Predicting CD62L expression during the CD8+ T cell response *in vivo*

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Abstract

Acute infection leads to CD8+ T cell activation, division, and differentiation. Following clearance of infection, cells revert to two distinct subsets of memory, central (T_{CM}) and effector (T_{EM}) memory. Adoptive transfer of naïve T cell receptor transgenic (TCR-tg) T cells has been used to study the differentiation of these memory subsets, which are often discriminated by expression of CD62L. Naïve CD8+ T cells are CD62L^{high}, and CD62L expression is lost during the 'effector' phase. Adoptive transfer studies show that higher transfer frequencies result in diminished T cell expansion and a higher proportion CD62L^{high}. This suggests a relationship between CD62L expression and cell division, where division leads to conversion from CD62L^{high} to CD62L^{low} phenotype. To address this hypothesis we adoptively transferred graded numbers of OT-1 TCR-tg T cells from naïve donors and tracked the kinetics and phenotype of the immune response following infection. We developed a simple mathematical model of division-linked CD62L differentiation which we compared to the experimental data. Our results show that division-linked differentiation predicts the differences in proportion of cells CD62L^{high} observed between responses of different adoptive transfer number, and within individual mice. We calculate that approximately 20% of CD62L^{high} cells convert to CD62L^{low} during each division.

Keywords

CD8 T cells; Cell Differentiation; Cellular proliferation; Mathematical model

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Conflict of interest: The authors declare no financial or commercial conflict of interest

Introduction

The CD8+ T cell response is important for control of infection with a number of pathogens including viruses and intracellular bacteria. Prior to infection, the naïve CD8+ T cell repertoire contains a diverse array of specificities and only approximately 100 to 1000 naïve precursors are available to respond to any given pathogen epitope (1, 2). These naïve cells lack functional capacity to mediate anti-pathogen effector functions. After infection and presentation of pathogen antigens by dendritic cells, naïve CD8+ T cells are activated and divide extensively, expanding up to >10,000 fold to form a large 'effector' response. Coincident with this expansion, CD8+ T cells differentiate and acquire effector functions and homing characteristics that allow them to control infection. The expansion phase is followed by a contraction (death) phase, where 90–95% of antigen specific CD8 T cells are eliminated. The remaining antigen specific CD8 T cells form the initial memory pool, which can be stably maintained for life.

The mechanisms and dynamics of CD8+ T cell differentiation are not well understood. On a population level, CD8+ T cells acquire effector function following infection. However, on the individual cell level a great diversity of phenotypes is observed (3–5).

Thus, cells do not have a simple 'on / off' switch for activation and differentiation, where all stimulated cells follow a similar 'program' of behavior. Rather, individual cells acquire different phenotypes of cytokine secretion over time (3, 6). This apparently 'individualistic' behavior on the single cell level has been explained by factors such as asymmetric division of cell contents and receptors (7), or stochastic effects mediated by low levels of signaling (8). The 'average' effect of these behaviors is observed at the population level.

Another proposed explanation for acquisition of mixed individual phenotypes over time is stochastic 'division-linked differentiation' seen for both B cells and T cells stimulated *in vitro* (9–17). Lymphocytes show unique patterns of acquisition and loss of cell surface markers and effector function that correlate closely with division number rather than time of stimulation. In some systems cellular heterogeneity emerges according to simple rules of probabilistic assortment (10, 11, 18, 19).

Stochastic events are also a feature of cell division and death times. Despite the element of randomness that occurs on a single cell level, modeling of population behavior allows prediction of, for example, the proportion of cells that will commence dividing or die, or the proportion of cells that will acquire a given phenotype on each division following stimulation (18, 20, 21).

The dynamics of CD8+ T cell activation, expansion and phenotype have been studied using adoptive transfer systems in mice, where large numbers of antigen-specific T cell receptor transgenic (TCR-tg) T cells are intravenously injected into naïve mice and tracked over the course of an infection (22). In particular the division, differentiation, effector function, and survival capacity of T central (CD127^{high} CCR7 + CD62L^{high}) (denoted T_{CM}) and effector (CD127^{high} CCR7-CD62L^{low}) (denoted T_{EM}) memory subsets have been intensively studied. T_{CM} cells have increased division potential, diminished effector function and increased chance of survival when compared to the T_{EM} population (23–25). However, the

precise mechanisms by which these lineages form, are not well understood. Adoptive transfer of TCR-tg T cells after sorting on CD62L expression has led to proposed differentiation pathways where the T_{EM} population generates the T_{CM} population, and alternatively where the T_{CM} population generates the T_{EM} population (25–27). On the other hand, T cell receptor (TCR) repertoire analysis and studies involving adoptive transfer of single cells show that various phenotypes can arise from a single progenitor or clonotype (28, 29).

Recently it has become clear that the number of TCR-tg T cells used in adoptive transfer studies can affect the phenotype and kinetics of responding cells (30–33). In particular, higher adoptive transfer populations exhibit reduced expansion following infection. This is in contrast to lower adoptive transfer populations whose robust expansion reflects that of the endogenous response (30, 31, 33). That is, although the total number of antigen specific cells at the peak of the response is higher in larger adoptive transfer populations, the ratio of expansion (peak number of cells divided by the adoptive transfer amount) is lower. The phenotype of populations derived from larger adoptive transfer numbers also have an increased proportion of cells retaining the $CD62L^{high}$ and $CD127^{high}$ phenotype (30, 32). This suggests a mechanism whereby the extent of cell expansion and level of differentiation to the $CD62L^{low}$ and $CD127^{low}$ phenotype maybe mechanistically linked. We have previously demonstrated how a division-linked mechanism controlling CD62L phenotype differentiation reproduces TCR repertoire structures in response to influenza in mice (34). Here, we analyze the interplay between division, differentiation and death of transgenic CD8+ T cells after adoptive transfer of different numbers of cells. We measure the rate of division-linked $CD62L^{hi}$ to $CD62L^{lo}$ conversion, and show how this parameter alone can accurately predict the proportion of $CD62L^{high}$ and $CD62L^{low}$ cells observed between adoptive transfer groups and within individual mice *in vivo*. In contrast to CD62L, our analysis concludes that one-way division-linked CD127 differentiation is not compatible with the experimental results.

Results

Cell division predicts CD62L expression at peak response

Previous studies have shown that larger adoptive transfer populations of TCR-tg T cells do not give proportionally higher levels of TCR-tg T cells at the peak of the response (30, 31). This indicates that larger transfer populations undergo reduced expansion, likely because of cells completing fewer divisions (we show later why increased death is inconsistent with the data). To study the interaction of division history on CD62L and CD127 expression, and on survival of effector cell populations, we transferred graded numbers of Thy1.1 OT-1 cells from naïve donors into C57BL/6 (B6 Thy 1.2) recipients which were subsequently infected with LM-OVA and bled at various times. This allows tracking of the levels and phenotype of the Ova-specific CD8+ T cell response longitudinally in the blood in individual mice (Sup. Fig. 1, Fig. 1). As previously reported we observed expansion of OT-1 T cells, and loss of CD62L expression during this expansion (Fig. 1). In addition, we observed that higher adoptive transfer amounts did not experience as vigorous expansion post infection as lower adoptive transfer amounts (30), and the proportion of cells remaining $CD62L^{high}$ at

the peak of the response was also higher with higher adoptive transfer amounts (Fig. 2A). Thus, there is a relationship between cell expansion and phenotype between different adoptive transfer amounts.

Using this data, we investigated the accuracy of a division-linked mechanism of differentiation to predict differentiation of OT-1 cells from CD62L^{high} to CD62L^{low} phenotype during the expansion phase of infection. Individual animal data on the adoptive transfer amount and size of the response at peak, was used to estimate the relative number of divisions cells had undergone in each mouse. We found that the relative number of divisions was well correlated with the observed proportion of cells remaining CD62L^{high} (Fig. 2C). Using the relative number of divisions and the differences in CD62L expression for each mouse and equation 1, we estimated the rate of differentiation from CD62L^{high} to CD62L^{low} per division (*c*) for each mouse. The median differentiation rate over all mice was calculated as 21.2% per division (Fig. 3). That is, approximately 20% of CD62L^{high} cells become CD62L^{low} upon each division. Using this median differentiation rate per division, a division-linked model of CD62L differentiation accurately predicts the proportion of cells CD62L^{high} among different mice at the peak of infection (Fig. 2C).

Progressive differentiation during the acute response

The results above demonstrate that division linked differentiation can explain the observed relationship between cell expansion and cell phenotype in different mice measured cross-sectionally at the peak of the response. However, this may have arisen due to differences in the cellular environment following transfer of different numbers of cells that fortuitously conform to the model. It does not demonstrate that differentiation occurs progressively with division in individual animals over the course of the response. Therefore, we analyzed the longitudinal data for each mouse to look at the relationship between expansion and CD62L expression over time within an individual. For each mouse we identified the first day when OT-1 cells and phenotype were measureable in blood, and then compared this with the levels and phenotype at the peak of the response. The difference in OT-1 T cell levels was used to calculate the number of divisions in this time, and the difference in CD62L expression was directly observed. Using these data the differentiation rate (*c*) was then computed for each animal over these days. This technique of measuring the differentiation rate gave very similar values to when relative division number was calculated across transfer groups (median of median value was 19.9% and 21.2% for longitudinal vs. cross-sectional measurements respectively)(Fig. 3), indicating that division linked differentiation also explains the dynamics of differentiation within individual mice over time. This is demonstrated in figure 4A and 4B, where we use the previous median differentiation rate (calculated from the cross sectional data), and show that this predicts CD62L phenotype levels in individual animals over time during the expansion phase of infection.

Calculating the CD62L conversion rate in the spleen

Both methods described above provide similar estimates for the conversion rate per division from CD62L^{high} to CD62L^{low} status, indicating that division-linked differentiation explains phenotypic variation within a mouse and across adoptive transfer groups. However, a major limitation of these calculations is that they assume that blood lymphocytes proportions

accurately reflect the total cell numbers in other areas of the body. The spleen would provide a better estimate of the total size of the OT-1 population. However, splenic sampling has the problem that it precludes longitudinal sampling within an animal. To investigate whether the blood provides a good measure of total OT-1 cells in the body, we studied cell numbers in the spleen of mice which were adoptively transferred with the highest and lowest levels (400 000 and 3200 TCR-tg T cells) and were subsequently infected. We also used a control group of mice receiving 400000 cells but remaining uninfected. Spleens were extracted the day before, the day of, and the day after the peak of the response, with OT-1 levels and the proportion CD62L^{high} recorded. For uninfected mice, spleens were extracted on day 4 post transfer.

By comparing the number of OT-1 cells in the spleen of infected and uninfected mice on day five that received 400 000 cells, we were able to estimate the total expansion of OT-1 cells (and therefore number of divisions undergone) in infected animals. However, since this occurred in different mice, we took the average number of cells in uninfected animals (n=3) and in infected animals (n=3) to derive a single value for the average number of divisions undergone. Similarly, for animals receiving 3200 OT-1 cells, we compared the average number of OT-1 cells on day five (n=3) and day six (the peak of infection)(n=3) to obtain a single value for the number of divisions undergone. Comparing the number of divisions and phenotype in different animals we estimated the differentiation rate per division in each animal. The results are shown in figure 3. The mean conversion rate per division was 21.8%, in close agreement with the estimates from blood (Fig. 3).

More complex regulation of CD127 phenotype

The expression of CD62L appears predictable based on a simple model of division-linked differentiation. Expression of the interleukin-7 receptor CD127 is high on naïve and memory T cells, but down-regulated during the 'effector' phase of the immune response. CD127 expression is high on both T_{CM} and T_{EM} memory T cells. We next sought to determine whether the pattern of expression of CD127 could be explained with a similar division linked process. As with CD62L, transfer of higher numbers of OT-1 cells resulted in a significantly higher proportion of cells remaining CD127^{high} at the peak of infection (Fig. 2B)(p=0.0038). However, although the proportion of CD127^{high} cells at peak was significantly correlated with division number (p = 0.0148), the relationship between division number and CD127 expression was much weaker than that observed with CD62L ($r^2 = 0.37$ versus 0.83, respectively) (Fig. 2D). Moreover, when we attempted to correlate expansion with CD127 expression longitudinally in individual animals during the acute phase of the response, the fit was extremely poor since CD127 expression actually increased in the high transfer amounts (Fig. 4C). This indicates that a simple one-way division-linked CD127 differentiation model is not compatible with the experimental results.

Higher adoptive transfer results in better memory formation

The modeling above assumes that the difference in OT-1 expansion in different animals is determined by the number of divisions the cells have undergone, rather than an increased death rate of cells with increasing transfer amounts. To investigate the death rate of cells, we looked at the contraction phase of the response after peak. As shown in Figure 5A, larger

adoptive transfer populations experienced reduced contraction from peak to memory. Although the death rate of T cells after the peak is most likely different to the death rate during expansion, the fact that the death rates after the peak is lower in the high adoptive transfer group does not support a model where reduced contraction (before the peak) is due to increased death. This supports our assumption that reduced expansion is most likely due to diminished proliferation. The differences in contraction raises questions about the basis of memory formation in different adoptive transfer groups.

Memory formation is affected by division history and CD62L phenotype

The variation in contraction between adoptive transfer frequencies could have arisen from either (i) differences in the number of divisions the cells had completed (with lower adoptive transfer amounts undergoing more divisions and perhaps being 'senescent' and dying faster) or (ii) the phenotype of OT-1 cells or some other property altered by adoptive transfer (for example lower adoptive transfer amounts having a higher proportion of cells CD62L^{low}). To investigate these possibilities we first looked at the correlation between the number of divisions and memory formation. The proportion of cells remaining from the peak is significantly negatively correlated with the relative number of divisions in different animals (Figure 5B, $p = 0.001$), which would appear to support the 'senescence' argument. However, a higher proportion of CD62L^{high} cells is also associated with a higher proportion of cells surviving into memory (figure 5C, $p = 0.003$), in support of the 'phenotypic' argument. To attempt to tease apart the effects of cell phenotype versus number of divisions undergone, we looked at the contraction of CD62L^{high} and CD62L^{low} cells, and how this was affected by the number of divisions. The survival of OT-1 cells from peak to memory was very different in the CD62L^{high} and CD62L^{low} compartments, with significantly less contraction in the CD62L^{high} compartment (mean 27.01% of peak remaining at day 53) compared to the CD62L^{low} (mean 6.25%, $p < 0.0001$ paired t-test).

We then analyzed the effect of cell division on the CD62L^{high} and CD62L^{low} compartments independently. The contraction of CD62L^{high} cells was not significantly correlated with adoptive transfer amount or division number (Figures 5D and 5E). However, the contraction of CD62L^{low} cells was significantly correlated with the adoptive transfer amount ($p \approx 0.04$, $r \approx -0.57$ Spearman tied-rank correlation), and had a trend towards correlation with division number ($r \approx -0.50$, $p \approx 0.08$). Thus, although it is clear that the adoptive transfer amount and the number of cell divisions the transferred cells have undergone affects the ability of cells to enter the memory compartment, whether this occurs directly as a result of the 'age' of the cells, or is mediated by some phenotypic characteristic that is dependent on division history (such as CD62L expression) is unclear.

Unrecruited cells do not impact on calculations

Our analysis assumes that all cells are recruited into the immune response and undergo division-linked differentiation. However, it is possible that only a small fraction of cells are recruited and the remaining cells undergo no division and remain 'naïve'. As these cells are CD62L^{high} and are likely to be long lived, they have the potential to effect both the division-linked differentiation and memory formation analysis. We find that even at the highest adoptive transfer amount, less than 0.1% of the total OT-1 population is comprised of

unrecruited cells (calculated from the difference in OT-1 cell numbers in the spleen in uninfected and infected mice, Supplementary Figure 2E). Since the percentage of CD62L^{high} cells varies from approximately 10–50%, these unrecruited cells cannot make any significant contribution to this level.

Discussion

The study of CD8+ T cell memory differentiation has produced a number of proposed lineages (27, 34). These include linear relationships, where cells progress though effector (CD127^{low}/CD62L^{low}), effector memory (CD127^{high}/CD62L^{low}) and central memory (CD127^{high}/CD62L^{high}) phenotypes, and divergent relationships, where a single progenitor gives rise to multiple phenotypes early on which then maintain this commitment (35). A significant limitation of these heuristic lineage hypotheses is that they only describe general population dynamics and do not make quantitative predictions about cellular behavior. The deterministic 'switch' between phenotypes, or between 'effector' and 'memory' stages of an immune response, often proposed in these models is increasingly being replaced by a more complex understanding of random events at the molecular level leading to diverse behaviors on a single cell level, and ultimately predictable behaviors on a cell population level (8, 36). As the understanding of these mechanisms grow, more rigorous analysis must be applied so that competing hypothesis can be distinguished and tested. The use of mathematical models allows us to make and test quantitative predictions about cell behavior. The present analysis gives insight into how the same CD8+ T cell clonotype can give rise to both 'central memory' and 'effector memory' populations (28, 29), and how these populations evolve during the course of an infection. It also demonstrates the link between population size and phenotype, and how this can be predicted *in vivo*.

Adoptive transfer studies allow the tracking of antigen specific TCR-tg T cell levels and phenotype over the course of an immune response, and have been applied to help understand memory T cell lineages. Much of this work has employed a high adoptive transfer frequency, resulting in 1 000 – 10 000 fold greater precursors than naïve antigen specific endogenous estimates (30–33). Although these studies do show that adoptive transfer can dramatically change the immune response, this does not diminish the usefulness of the adoptive transfer procedure. Rather it emphasizes the need for careful experimental design and data analysis. We adoptively transfer TCR-tg T cells in 4 quantities ranging from 3 200 to 400000 donor cells. This allows us to study the kinetics and phenotype of an immune response under various conditions. We observe that CD62L expression is lost during the acute expansion phase of the response and that higher adoptive transfer population's experience a diminished expansion (and in turn, an increased proportion of cells CD62L^{high}). A simple mathematical model, where differentiation from CD62L^{high} to CD62L^{low} occurs on division and the reverse conversion does not occur accurately predicts the proportion of CD62L^{high} and CD62L^{low} cells between mice and within individual mice during the expansion phase of the response. In addition, this model of division linked differentiation is also consistent with the observed distribution of CD8+ T cell clonotype sizes and phenotypes in the endogenous repertoire following influenza virus infection *in vivo* (34). Interestingly, such a model is inconsistent with the kinetics of the CD127

phenotype, which has been shown to be more dependent on the activation stimulus provided (37).

An inherent limitation of our study is that it implicitly assumes that all cells divide the same amount over time. That is, if the total population of cells double, it assumes all cells divided once. However, in vitro studies of cell division have suggested that cells are not recruited synchronously into division, and also have a distribution in their time to divide(20). An important question is whether such asynchronous recruitment and division would produce a different outcome in terms of the observed division-linked differentiation. To further investigate this, we applied a well established model of cell division (the 'Cyton model') that has been developed to incorporate factors such as a distribution in the time to first division and subsequent division and differentiation rates (36). By incorporating a known rate of division linked differentiation into this explicit model, we then tested how well a simple estimate of differentiation based upon total cell number and cell differentiation state would perform. We find that estimates of differentiation rate based upon total cell populations are remarkably accurate, and thus that our estimates of division-linked differentiation based on total population number are unlikely to be affected by asynchronous recruitment and division within the T cell population.

Quantifying CD8+ T cell division, differentiation, and memory formation is important for vaccine design and viral control in chronic infection. Here we measure how the extent of cell division determines CD62L expression, and observe that division is associated with the extent of memory formation *in vivo*. It seems highly likely that other phenotypic characteristics such as cytokine expression or cytolytic function may also be determined by the extent of cell division. The possibility that the regulation of immune effector functions such as cytokine production and immunoglobulin isotypes have evolved to change with division to optimize the effectiveness of the immune response has been proposed (10, 11, 14, 15, 38) and if correct would explain the existence of stochastic division-linked processes inherent to lymphocyte behavior. Recent work has focused on how the 'quality' (functional characteristics) of a response may be more important determinants of outcome than the simple 'quantity' of the response (39). Thus, understanding and predicting how different approaches to vaccination affect both number and phenotype of cells may be critical to optimizing vaccine efficacy.

Materials and Methods

Adoptive transfer and experimental infection of mice

Recipient mice were 6–10 week old C57BL/6 mice from the National Cancer Institute. They were given graded numbers of Thy1.1 OT-1 cells (400 000, 80 000, 16 000, 3 200 cells) purified from the spleens of naïve donors. Mice were infected with 8×10^6 CFU of Att LM-Ova bacteria (prepared as previously described (37)) i.v. one day after cell transfer. Mice were bled by small tail snips to obtain ~20μl blood on various days after infection, and the transferred population identified with Thy1.1 (OX-7, Pharmingen), and stained with antibodies for CD62L (MEL-14, eBioscience) or CD127 (A7R34, eBioscience). Experimental protocols were approved by the University of Iowa Animal Care and Use committee.

Modeling division-linked differentiation

The simplest model of division-linked differentiation assumes that a fixed proportion of CD62L^{high} cells differentiate to the CD62L^{low} phenotype upon each division and that the reverse conversion does not take place. If P_0 is the proportion of cells that are CD62L^{high} at the start of division, and $P(n)$ is the proportion of cells CD62L^{high} after n divisions, then we can calculate the rate of differentiation (c) to CD62L^{low} phenotype per division as

$$c = 1 - \left(\frac{P(n)}{P_0}\right)^{\frac{1}{n}} \quad (1)$$

P_0 and $P(n)$ are easily read from the experimental data. However, calculating the absolute number of divisions that cells have undergone following transfer is difficult, as it requires assumptions about the 'take' of transferred cells and the total number of cells in the whole animal. Instead we calculated the relative number of divisions in different animals or in individual animals across time based on the sequential sampling in blood.

We first calculated the relative number of divisions at the peak of the response in different mice. To do this we determined which mouse had undergone the least divisions (our 'baseline mouse', B) – that is, the mouse that received the highest adoptive transfer dose and had the smallest peak level of OT-1 cells. We then computed the relative extra number of divisions required by the remaining mice to achieve the peak of their response. For example, a mouse with the same peak level of cells that received half the adoptive transfer amount of mouse B required one extra division to reach peak. Similarly, if a mouse received the same adoptive transfer number as mouse B but had twice amount at peak, then it required one extra division to do so.

We also estimated the number of divisions by comparing the change in number of cells in an individual mouse over time. That is, if the proportion of OT-1 cells quadrupled from day 4 to 5, we assumed two extra divisions, etc. Note that as the experimental data measured the OT-1 population is a percentage of blood lymphocytes, the calculation of the number of divisions the cells have undergone (n) requires additional assumptions about these populations (discussed in Extended Methods), which however had little impact on the estimated number of divisions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

TCR-tg	T cell receptor transgenic
T$_{CM}$	T central memory
T$_{EM}$	T effector memory
TCR	T cell receptor

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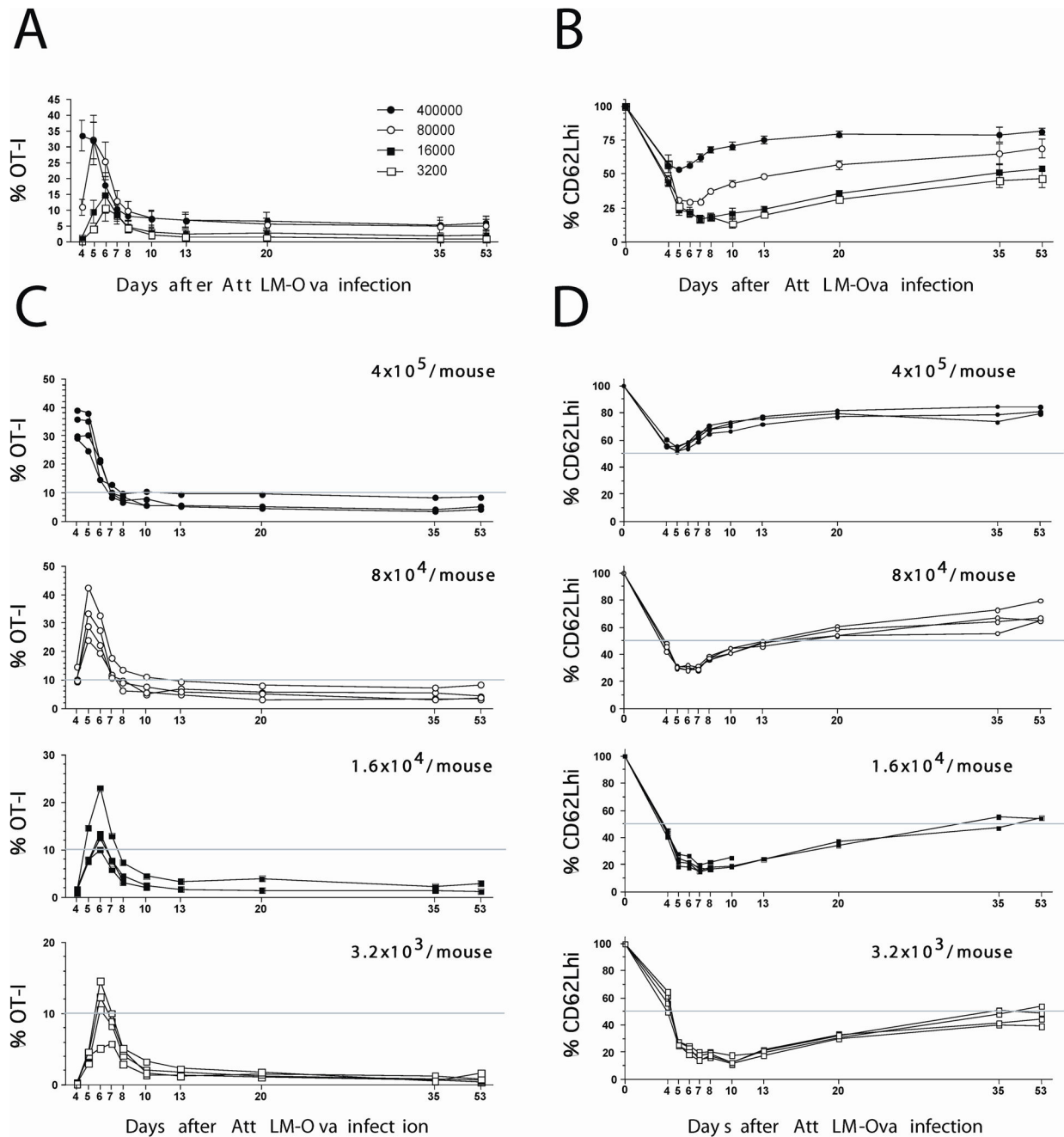
Figure 1. Kinetics and phenotype of OT-I CD8 T cells after infection
Purified naïve OT-I Thy1.1 cells at the indicated numbers were transferred into B6 Thy1.2 mice, and 1 day later, the recipient mice were immunized with Att LM-Ova. Kinetics and phenotype (CD62L) of OT-I Thy1.1 cells were followed in the blood at indicated days p.i. A, C) Kinetics of OT-I CD8 T cell response after infection. B, D) Expression of CD62L on gated OT-I Thy1.1+ / CD8+ cells on indicated days after infection.
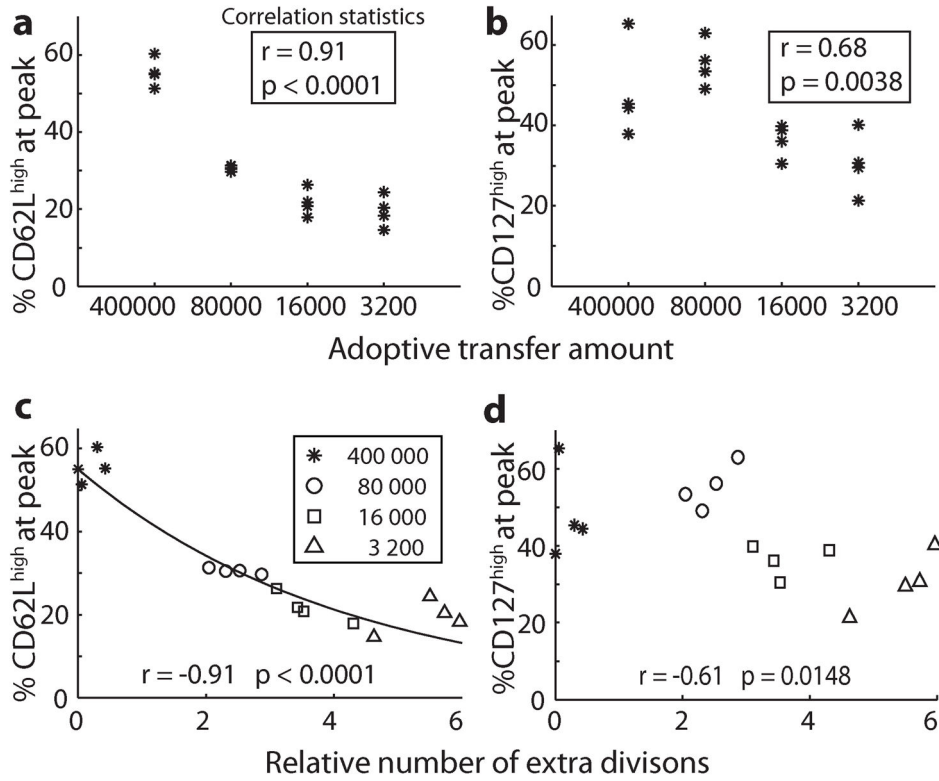
Figure 2. Adoptive transfer number and cell division affects phenotype
400000, 8000, 1600 and 3200 naïve OT-1 T cells were transferred into naïve mice which were subsequently infected with LM-OVA. The percentage OT-1 cells and phenotype were measured at the peak of the response in each animal. There was a significant positive correlation between the proportion of cells CD62L^{high} and adoptive transfer amount (A), and the percentage of cells CD127^{high} and adoptive transfer amount (B) (Spearman's tied-rank correlation). The relative number of extra divisions required by different mice to achieve the peak of their response (compensating for different adoptive transfer numbers) was calculated (C, D). There was a significant negative correlation between the relative number of extra divisions, and both the percentage of cells CD62L^{high} (C) and the percentage of cells CD127^{high} (D) (Spearman's rank correlation). The predicted relationship between CD62L expression and division number using a division-linked mechanism where 21.2% (median value from between mice, figure 3) of the CD62L^{high} population becomes CD62L^{low} with each division is overlaid in panel (solid line in panel C).
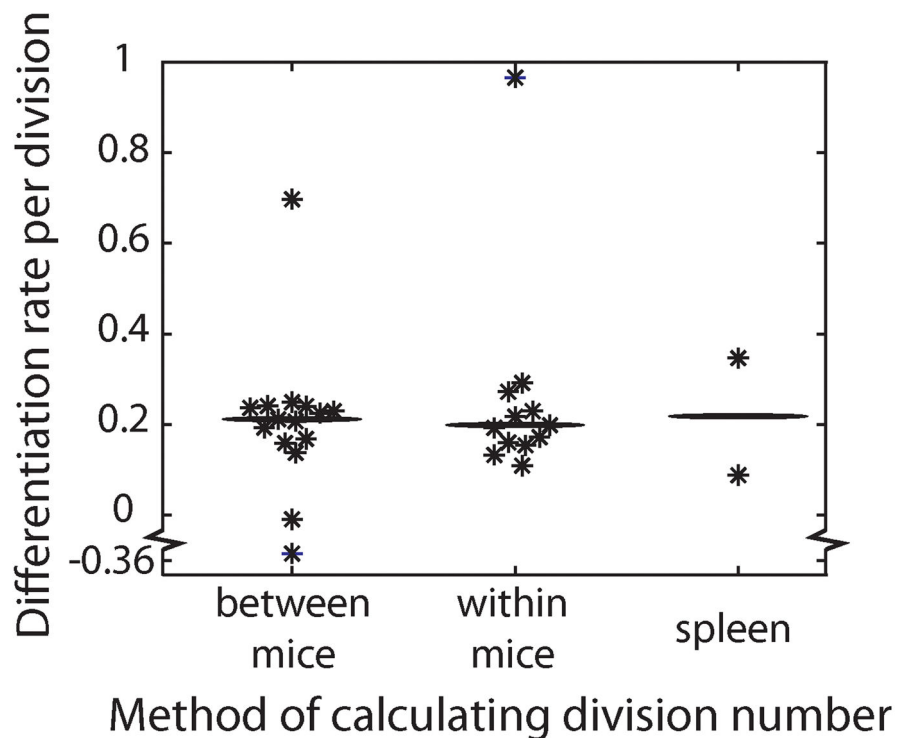
Figure 3. Estimating the proportion of CD62L^{high} cells that become CD62L^{low} on each division
The rate of differentiation (c) from CD62L^{high} to CD62L^{low} per division was calculated with three methods. In the first, the relative extra number of divisions to reach the peak proportion of OT-1 cells calculated between mice taking into consideration the adoptive transfer amount (n = 15, median c value = 0.212 [indicated by horizontal bar]). The second method calculates the number of divisions from the first day of measurement to the peak of infection in individual mice (n = 12, median c value = 0.199). The third calculates the number of divisions cells undergo in the spleen (n = 2, mean c value = 0.218).
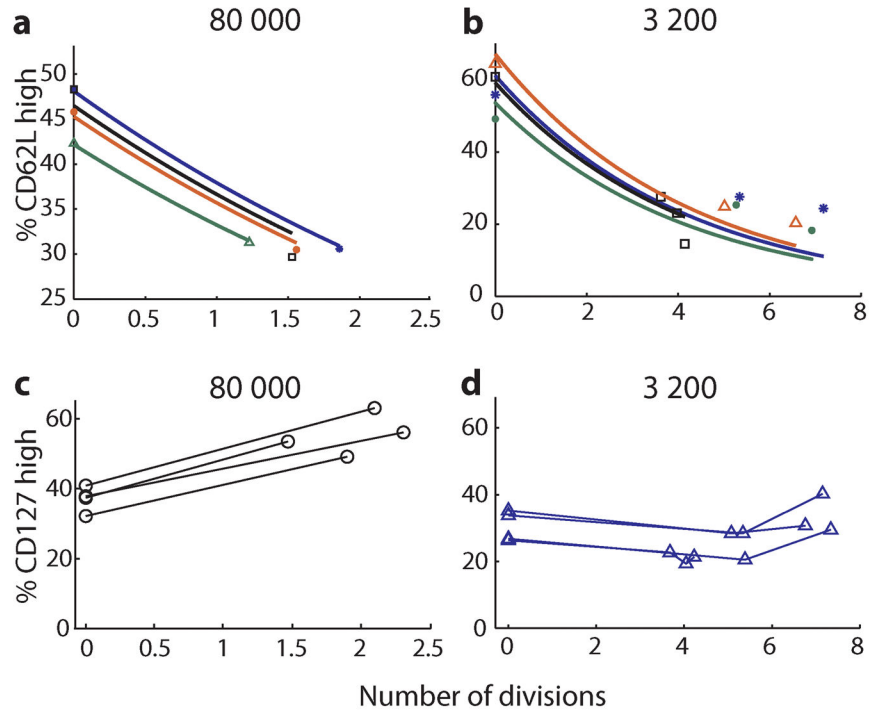
Figure 4. Predicting differentiation during the acute response
The relationship between the number of cell divisions and cell phenotype is studied. (A, B) The number of divisions is plotted against the percentage of cells CD62L^{high} for the 80 000 (A) and 3 200 (B) adoptive transfer amounts. Overlaid is the prediction of a mechanism whereby 21.2% (median differentiation rate in cross-sectional study, figure 3) of the CD62L^{high} population becomes CD62L^{low} with each division (–). Different symbols and colors are used to distinguish individual mice. The percentage cells CD127^{high} increased with division in the 80 000 adoptive transfer group (C), but did not consistently increase or decrease with division in the 3 200 adoptive transfer group (D). Lines connect points from individual animals.
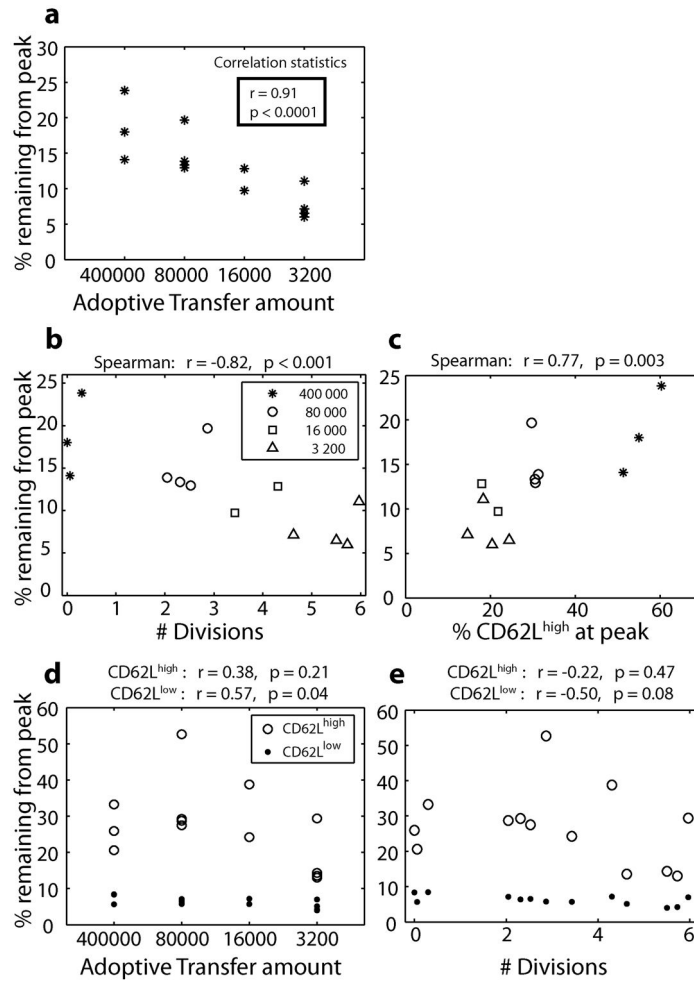
Figure 5. Division history, CD62L expression, and memory formation *in vivo*
The dynamics of the T cell response to infection were tracked from day 4 out to day 53 post-infection. Memory formation was studied by measuring the proportion of OT-1 cells at the peak that remained at day 53 post-infection. Survival of total OT-1 cells is positively correlated with adoptive transfer frequency (A) (Spearman's tied rank correlation test), indicating that higher transfer amounts lead to better memory formation. This may have occurred because these cells underwent fewer divisions (were 'younger') or because a higher proportion of these cells were CD62L^high. Both division number (B) and CD62L expression (C) are significantly correlated with survival (Spearman's rank correlation test). The average survival of the CD62L^high population (mean survival = 27.01%) was significantly higher than the CD62L^low population (mean survival = 6.25%, $p < 0.0001$ paired t-test), regardless of the adoptive transfer amount (D, E). When the CD62L^high and CD62L^low populations were analyzed separately, the survival of the CD62L^high population was not significantly correlated with adoptive transfer amount. However, there was a significant positive correlation between the survival of the CD62L^low population and adoptive transfer amount ($p = 0.04$, $r = 0.57$ Spearman tied-rank correlation) (D). There was

also a trend towards reduced survival of CD62L^{low} cells that had divided more times (E), although this was not significant (p=0.08).