Assessing T cell differentiation at the single-cell level

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THE DESCENT OF MEMORY T CELLS

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Our T cell repertoire is shaped by antigen encounter. From a naive T cell pool that contains millions of different T cells with unknown specificities, pathogen infection leads to selection of those T cells that can detect pathogen-derived antigens. Following clearance of infection, a population of memory T cells remains and protects the individual from severe reinfection. A central question in the field has been how the generation of long-lived memory T cells, versus short-lived ("terminally differentiated") T cells, is controlled. In this review we discuss the models that have been put forward to explain the generation of memory T cells after infection and the experimental evidence supporting these hypotheses. Based on the available data we propose a new model that stipulates that during immune responses T cells do not acquire different *fates* that determine their subsequent long-term survival but rather T cells assume different *states* that simply reflect the likelihood of future survival, states that can still be modulated by external signals.

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INTRODUCTION

The formation of T cell memory has been an area of intense research, both because it concerns a fundamental aspect of adaptive immunity and because the induction of robust T cell memory is a goal of vaccination strategies. Kinetically, T cell responses can be subdivided into three distinct phases. During the initial expansion phase, naive antigen-specific T cells get activated and respond by extensive proliferation, leading to a massive (up to 10^4–10^5-fold) increase in antigen-specific T cell numbers. After antigen clearance, this population of antigen-specific T cells goes down in size (in immunological slang "contracts") due to apoptosis. When this contraction phase has ended, a small but stable pool of memory T cells remains behind. Enumeration of antigen-specific T cells at different times after infection by MHC multimer staining easily identifies the onset of this memory phase (i.e., the time point at which antigen-specific T cell numbers stay constant). However, it does not provide a clue on which individual T cells commit to longevity and at what point in their development these cells—or their ancestors—made this commitment. With the aim to establish this "descent of memory T cells," a multitude of studies have been performed over the past decades that followed T cell populations at the bulk level, and these studies have resulted in the proposition of a variety of models. Furthermore, in recent years, novel technologies that follow T cell history at the single cell level have been developed and used, thereby providing much better insight into memory T cell descent.

Below, we will discuss the different models for memory T cell generation and the predictions they make with respect to three central questions in the field:

1. Can long-lived memory T cells arise from effector T cells?
2. Which factors determine whether an individual T cell survives long-term?
3. When does an individual T cell commit to long-term persistence?

It is important to point out that these should be considered three entirely separate questions and—contrary to what has been done occasionally—data on one of these provide very little insight into the others. To provide some examples, models that predict that T cells commit to long-term persistence before the first cell division do not provide any insight into the question whether the daughter cells might transiently acquire certain effector properties prior to forming long-term memory. Similarly, models that invoke signal strength as a determining factor in the control of T cell fate do not inform us when exactly after T cell activation such signals act.

DEFINITIONS

Before discussing these different models, it is important to clarify some key terminology.

- » Strictly speaking, effector T cells are those T cells that are responsible for pathogen removal, and depending on the type of pathogen, this could either involve functionalities such as cytokine secretion or direct lysis of infected cells. However, as it

would be impractical to use a definition of effector T cells that varies between infections, a pragmatic solution is to identify any T cell capable of producing cytokines such as IFN-γ, capable of producing molecules involved in target cell lysis such as granzyme B or perforin, or capable of killing infected cells as an effector cell.

- » Terminally differentiated cells are by definition cells that can no longer differentiate further to adopt another identity. There is no *a priori* reason that a terminally differentiated cell should be short-lived. However, for terminally differentiated effector T cells this is the general assumption. The terms "effector" cell and "terminally differentiated" cell are not synonymous: the fact that a T cell displays effector cell properties does not imply that it is terminally differentiated.
- » Memory T cells are those cells that survive beyond the contraction phase and persist long-term in the absence of pathogen.[1] Memory T cells can be subdivided, for instance based on their localization to lymphoid or peripheral tissues.[2,3] However, for the sake of simplicity we will here treat them as a single entity, characterized by the ability to survive long-term.
- » Cell state is the phenotypic and functional state of a cell, which can be transient or permanent.
- » Cell fate is a permanent and heritable cell state. In other words, a T cell that would commit to a memory T cell fate would only produce progeny that can survive in the absence of antigen. From the moment a T cell adopts a particular fate it is considered "committed," its future and that of its progeny is "programmed."
- » T cell priming is the event of T cell activation, leading to its first cell division.

CAN LONG-LIVED MEMORY CELLS ARISE FROM EFFECTOR T CELLS?

Fifteen years ago, little more was known about the generation of memory T cells than that these cells survived beyond the contraction phase and persisted at relatively stable numbers in the absence of antigen. From this, it was assumed that a fraction of effector T cells resisted apoptosis and differentiated further into long-lived memory T cells. Thus, in this linear differentiation model (Fig. 1A), memory T cells arise directly from effector T cells. Only relatively recent it was realized that it is impossible to deduce a precursor–product relationship between effector and memory T cells from the overall response kinetics. Specifically, it is possible that all effector T cells would be terminally differentiated (i.e., unable to convert to memory T cells) and thus die during contraction phase, whereas a dedicated subset of memory precursor cells that lack effector function would survive. To dissect whether long-lived memory T cells can directly arise from effector cells, several approaches have been taken, in particular the cell-tracing experiments performed in recent years have been informative.

In early experiments, proliferating antigen-specific T cell receptor transgenic (TCRtg) $CD4^+$ or $CD8^+$ T cell populations generated through *in vitro* peptide stimulation[4,5] or *in vivo* lymphocytic choriomeningitis virus (LCMV) infection[6] were adoptively transferred

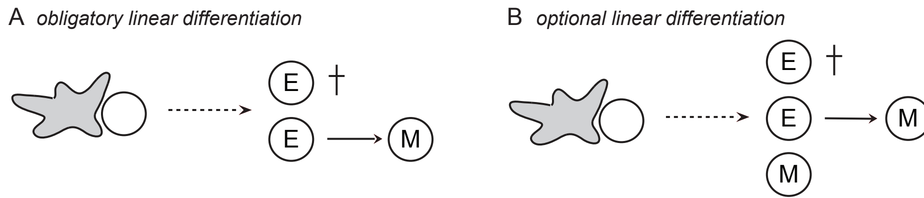

Figure 1: Linear differentiation model. According to the linear differentiation model, some effector T cells die after antigen clearance, while others give rise to memory T cells. (**A**) Linear differentiation might be obligatory in the sense that all memory T cells are direct descendants of effector cells. (**B**) Linear differentiation might also be optional in the sense that memory T cells can arise from effector cells, but can also arise independently of effector function.

into mice, and the potential of the transferred population to form persisting memory cells was monitored. As memory cells did develop in these experiments, the data were used as evidence for linear effector to memory differentiation. However, in all these early studies a heterogeneous population of dividing cells was transferred, of which the functional characteristics remained undefined, and the possibility remained that the memory cells arose from a subset of transferred cells that were not effector cells. Subsequent work demonstrated that the transfer of an essentially pure population of cells showing expression of perforin[7] or granzyme B[8] also resulted in the formation of T cell memory. To exclude the formal possibility that the memory T cells that developed in these experiments arose from a few contaminating non-effector T cells, several groups have subsequently used technologies that specifically tag effector T cells. Specifically, a number of reporter mouse strains have been created in which IFN-γ or granzyme B promoter activity leads to the long-term expression of a fluorescent or cell surface marker.[9–11] Using these mouse models, the groups of Baltimore, Weaver, and Fearon demonstrated that upon LCMV and influenza A infection long-lived $CD4^+$ and $CD8^+$ memory T cells can arise from T cells that have previously transcribed IFN-γ or granzyme B genes. It may be argued that the activity of the truncated human granzyme B reporter in an early study in this field[9] would not necessarily reflect the function of the endogenous murine promoter. However, as the same result was obtained in subsequent studies using reporter systems that are based on murine control elements,[10,11] these concerns are alleviated. Thus, the data obtained to date provide strong evidence that memory T cells can be formed from cells that previously transcribed genes associated with effector T cell function.

Two important questions remain, however. First, does the activity of the IFN-γ or granzyme B promoter reflect the actual production and secretion of these proteins? In other words, did the cells that are marked in these studies actually function as an effector cell? Second, do all memory T cells during natural infection arise from cells with effector properties (Fig. 1A), or can memory T cells also develop independently of effector function (Fig. 1B)? *In vitro* data in which peptide-stimulated TCRtg $CD8^+$

T cells were cultured in different cytokine environments have been used as evidence that memory T cells do not necessarily need to pass through an effector stage.[12] This conclusion was based on the observation that a $CD8^+$ T cell population cultured in high doses of IL-2 was able to lyse target cells more efficiently than a T cell population cultured in IL-15 and low doses of IL-2, while both populations were able to form memory. These data suggest that the capacity to kill infected cells may not be a necessary prerequisite to becoming memory cells. It should be noted though that cells grown under either condition produced IFN-γ, indicating that this particular effector function was possessed by both cell populations. Furthermore, the data do not address whether memory T cell formation without the transient acquisition of cytolytic capacity forms a physiological route for memory T cell production. Indirect evidence against the model that memory T cells are also formed *in vivo* without the transient acquisition of effector properties has been obtained in the reporter mouse studies. The tagging efficiency in these reporter mice is not absolute and consequently only a fraction of the cells transcribing the IFN-γ or granzyme B genes is marked by the reporter. However, the percentage of reporter-positive cells was similar in the effector phase as in the memory phase.[11] Thereby, these data suggest that a large fraction of memory T cells pass through an effector T cell stage. The possibility that a fraction (or specific subset) of memory T cells never displayed effector cell properties remains a possibility though,[13] and deserves further study.

WHICH FACTORS DETERMINE LONG-TERM T CELL SURVIVAL?

The above data indicate that at least part of the memory T cells are derived from the pool of T cells that display effector cell properties. Is there heterogeneity within the effector population that explains why some effector cells continue to develop into memory cells, while others die during contraction?

In an attempt to explain why some activated T cells would survive beyond the contraction phase and others not, Ahmed and Gray proposed the decreasing potential hypothesis, in which the level of antigenic stimulation was seen as the crucial factor dictating whether a cell would commit to a long-lived memory fate (weak stimulus) or undergo apoptosis after terminal differentiation (strong stimulus)[14] (Fig. 2A). Such variation in the strength of signals received by individual antigen-specific T cells was thought to be the result of exposure to different amounts of antigen or inflammatory environments, for example, due to difference in tissue localization, or asynchronous recruitment of naive T cells into the response (with early recruits receiving distinct signals from T cells activated later during infection). Also, Lanzavecchia and Sallusto argued that the strength of stimulation—the combination of TCR triggering, engagement of costimulatory molecules, and the duration of stimulation—that a T cell receives is fate determining. Their progressive differentiation model (Fig. 2B) proposes that T cells undergo progressive degrees of differentiation by accumulating increasing amounts of activation signals.[15,16] Weakly stimulated cells would initiate some proliferation but

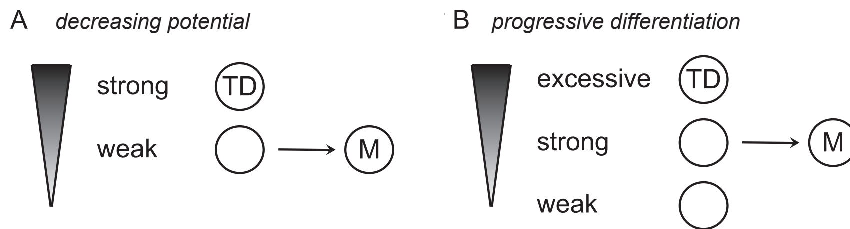

Figure 2: Comparison of decreasing potential and progressive differentiation models. (**A**) The decreasing potential model suggests that weakly stimulated T cells commit to a memory fate (cell marked M), while a strong stimulus leads to the fate of terminal differentiation (cell marked TD). (**B**) Also, the progressive differentiation model predicts that the most strongly (excessively) stimulated T cells undergo terminal differentiation. An intermediate stimulus leads to a memory fate, while weakly stimulated cells remain uncommitted (unmarked cell).

eventually die by neglect after antigen withdrawal, and without further differentiation into effector or memory cells. Cells that receive a strong signal would have the capacity to develop into memory cells, while excessively stimulated T cells were thought to undergo activation-induced cell death after terminal differentiation. For reasons that are unclear, the progressive differentiation model has sometimes been interpreted as opposing the decreasing potential hypothesis. It does not. To draw an analogy with T cell selection within the thymus, the decreasing potential model from Gray and Ahmed focuses on how the difference between a medium- and a high-strength signal can mean the difference between survival and negative selection, whereas the progressive differentiation model from Lanzavecchia and Sallusto also takes into account how the difference between a weak signal and a medium-strength signal can mean the difference between neglect and positive selection. Thus, both the progressive differentiation model and the decreasing potential model suggest that T cells that receive an intermediate activation signal can survive into the memory phase, whereas T cells that receive a stronger signal will die during contraction.

What is the experimental evidence supporting the hypothesis that high signal strength leads to the preferential development of terminally differentiated T cells? Most evidence comes from experiments in which the ratio between memory T cells and terminally differentiated cells (or the total number of antigen-specific T cells present at the peak of the response, which primarily consists of terminally differentiated cells) was monitored under diverse inflammatory conditions, or in situations of altered antigen availability. With one exception,[17] the diminished inflammation and antigen presentation that occurs upon treatment of bacterially infected animals with antibiotics has been shown to lead to a reduced magnitude of the peak T cell response.[8,18–20] In contrast, the absolute number of memory T cells that developed[19] or the number of KLRG1$^{lo/int}$CD127^{hi} cells[8]—presumed precursors of memory T cells[8,21]—was hardly affected. These data demonstrate that antigen-derived and/or inflammatory signals can influence the relative size of the memory population. In these studies,

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the effects of antigen availability and inflammatory signals have also been investigated independently, and both reduced antigenic stimulation and limited inflammation were shown to result in an increase in the proportion of KLRG1$^{lo/int}$CD127^{hi} cells.[8,21] On the contrary, a strong inflammatory stimulus through the administration of exogenous IL-12 led to an increased percentage of antigen-specific T cells that expressed CD25, a phenotypic property linked to terminal differentiation.[22] In line with this, mice lacking functional IL-12 generated larger memory populations after *Listeria monocytogenes* infection than did wild-type mice.[23] Furthermore, exposure to the inflammatory cytokines IL-12 and IL-4 has been shown to induce expression of the transcription factors T-bet and Blimp-1,[8,24,25] which in turn induce the expression of KLRG-1 and CD25, molecules that are associated with terminal differentiation.[8,21,22,26] Thus, these studies suggest that both antigen and inflammation can form signals that lead to terminal T cell differentiation.

Nevertheless, not all studies support the hypothesis that increased signal strength promotes terminal differentiation. Specifically, the ratio between terminally differentiated and memory T cells was not affected when the duration of antigen availability was shortened through specific depletion of antigen presenting cells at different times after infection.[27] Similarly, recent work by Bevan and colleagues indicates that differences in TCR affinity for antigen do not alter the ratio between the number of effector phase and memory phase T cells.[28]

Taken together, a majority of studies suggest that at least some types of prolonged or increased stimulation favor the formation of terminally differentiated cells, while weak stimulation is sufficient for the generation of long-lived memory cells. Intuitively this makes sense, as it provides the system with the flexibility to alter output depending on the magnitude or duration of infection. However, our knowledge of the exact molecular interactions that do and do not determine “signal strength”—and thereby the ratio of terminally differentiated to memory cells—is at present still sketchy.

WHEN DOES AN INDIVIDUAL T CELL COMMIT TO LONG-TERM PERSISTENCE?

While there is now substantial evidence that the strength of at least some signals received by T cells can influence the balance between the number of terminally differentiated and memory T cells, this does not inform us at what point in development an individual T cell commits to either terminal differentiation or to long-term persistence as a memory T cell.

A number of studies have demonstrated that already early after infection the responding antigen-specific T cell population shows remarkable heterogeneity in terms of surface molecule expression as well as cytokine secretion. This raises the question whether this heterogeneity can serve as a reliable marker for T cell survival into the memory phase. If so, this would also indicate that the commitment to form memory or to undergo terminal differentiation occurred prior to the moment of first marker expression.

One of the molecules that are differentially expressed in responding $CD8^+$ T cells is killer cell lectin-like receptor G1 (KLRG1), which appears on the surface of a subset of antigen-specific T cells 4–5 days after LCMV infection[8,21,29] and is induced through the transcription factor T-bet.[8] Furthermore, heterogeneity has also been observed with respect to IL-2 production[21] and expression of CD62L[30,31] and the IL-7Rα (CD127)[21,26,31–33] and IL-2Rα (CD25) chains.[26] Specifically, IL-2, CD62L and CD127 were primarily produced by/expressed on the KLRG-$1^{lo/int}$ population,[8,21,30] while CD25 was predominantly expressed on KLRG-1^{hi} cells.[26] To determine whether these molecules could serve as markers of T cell fate, two different approaches have been taken. First, the percentage of cells expressing a certain marker has been followed during the course of an immune response. These experiments have led to the suggestion that T cells expressing CD62L or CD127 and low levels of KLRG-1 early during the response were precursors of memory T cells, as the percentage of cells within the antigen-specific population that displayed this phenotype was increased during the memory phase.[8,30,31,33] The evidence obtained by this type of analysis is obviously highly indirect: as marker expression can change over time it is uncertain whether a T cell expressing such a marker late during the response is the (progeny of a) cell that expressed this molecule earlier during the response. In a second—more informative—approach that has also been followed in most of the studies, T cell populations have been sorted based on marker expression and have subsequently been transferred into different hosts. Provided that cell isolation affects survival to an equal extent for both populations, this approach allows one to directly compare the capacity of the two sorted populations to persist as memory T cells.

In early experiments, $CD127^{hi}$ and $CD127^{lo}$ populations were sorted during the contraction phase and their potential to survive long-term was evaluated after transfer into naive recipients.[31,33] $CD127^{hi}$ cells were shown to preferentially survive, but it was not addressed to what extent survival would differ when transferred into an infectious environment. More recently, transfer of KLRG-1^{hi} and KLRG-$1^{lo/int}$ TCRtg $CD8^+$ T cells 4 or 8 days after LCMV infection into timed recipients has revealed that KLRG-$1^{lo/int}$ cells were ~5–12 times more efficient in surviving for several months after transfer,[8,21,33] suggesting that low KLRG-1 expression is associated with persistence into the memory phase. Similarly, it has been observed that the extent of IL-2 signaling, a property that is directly linked to CD25 expression, influenced expression of effector molecules and surface markers associated with terminal differentiation.[22,26,34] Specifically, analysis of $CD25^{hi}$ and $CD25^{lo}$ cells 3.5 days after LCMV infection revealed preferential expression of CD127, CD62L, and IL-2 in the $CD25^{lo}$ population.[26] Transfer of sorted $CD25^{hi}$ or $CD25^{lo}$ cells, as well as of cells previously activated in high or low IL-2 concentrations, demonstrated that limited IL-2 signaling through CD25 favored long-term persistence, presumably through higher resistance to apoptosis.[26] One of the transcriptional regulators involved in this process is B lymphocyte-induced maturation protein-1 (Blimp-1), which is induced in $CD4^+$ and $CD8^+$ T cells after IL-2 signaling. Blimp-1 activity induces CD25[24,35] and T-bet expression,[36] and in line with this Blimp-1

has been shown to promote terminal differentiation and T cell exhaustion.[36–39] Taken together, the above data indicate that terminal differentiation is associated with the expression of KLRG-1 and CD25 at 3–4 days after infection.

What does this early heterogeneity tell us about the moment of fate commitment? Does it suggest that T cells commit to longevity or terminal differentiation early during the response, and, if so, can this be linked to a defined event of cell division? Thus far, several models have been proposed that aim to explain at which point in development an individual cell commits to persistence or to death after antigen clearance. These models can be categorized into models that predict fate determination before the first cell division (D0), during the first cell division (D1), or after the first cell division (D1+) (Fig. 3). The evidence for each of these models will be discussed in relation to the strengths and weaknesses associated with the technologies used for the tracking of T cell fate.

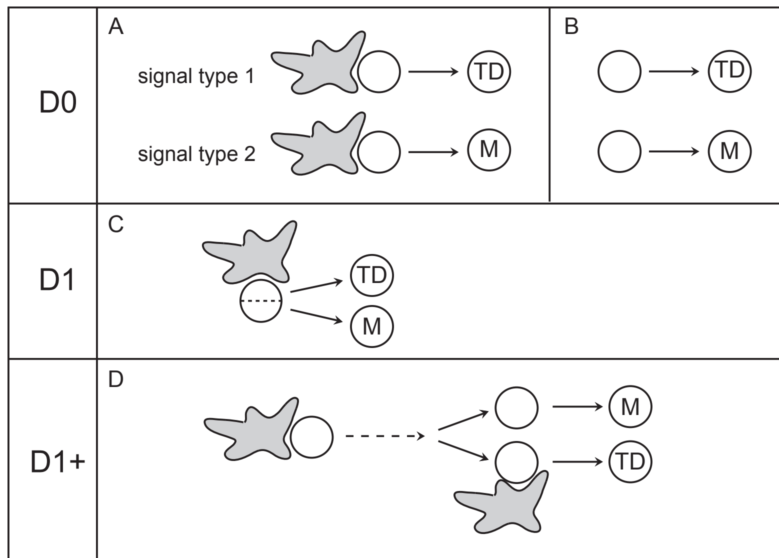

Figure 3: Models predicting the moment of fate commitment. (**A, B**) T cells commit to the fates of terminal differentiation (TD) and memory (M) before the first cell division (at division 0, D0). (**A**) Signal type 1 given to the naive T cell upon priming leads to terminal differentiation of itself and essentially all its progeny, whereas signal type 2 in turn programs a memory fate. (**B**) Naive T cells are intrinsically different and thus already committed even before activation. (**C**) Fate commitment occurs during the first cell division (D1) through asymmetry of the division. The daughter cell located proximal to the priming DC commits to terminal differentiation and the daughter cell distal to the DC commits to a memory fate. (**D**) Fate commitment as a late event in T cell proliferative bursts (D1+). Daughter T cells receiving additional stimuli after priming commit to terminal differentiation. Daughter cells that do not acquire additional stimuli or only receive weak stimuli adopt a memory fate. Uncommitted cells are depicted as empty.

Models predicting fate determination at the D0 stage

When the progressive differentiation and decreasing potential models from Lanzavecchia/Sallusto and Gray/Ahmed were first brought forward, the prevailing view was that proliferation of activated T cells required their continuous stimulation by antigen. However, subsequent work by the groups of Pamer, Schoenberger, and Ahmed demonstrated that a brief encounter with antigen is sufficient to induce naive T cells to undergo several rounds of division.[40–43] Furthermore, the progeny of T cells that were activated by such a brief stimulus acquired effector functions and could survive as memory T cells.[40–42,44] These results opened the interesting possibility that T cell fate (i.e., terminal differentiation or long-term persistence) may already be programmed at the level of the naive T cell (Fig. 3A), as suggested previously by Farber.[45] T cells could for instance acquire heterogeneous fate determining signals before the first cell division by interacting with multiple antigen presenting cells of potentially variable maturation states before they form the stable contacts that lead to initial cell division.[46,47] Furthermore, the time after infection at which an individual T cell would be primed could affect the signals (the amount of antigen or the nature and amount of inflammatory signals) that different T cells receive during initial activation. As an alternative possibility for T cell commitment at the D0 stage and that does not invoke differential signals during T cell priming, it may also be postulated that individual naive T cells are intrinsically different, and that this heterogeneity would influence the fate of its daughters (Fig. 3B).

Indirect evidence. Some evidence for models predicting fate determination at the D0 stage comes from studies in which the formation of long-lived memory cells was assessed after manipulation of antigen availability during priming. One study demonstrated that limiting the available amount of antigen through injection of antibody specific for peptide-MHC complexes before vesicular stomatitis virus (VSV) infection reduced the formation of antigen-specific $CD4^+$ memory T cells, compared to mice injected with control antibody, while the height of the peak T cell response was unaltered.[48] A similar reduction in the formation of persisting $CD8^+$ memory T cells was observed when *Listeria monocytogenes*-infected mice were treated with ampicillin 24 h after infection.[17] These studies suggest that the development of long-lived memory cells occurs more efficiently after a strong stimulus early during the T cell response. However, it is difficult to judge whether the manipulations used in these studies only affected the strength of signals received by T cells during priming (i.e., before the first cell division), or—perhaps more likely—during the course of T cell proliferation. Furthermore, such a reduction in the formation of T cell memory relative to the peak T cell response was not observed when antigen availability was altered through depletion of antigen presenting DCs via stimulation of the diphtheria toxin receptor selectively present on transferred antigen-loaded DCs as early as 1 h after DC transfer.[27] Furthermore, an opposite result, in which a strong stimulus during priming increased the magnitude of the peak T cell response rather than memory T cell

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frequencies, was obtained in *in vitro* T cell priming experiments in which the duration of antigenic stimulation was tightly controlled and restricted to the time before the first cell division. In these experiments, memory T cell development *in vivo* was equally efficient for a population of briefly (4 h) and longer (20 h) stimulated T cells, while the magnitude of the peak T cell response was reduced after short antigen exposure.[49]

Indirect support for fate commitment at the D0 stage has also been provided by the finding that CD25 is already differentially expressed on *in vitro* activated but still undivided T cells.[22] As CD25 preferentially marks T cells with limited memory potential,[22,26] these data suggest the possibility that the capacity to persist long-term is programmed before the first cell division. However, since IL-2 signaling at later stages after activation, and thus after the first cell division, can further regulate CD25 expression, it remains possible that signals received after priming can modulate the potential for long-term persistence. Sorting of undivided $CD25^{hi}$ and $CD25^{lo}$ cells, followed by transfer into infected recipients, would allow one to assess whether different fates have already been programmed in these subsets before the first cell division.

Finally, a recent report assessed the role of TCR signaling on the formation of memory cells by generating T cells expressing a TCR harboring a point mutation in the β transmembrane domain.[50] These T cells failed to persist as memory cells, whereas their differentiation into functional effector cells was not impaired. These data suggest that different signaling events are required for terminal differentiation and for long-term persistence. At present, it is unclear whether any physiological stimuli exist that convey a TCR signal similar to the signal transmitted by this mutant TCR, and that could therefore control memory and effector T cell fate. Furthermore, although these data have been taken as support for the model that fate determination occurs during T cell priming,[50] it is unknown at which point in development T cells expressing the mutant TCR fail to receive a signal required for long-term survival. As a consequence, the experiments do not appear informative with respect to the question at which point in development commitment to long-term persistence occurs.

Taken together, a number of studies are consistent with fate commitment at the D0 stage. However, not all data can be reconciled with this model and—most importantly—in these studies it has not been directly assessed at what point in development the signal that influenced long-term survival was received by the T cells; consequently the evidence remains indirect.

Direct evidence. To directly assess at what stage a T cell commits to either long-term persistence or to terminal differentiation, it is valuable to follow individual T cells rather than T cell populations through time. Specifically, by tracing individual T cells and their progeny, one can reveal whether this cell still has the potential to yield both terminally differentiated offspring and memory T cells, or whether the cell can only generate one type of output and is therefore committed. Over the past years, several techniques have been developed to allow cell tracing at the single cell level and the general concept and available technology are discussed in a recent review.[13]

As a first approach, the sequencing of TCR genes has been used by a number of groups to dissect the development of antigen-specific T cell responses. TCR sequencing offers the substantial advantage that it allows one to follow the endogenous T cell response, rather than that of transferred TCRtg T cells. Sequence analysis of the CDR3 region of TCRβ-chains has revealed that close to half of the identified sequences were shared between primary expansion, resting memory phase, and recall responses within the same mouse.[51,52] However, sequences that appeared unique for the effector phase T cell pool or the memory phase T cell pool were also observed. At first glance, these data appear to indicate that some naive T cell clones contribute to the formation of long-lived, as well as short-lived, cells, while other clones only had the potential to produce progeny with either only long-term or only short-term survival potential. However, the interpretation of these data is complicated by two factors. First, TCRβ-chains are shared by multiple naive T cell clones.[53] Consequently, if the same TCRβ sequence is observed in both terminally differentiated and in long-lived memory T cells this does not indicate that this involved the progeny of the same naive T cell.[13] Second, in at least some studies that have used TCRβ-chain analysis to track the developmental potential of naive T cells, it was not assessed whether the repertoire present in a given sample was representative of that population (something that can readily be determined by multiple sampling). In the absence of such a control, any statements on the lack of kinship between cell populations are precluded.[13]

An elegant study that was able to monitor the developmental potential of naive T cells truly at the individual T cell level has been performed by the group of Busch. In this study, single naive TCRtg $CD8^+$ T cells were obtained by micromanipulation, and each individual cell was injected into a separate mouse that was subsequently infected with *Listeria monocytogenes*.[54] The progeny of this single naive T cell had the capacity to develop into a heterogeneous effector population, and the progeny of a single transferred T cell was capable of responding to secondary infection. These experiments suggest that individual naive T cells can yield both effector and memory T cell progeny. However, as the throughput of this experimental system is limited (the potential of only one antigen-specific T cell can be studied per mouse), the experiments do not address whether all naive antigen-specific T cells primed during infection can produce effector and memory cell progeny.

To enable cell tracking of large numbers of individual naive T cells at the single-cell level, we have recently developed a cellular barcoding technology. In this technology, individual naive T cells are provided with unique genetic tags (barcodes) that are transmitted to all progeny.[18,55,56] These barcodes may be introduced into peripheral T cells by standard retroviral infection. However, to avoid the potential effect of *in vitro* T cell activation on subsequent fate, one can also perform retroviral transduction of TCRtg thymocytes. The resulting barcode-labeled thymocytes are subsequently injected into the thymus of unmanipulated recipient mice, and this allows their differentiation into mature barcode-labeled naive T cells.[56] Provided the essential controls are performed to assess the boundaries in which kinship mapping can be

performed,[13] this technology allows one to distinguish whether any cell populations of interest are derived from common or from distinct progenitors.[18,55,56]

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To test whether the progeny of individual naive T cells would be able to develop into both short-lived and long-lived T cells, or only one of these subsets, naive OT-I $CD8^+$ T cells, each harboring a unique genetic barcode, were transferred into recipient mice and challenged by recombinant *Listeria monocytogenes* or influenza infection.[56] Subsequent barcode analysis of T cells participating in the primary response and those persisting long-term revealed that the same barcodes were present in both populations and at roughly similar frequencies. Also KLRG-1^{hi}CD127^{lo} and KLRG-1^{lo}CD127^{hi} populations isolated at the peak of the primary T cell response harbored largely the same barcodes. These data indicate that both during systemic and local infections, memory and effector phase $CD8^+$ T cells are progeny of the same individual naive T cells. As it can be assumed that not all barcode-labeled naive T cells were primed simultaneously but rather over a physiological range of time, these data also demonstrate that T cells primed at distinct time points during infection produce a similar ratio of terminally differentiated and long-term persisting progeny. Furthermore, within the range tested, the affinity of TCR-pMHC interactions did not influence the capacity of individual T cells to yield both short- and long-lived progeny.[56]

What are the limitations of the types of analysis performed in the single-cell transfer and cellular barcoding studies? A first limitation that could be noted is that cell tracking was performed on monoclonal T cells. This argument is actually not particularly compelling; when analyzing T cell responses at the single cell level, one, by definition, examines T cell reactivity at the clonal level. A second argument that could be made is that the response of the adoptively transferred T cells is not representative of endogenous T cell responses. However, as very low numbers of adoptively transferred cells were used in these studies (in the case of Busch at n= 1 per mouse) and as these cells responded amidst polyclonal endogenous antigen-specific cells, this concern does not seem significant.

While the single-cell transfer and barcoding studies demonstrate that under physiological conditions of infection, individual naive T cells yield functionally diverse progeny, this does not necessarily imply that all recently primed T cells are identical. Interestingly, recent multi-photon microscopy experiments suggests that heterogeneity in at least some functional properties can be acquired during priming and can be passed on to early daughter populations under conditions in which further antigenic and inflammatory stimuli are absent.[57] This finding may potentially be reconciled with the *in vivo* lineage tracing studies discussed above by assuming that the cumulative signals that T cells receive during further *in vivo* differentiation cancel out the early heterogeneity observed by Bousso and colleagues.

In summary, there is now strong evidence from both single-cell transfer and barcoding studies that single naive T cells yield heterogeneous progeny: fate is not fix at the D0 stage.

Models predicting fate determination at the D1 stage

At which point in development could commitment to terminal differentiation or to long-term persistence occur if naive T cells are still bipotent? Recently, it has been proposed that these different T cell fates may be programmed during the first cell division through asymmetry of this division[58] (Fig. 3C). Asymmetric cell division has so far mainly been associated with the ability of stem cells to simultaneously self-renew, while also producing progeny that commits to differentiation.[59–61] Generally speaking, unequal daughter fates (as must take place in any self-renewing system that generates differentiated progeny) can be generated through symmetric or through asymmetric divisions (Fig. 4). In the first case, initially equal daughter cells adopt different fates through subsequent encounter of different fate-determining signals.[62] In the second case, acquisition of unequal daughter fates is achieved during division through defined cell intrinsic or niche-controlled mechanisms that have been described in detail in invertebrate model systems.[59,60,62,63] Cell intrinsic mechanisms rely on the establishment of a polarity axis that enables unequal distribution of intrinsic cell fate determinants over the two daughter cells during mitosis, which directs the daughters into different fates. Alternatively, originally equipotent daughter cells can acquire unequal fates if niche-derived signals are required to maintain self-renewal capacity and if one of the daughter cells is forced to lose contact with the niche during mitosis.

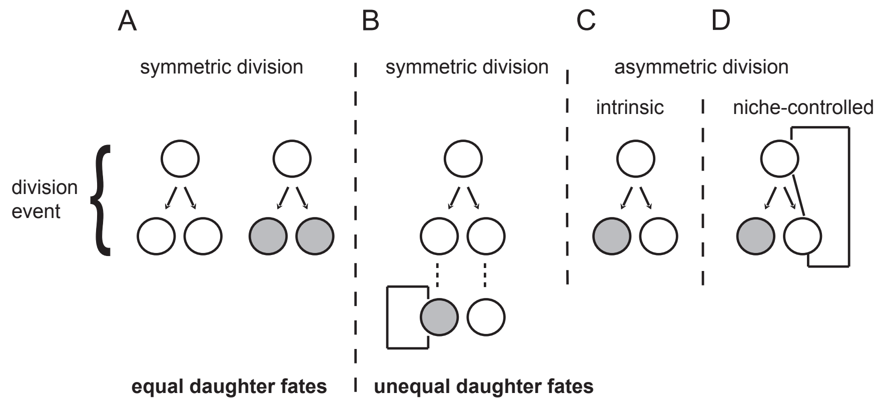

Figure 4: Mechanisms leading to equal or unequal daughter fates. (**A**) Equal daughter fates are by definition generated through symmetric cell division. During this division, either two uncommitted (white) or two committed (gray) cells can be formed. Unequal daughter fates can be the result of either symmetric or asymmetric division. (**B**) Cell division produces two initially equal daughters, of which one remains uncommitted, while the other initiates commitment through encounter of particular environmental factors. (**C**) Unequal distribution of cell fate determinants over the two daughter cells during the mitotic event results in unequal daughter fates. (**D**) Following mitosis, one of the two daughter cells loses contact with a niche that is required for maintaining an uncommitted state, and this results in a niche-controlled asymmetric cell fate. Note that in B, commitment to differentiation is random, whereas in C and D commitment invariably occurs for one of the two daughter cells.

3

Many of the proteins involved in asymmetric cell division in invertebrates are conserved in mammals. In line with this, intrinsic asymmetric division has been shown to occur in the mammalian brain,[60] and for long it has been postulated that also hematopoietic stem cells could generate diverse progeny through asymmetric division. However, evidence that diverse progeny may result from intrinsic asymmetric divisions of hematopoietic stem cells has only been provided recently. Specifically, using a time lapse microscopy setup in which GFP is expressed after Notch signaling,[64] it was demonstrated that GFP-expressing hematopoietic stem cells can give rise to two GFP^+ daughters, two GFP^- daughters, or to one GFP^- and one GFP^+ daughter.[65] Notch signaling has previously been shown to be fate determining,[64] and the loss of GFP expression correlated with acquisition of lineage markers and differentiation on a population level.[64,65] In combination with the finding that Numb, an inhibitor of Notch signaling, was asymmetrically localized during mitosis, these experiments suggest that murine hematopoietic stem cells can undergo both symmetric divisions and intrinsic asymmetric divisions.[62]

Strikingly, Reiner and colleagues have suggested that not only hematopoietic stem cells but also naive $CD8^+$ and $CD4^+$ T cells may divide asymmetrically after activation, and that this asymmetry would determine the fate of subsequent progeny.[58,66,67] In a first set of experiments, adoptively transferred $CD4^+$ or $CD8^+$ TCRtg T cells, primed *in vivo* by infection with *Leishmania* or recombinant *Listeria monocytogenes*, were isolated before their first cell division.[58] In more recent experiments, $CD8^+$ T cells were activated *in vitro*, using peptide-pulsed DCs.[67] Confocal microscopy of cells obtained in both systems revealed polarized expression of several molecules, but with remarkable differences between the two systems. After *in vivo* priming, ~60% of cells showed polarized expression or unequal inheritance of molecules that have been associated with the immunological synapse (CD8, LFA-1, IFN-γ receptor (IFN-γR)), molecules involved in establishing cell polarity (PKCζ, Scribble) and polarized expression of the cell fate determinant Numb.[58] With the exception of PKCζ, all of these molecules were localized at the same side of the cell as the microtubule organizing center (MTOC), and thus assumed to have been facing the immunological synapse. Also *in vitro* priming of T cells induced polarized expression and asymmetric inheritance of the polarity proteins Par3, Scribble, and atypical PKC, but no polarization of CD8 was observed in this system.[67] Furthermore, in this case the cell fate determinant Numb was observed to be enriched in the DC-distal daughter cell rather than the (putative) DC-proximal daughter. To test whether the unequal inheritance of molecules had an effect on the fate of the two initial daughters, *in vivo* primed TCRtg $CD8^+$ T cells that had divided once were sorted into $CD8^{hi}$ and $CD8^{lo}$ populations and transferred into recipient mice. Subsequently, the potential of the two cell populations to reduce bacterial burden was assessed by bacterial challenge either shortly after infusion or at a late time point after transfer. Both $CD8^{hi}$ and $CD8^{lo}$ populations were able to convey protection against infection at early and late time points. However, the bacterial load after late infection was reduced to a larger extent in mice that had received $CD8^{lo}$ cells. Based on the

critical assumption that $CD8^{hi}$ and $CD8^{lo}$ cells represent the DC-proximal and DC-distal daughters respectively, this experiment suggests that DC-distal daughters are superior in their capacity to protect against reinfection.

However, as the $CD8^{hi}$ (i.e., the putative DC-proximal) cell population displayed a potential to control infection at a late time point, these experiments suggest that the two cell populations used do not display an absolute difference in fate but rather are either composed of a mixture of cells with different fates, or—as will be discussed more extensively below—represent subsets that differ in their propensity to persist long-term. Experiments in which single cell tracing is performed on cells that have undergone their first division will be helpful to directly reveal to what extent asymmetry during the first division results in an asymmetry in fate. Conceptually this should be feasible, using either single cell transfer or cellular barcoding. However, the technological hurdle is significant.

Models predicting fate determination later than D1

Models for early determination of T cell fate can be intuitive if one assumes that T cell priming initiates a fixed program of differentiation. However, the finding that a short period of antigen exposure is sufficient for survival, proliferation, and the formation of effector and memory T cells[40–42,68] does not imply that primed T cells can no longer integrate signals that influence the capacity of this cell and its progeny to persist as a memory T cell.

Direct evidence for the hypothesis that T cells can still integrate signals acquired after priming is provided by experiments in which T cells were isolated 3–5 days after infection, a time point at which most T cells have already divided as based on CFSE dilution. Transfer of these cells into hosts that were either pathogen-free or infected with pathogens that did or did not express cognate antigen showed that both further proliferation and the phenotype of the transferred T cells can be influenced by late-acting signals.[18,21] It should be noted though that these experiments have thus far not addressed whether such late-acting signals are (partially) responsible for the commitment to either terminal differentiation or long-term persistence.

T CELL HETEROGENEITY AS AN INDICATOR FOR CELL STATE RATHER THAN CELL FATE?

Heterogeneity within the responding T cell population has been interpreted as evidence for the adoption of divergent fates early during the response, but do the available data indeed allow the conclusion that T cells expressing defined molecules are already committed to a particular fate? Whenever the memory potential of T cell populations, sorted on the basis of a distinct marker profile, was evaluated after transfer, the populations only showed a gradual difference in their ability to survive long-term.[21,26,58] Thus, the marker expression profiles used were unable to distinguish cells with an absolute difference in fate. Ignoring the "dull" and unlikely explanation

of an impure sort for now, we see two possible explanations. First, T cells are already truly committed at this stage, but the expression levels of CD25, KLRG-1, or CD8 after the first cell division are simply not sufficient to unambiguously separate the two committed T cell populations. At the moment, a new marker (not necessarily a cell surface marker; it may, for instance, involve histone modifications) or a combination of several molecules would be identified that unmistakably identify committed cells, the pure isolation of terminally differentiated or long-term persisting clones would become feasible. Second, the fact that current markers only partially separate long-term and short-term persisting cells might simply mean that T cells do not commit to a particular fate early during the response, or perhaps do not commit at all. Rather than signifying cell fate, marker expression could signify the current cell state and thereby the probability that an individual cell will either survive long-term, or will die during the contraction phase (Fig. 5). To draw a simple analogy, a high cholesterol level increases the likelihood of a heart attack. However, at no point in time is there a "fate decision" that segregates individuals into two groups (the equivalent of T cell fates), one that will and one that will not die from heart disease. Instead, the phenotype (cholesterol level) at a given point in time reports on the current state, but subsequent signals (a low cholesterol diet) may still influence future state and thereby the probability of heart disease.

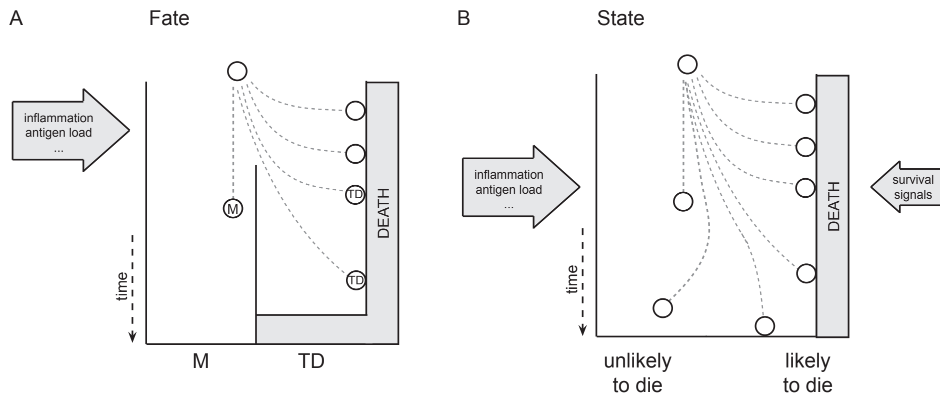

Figure 5: Cell fate versus cell state. (**A**) Activated T cells commit to the fates of terminal differentiation (TD) or memory (M). Before commitment takes place (committed cells are marked by "TD" or "M"), T cells can integrate external signals (inflammation, antigen load), and the extent of this stimulation determines the fate that is adopted by the cell. Once a cell is committed (i.e., ends up to the right or to the left of the line separating TD and M), external signals can no longer influence whether a cell will die after pathogen clearance or will survive as a memory cell. (**B**) Activated T cells do not commit to a particular fate. The cells integrate external signals that influence their likelihood to die, but commitment never takes place. At any point in time—before the cell initiates apoptosis—external signals can act to modulate the cell state, and thereby the likelihood to die.

How could such a regulation of T cell state be envisioned? At the superficial level, a given T cell state would reflect the balance between intracellular mediators that favor survival and those that predispose to death, and this balance could be modulated after additional antigenic or inflammatory signals. What would this mean molecularly? It is evident that the balance between anti-apoptotic and pro-apoptotic molecules forms a likely candidate as the principal indicator of T cell state. Interesting in this respect is the observation that TCRtg $CD8^+$ T cells lacking the pro-apoptotic molecule Bim form a stable pool of memory T cells, while hardly undergoing contraction.[69] With the caution in mind that this study was not performed at a single cell level, these experiments suggest that all cells taking part in the primary response, including KLRG-1^{hi}CD127^{lo} cells, can form memory cells if they are simply prevented from dying.

CONCLUSION

To date, there is strong evidence that memory T cells can be direct progeny of cells that display effector gene expression, but it is still uncertain whether effector function is a prerequisite for memory development. Less clear is the exact timing at which individual T cells commit to terminal differentiation or memory, and whether such commitment actually takes place at all. Studies in which the developmental potential of individual naive T cells was examined at the single-cell level clearly demonstrate that fate commitment does not take place before the first cell division. This rules out the possibility that naive T cells are predisposed toward memory or terminal differentiation, or that fate is dictated through differential priming by APCs. The scenarios in which T cell fate is either determined through asymmetric division or through cumulative signals acquired after priming remain distinct possibilities. However, as the heterogeneity observed thus far within T cell populations has not led to the identification of T cell populations with a full commitment to terminal differentiation or to long-term persistence, it also seems possible that T cells do not make a fate decision that determines whether they will survive or die. Instead, T cells might assume a continuum of cell states that indicate the likelihood by which an individual cell will either die during contraction or will persist. In this model, signals in the form of antigen or inflammation acquired early during the response or at later points in time influence cell state and, thereby, the likelihood of survival or death, but do not induce commitment. At present, evidence for this "no-fate-but-state" model of T cell differentiation is still limited. However, the flexibility it allows appears attractive from an evolutionary point of view.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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