assumed to be due to killing by spleen-resident CTL. doi:10.1371/journal.pone.0001301.g001](filename)
The killing rate \( k \) was then estimated by fitting eqn. (7) to the measured target cell frequencies \( T_i\) at each timepoint. This discards information, however, since the data comprise paired measurements of \( T_i\) and \( U_i\) for each animal. Using this paired information improves the fitting procedure since a further source of variation is removed—the initial ‘take’ of injected cells [i.e. the proportion of the injected targets that migrate to the spleen, \( \sigma/(\sigma+d) \)]. Since we expect the uncertainty in the take to be identical for pulsed and unpulsed targets in the same inoculum, the paired information can be used to remove this uncertainty.

Substituting eqn. (6) into eqn. (7) gives

\[
T_i(t) = f_i \frac{U_i(t)}{(N_i)} \left( \frac{d}{kC_i - d} \right) \left( \frac{e^{-dt} - e^{-kC_i t}}{1 - e^{-dt}} \right),
\]

removing the unknown quantity \( c = \sigma N_i(0) \) from the calculation, and where \( d = \sigma + \delta \). Thus \( k \) can be estimated using the measured values \( T_i, U_i, (N_i)_{i=1}, f_i \) and \( C_i \) together with the previous estimate of \( d \).

An alternative is to work with the proportion of the pulsed targets in the spleen that have been killed, \( p(t) \).

\[
\text{Proportion of targets killed} = p(t) = 1 - \frac{\text{Pulsed targets}}{\text{Unpulsed targets}}.
\]

\[
= 1 - \frac{(N_i)T_i(t)}{f_iU_i(t)} \quad (9)
\]

\[
= 1 - \left( \frac{d}{kC_i - d} \right) \left( \frac{e^{-dt} - e^{-kC_i t}}{1 - e^{-dt}} \right).
\]

where unpulsed target cell numbers are multiplied by the ratio \( f \) to correct for deviations from a 1:1 ratio of pulsed to unpulsed targets in the inoculum. Working with the proportion of targets killed makes visualising the raw data more straightforward, as we see below.

In either case, a two-step process is used to estimate \( k \):

1. First estimate the loss rate of injected cells from the blood, \( d = \sigma + \delta \), using the unpulsed cell data alone. From eqn. (6), for each animal \( i \), the total number of unpulsed target cells in the spleen is

\[
U_i(t) = Z_i(1 - e^{-dt})
\]

where \( Z_i \) is proportional to \( N_i(0) \). The parameter \( d \) can be estimated with a non-linear least squares fit to the data from all animals simultaneously and assuming \( Z_i \) is the same (and unknown) for all animals.

2. Use this estimate of \( d \) to generate point estimates of \( k_{NP} \) and \( k_{GP} \) using either equation (8) or (9).

Confidence intervals can be generated by repeating steps 1 and 2 on resampled datasets to generate empirical (bootstrap) distributions of all three parameter estimates (\( d, k_{NP}, k_{GP} \)).

### Transforming the data

Ideally the data should be transformed such that the errors are at least approximately normally distributed, justifying a least-squares fitting approach. Since the influx and loss of targets from the spleen are modelled as exponential processes, an obvious approach is to fit the logarithm of the target cell frequencies. However, these frequencies are measured by FACS analysis and at late timepoints when frequencies are low they are subject to increasing fractional error. In particular, we do not expect the assumption of constant error variance to hold on a logarithmic scale. For this reason we argue that it is incorrect to fit to either the logarithm or the logit of target cell frequencies (the logit function being approximately equal to the logarithm for small arguments). A reasonable alternative is to transform all cell frequency measurements using the arcsine-square-root [18], and the results we present here are generated using this data transformation. However, parameter estimates do not differ substantially when we fit directly to the untransformed cell frequencies or proportions.

### Selecting an estimation method

Some of the uncertainty in \( k \) comes from variability in the injected cell numbers \( N(0) \). We see from eqns (8) and (9) that \( N(0) \) does not appear explicitly in the second step of our revised estimating procedures. However, variation in \( N(0) \) generates uncertainty in the estimate of \( d \) in the first step, which propagates into the second step. To investigate whether this could introduce a bias in the estimate of \( k \), we took a Monte Carlo approach. First we analysed the original data to estimate a distribution for the injected cell numbers \( N(0) \), and then generated artificial datasets using this distribution. We have

\[
N(0) = \frac{U(0) d}{\sigma (1 - \exp(-dt))},
\]

where \( U(0) \) is measured, and \( d = 0.021, \sigma = 6.6 \times 10^3 \). We constrain estimates of \( \sigma \) using the relations

\[
0.0013 \leq \frac{c}{N_{MAX}} \leq d = 0.02,
\]

where \( N_{MAX} \) is the approximate number of cells in each injection and so is an upper bound on the actual transferred number \( N(0) \). We used a value \( \sigma = 0.01 \), and then estimated \( N(0) \) for each animal using the observed values \( U(0) \) in eqn. (10). \( N(0) \) was well described by a lognormal distribution with \( \log(\text{mean}) = 13.3 \) and \( \log(\text{sd}) = 0.61 \). We then generated an artificial dataset by drawing a value \( N(0) \), from this distribution and a value of \( C_i \) from the empirical distribution of CTL frequencies, and using these numbers to generate \( U(0) \) and \( T(0) \) with eqns (6) and (7), using the original estimate \( k = 1.33 \) for NP targets killed by effector CTL.

Replicates were run to generate a cohort of simulated animals and this data was used with the two-step estimation procedure to re-estimate \( k \). We show the results of fitting the model with both methods in Table 1, as well as the results using the original method [13].
The discrepancies between all estimates and the true value $k = 1.33$ were all significant ($t$-test, $p<0.001$). On the basis of this analysis we show fits to the proportion of targets killed, since it provides our least biased estimator.

Note that eqn. (9) can easily be solved numerically to obtain direct estimates of $k$ for each animal. However, in this procedure the parameters governing target cell influx ($\sigma$ and $\delta$) are taken to be identical for each animal but the CTL killing rate $k$ is assumed to be variable, which we feel is an unreasonable assumption.

### DISCUSSION

Measuring CTL killing rates with this in vivo assay requires not only modeling killing itself but also the flow of targets into the spleen. We have improved our previous analysis by removing one major source of uncertainty (variation in the number of targets transferred intravenously) and using the natural pairing of measurements to make fuller use of the information contained in the data. In contrast to previous estimates we now predict that the second cohort are killed more slowly than the first.
Measuring CTL Killing *In Vivo*

**NP effectors**

- Proportion of targets killed
  - Time (mins)
  - Residuals
  - k = 3.71 (2.76, 4.84)

**GP effectors**

- Proportion of targets killed
  - Time (mins)
  - Residuals
  - k = 2.19 (1.47, 3.77)

**NP memory**

- Proportion of targets killed
  - Time (mins)
  - Residuals
  - k = 1.57 (1.18, 1.96)

**GP memory**

- Proportion of targets killed
  - Time (mins)
  - Residuals
  - k = 0.72 (0.53, 0.92)
Measuring CTL Killing In Vivo

Figure 2. The fits (square panels) of the basic killing model to the data using the proportion of GP and NP-pulsed target cells that are killed over time, by effector CTL (upper panels) and memory CTL (lower panels) in the spleen. In the square panels the black open circles show the measured proportion of targets killed, and the fitted values for each animal are shown offset to the right in red open circles. The residuals after arcsin square-root transformation are shown below each fit in the rectangular panels. Best fit estimates of \( k \) in units of \( \text{min}^{-1} \) are shown in each panel, with 95% confidence intervals.

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CTL effector function can be limited by regulatory T cells [20]. It is therefore possible that different levels of inhibition acting on CTL specific for different LCMV epitopes could influence CTL killing rates and that our estimates are not the ‘true’ killing rates of these populations. Clearly, combining our assays with direct visualization would be a powerful test allowing for the validation of both methods.

Despite the improvements to our method we have described, a small discrepancy between one of the four datasets and the fit (for the GP effector data) suggests that the killing model could be further refined. There are also other potential sources of error, both in the assay and the models.

First, our estimate of \( k \) assumes that the ratio of pulsed to unpulsed cells migrating into the spleen during the experiments is identical to the ratio in the initial inoculum. This assumption might be violated if there is differential sequestration of pulsed and unpulsed targets in other tissues (such as the lungs). If cells exposed to CTL in other organs in the body recirculate into the blood, this will lead to enrichment of unpulsed targets in the blood and overestimation of killing rates in the spleen. Conversely, if recirculation of cells exposed to CTL can be neglected, our estimate of the killing in the spleen is unaffected. The importance of extra-splenic killing and recirculation could be tested with non-destructive sampling of the blood immediately after transfer (that is, over the short timescale during which cells are migrating into the spleen) to test that the ratio of pulsed to unpulsed targets in blood is preserved.

A second potential problem is the assumption that killing can be described by a mass-action term. Our models assume a killing rate of \( k_{CT} \) where \( C \) and \( T \) are the CTL and target cell frequencies in the spleen, respectively. This is expected to hold for well-mixed populations at intermediate C:T ratios. When the C:T ratio is very low (=1) the net rate of killing is limited not only by the encounter rate of CTL and targets, as assumed by mass-action, but also by CTL recycling and/or the ‘dwell time’ taken for a single CTL to browse a potential target cell, deliver the cytolytic granules and detach from it. At high CTL densities and high C:T ratios, multiple CTL may bind to a single target and so mass-action may also break down. For GP memory cells the mean C:T (or ‘effector:target’) ratio over all timepoints was 1.46 (1.09, 2.08) and for NP memory it was 4.92 (3.43, 7.46), and so we might expect mass action to hold. For effectors, C:T was 917 (1350, 3040) for NP and 129 (87, 193) for GP. Particularly for the effector populations, then, estimates of \( k \) might be improved further by introducing the possibility of pulsed targets being hit by multiple CTL.

A third potential issue is that we identified specific CTL using tetramer staining only. It is possible that only a proportion of these cells express effector molecules such as perforin and granzyme and are capable of killing. Clearly, overestimating the number of functional CTL will underestimate killing rates. However, the relationship between killing in vivo and the expression levels of these molecules has not been definitively established. For example, exhausted CTL express significantly higher levels of Granzyme B than memory cells [22] and yet are unable to kill, and CTL can kill without perforin [23]. The method we propose smoothes over any heterogeneity in the epitope-specific CTL response and provides an average efficiency of killing for each specificity.

Finally, we note an important difference between our approach and those used in quantitative studies of cell lysis or virus dynamics. For example, in HIV infection, the analysis of the decay of circulating virus in the blood directly after treatment with anti-viral drugs allows the extraction of important biological parameters such as infected cell lifetime and the half-life of free virus [24].

Table 2. Original and revised estimates of killing rates, using paired estimates of pulsed and unpulsed targets.

|          | Original | Revised |
|----------|----------|---------|
| Effector | \( k_{UP} \) | 1.33 (0.70, 2.15) | 3.71 (2.76, 4.84) |
|          | \( k_{GP} \) | 0.70 (0.50, 0.97) | 2.19 (1.47, 3.77) |
| Memory   | \( k_{UP} \) | 1.45 (0.92, 2.33) | 1.57 (1.18, 1.96) |
|          | \( k_{GP} \) | 0.99 (0.43, 1.77) | 0.72 (0.53, 0.92) |

Confidence intervals are shown in parentheses and were calculated using the adjusted percentile method with 1000 (original results) or 2000 (revised estimates) bootstrap replicates.

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Figure 3. Estimates of the killing rate with 95% confidence intervals using the original procedure [13] and the revised method. This represents the data presented in Table 2.

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Author Contributions

Identified the issue with the earlier treatment of the data: A Yates, R Antia. Developed the paired measurement method: A Yates, R Antia. Helped identify the issue with the earlier treatment of the data: R Regoes. Contributed the bias analysis: R Regoes, F Graw. Contributed to the development of the new method: R Regoes, F Graw. Supplied the data: D Barber, R Ahmed. Contributed many helpful discussions on the killing assay: D Barber, R Ahmed. Performed the analyses: A Yates. Wrote the first draft of the paper: A Yates.