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T Cell Fate at the Single-Cell Level

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Abstract

T cell responses display two key characteristics. First, a small population of epitope-specific naive T cells expands by several orders of magnitude. Second, the T cells within this proliferating population take on diverse functional and phenotypic properties that determine their ability to exert effector functions and contribute to T cell memory. Recent technological advances in lineage tracing allow us for the first time to study these processes in vivo at single-cell resolution. Here, we summarize resulting data demonstrating that although epitope-specific T cell responses are reproducibly similar at the population level, expansion potential and diversification patterns of the offspring derived from individual T cells are highly variable during both primary and recall immune responses. In spite of this stochastic response variation, individual memory T cells can serve as adult stem cells that provide robust regeneration of an epitope-specific tissue through population averaging. We discuss the relevance of these findings for T cell memory formation and clinical immunotherapy.

INTRODUCTION

Antigen-specific T cells can provide highly efficient and long-lasting immunity against infections. In addition, endogenous and genetically engineered T cells are harnessed for the treatment of various cancers with increasing success (1–3). On the other hand, T cells can cause allergy and life-threatening autoimmune diseases. Over the last decades it has been well established that activation of T cell effector functions, as well as generation and maintenance of memory T cells, requires tight regulation in order to support protective immune responses while at the same time preventing excessive activation and chronic immunopathology (4, 5).

Primary T cell immune responses originate from a pool of naive T cells harboring a highly diverse repertoire of different T cell receptors (TCRs) that is generated during thymic development (6). Since most T cells express only one functional TCR, diversity or breadth of the receptor repertoire comes at the cost of limiting its depth—that is, the number of naive T cells that is available for any given epitope. Indeed, in both mice and humans the frequency of T cells carrying a TCR specific for a given epitope is generally very low, ranging between 1 to 10 per million $CD4^+$ T cells and 1 to 100 per million $CD8^+$ T cells (7). Because of these minute starting frequencies, an epitope-specific T cell population needs to expand both vigorously and rapidly in order to provide immune protection during an acute infection. This important concept of adaptive immunity was first introduced in 1957 by the Australian immunologist Frank Macfarlane Burnet as the clonal selection theory (8). Clonal expansion of antigen-specific T cells usually reaches its peak within one week after initial T cell activation by antigen and goes along with acquisition of potent effector functions and—generally—rapid clearance of the invading pathogen. Following this effector phase, the majority of antigen-specific T cells die. However, some survive beyond the contraction phase and stably persist in the absence of antigen. The resulting 100- to 1,000-fold increase of epitope-specific T cells, relative to the preimmune state, allows for stronger and more rapid recall immune responses in the event of antigen reencounter and thereby serves as the quantitative basis for adaptive T cell immunity. However, epitope-specific T cells present during the memory phase, i.e., after the end of contraction, are not only increased in numbers but also display functional characteristics, such as distinct migratory patterns (9, 10) or immediate effector cytokine production upon TCR triggering (11, 12), that clearly set them apart from their naive counterparts, thereby contributing to enhanced recall immunity in a qualitative manner.

For many years it has been realized that both during the effector and during the memory phase epitope-specific T cell populations harbor substantial phenotypic and functional diversity (13, 14). Furthermore, the generation of different T cell subsets during infection or in response to vaccination appears to be key for the quality of antigen-specific immunity (15–18). Because of this, two main questions have been at the center of recent research in T cell differentiation: (*a*) How is T cell subset diversification regulated? (*b*) Which of the emerging subsets provide the basis for long-lived T cell memory?

Addressing these questions requires not only the single-cell resolution of a T cell's current phenotype that is needed to detect diversity in the first place, but also the single-cell resolution of a T cell's developmental potential. In other words, it requires an understanding of the kinship between ancestor and offspring spanning multiple T cell generations and stretching out from the location of ancestral priming across to the various tissues to which T cells travel during a primary immune response and the ensuing memory phase. To this end, new technologies have been developed that allow the fate mapping of individual T cells in vivo. In this review, we summarize these technological advances and the novel findings derived from their use. In addition, we discuss how these findings influence our basic understanding of adaptive T cell immunity and T cell memory and touch upon their clinical implications in the context of immunopathology, vaccination, and adoptive immunotherapy.

DIVERSITY IN T CELL–BASED IMMUNE RESPONSES

To analyze the cellular composition of a T cell response, it is necessary to determine phenotypic and functional characteristics at the level of individual cells. A major breakthrough for establishing such single-cell resolution was the development of flow cytometry (19). With this technology, it suddenly became possible to analyze large amounts of individual cells with respect to multiple parameters and within reasonable time frames (20, 21). Different techniques for immunostaining have been developed to optimize identification of molecules located at the cell surface, in cytoplasmic compartments, or even within the nucleus. First attempts to visualize intracellular cytokines in stimulated T cells were less successful, since these molecules are rapidly secreted and therefore cannot be reliably detected within the producing cell. However, recently activated T cells coincubated with inhibitors of protein secretion, such as monensin or brefeldin A, accumulate proteins they would otherwise secrete, thereby allowing intracellular detection of cytokines at the single cell level (22–24). Further technological advances have made it possible to accumulate secreted proteins at the surface of the secreting cell (25), providing a strategy to purify viable T cells based on their functional (i.e., secretory) properties. Furthermore, conditional reporter genes provide the option to label individual T cells based on their transcriptional activity (26), and phosphoprotein staining can identify recently activated signaling components at the single-cell level (27). Finally, cytometry by time of flight with antibodies labeled with heavy metal isotopes (28), as well as single-cell RNA sequencing (29), allows analysis of T cell diversity in unprecedented detail.

Starting with the identification of distinct cytokine expression patterns of $CD4^+$ T cell clones described as T helper 1 (Th1) and T helper 2 (Th2) by Tim Mosmann and colleagues (30), technologies that provide single-cell resolution have identified many effector and memory T cell subsets. According to their patterns of cytokine production, transcription factor expression, and cell surface marker expression, $CD4^+$ T helper cells are currently subdivided into multiple lineages, encompassing at least Th1, Th2, Th17, follicular T helper (Tfh), and regulatory T (Treg) cells (5). The importance of diversification in the context of immunological memory first became apparent with observations by Federica Sallusto and Antonio Lanzavecchia (31), showing that memory T cells can be subdivided by distinct expression patterns of adhesion molecules and chemokine receptors that—crucially—translate into migratory differences: This seminal work led to categorization of central memory T cells (TCMs), which continuously recirculate—like naive T cells—via the bloodstream to lymphoid organs, and effector memory T cells (TEMs), which preferentially migrate to nonlymphoid tissues (9). The recent identification of tissue-resident memory T cells (TRMs) (32, 33), which might be further subdivided depending on the organ they reside in (34), further adds to the complexity and diversity of the memory T cell compartment.

With the number of T cell subtypes increasing, it becomes a key question how such phenotypic and functional diversity is generated. Is the diversification of T cell fates mainly determined by extrinsic cues provided by a certain tissue microenvironment? Or does it happen autonomously, following a cell intrinsic developmental program? A mechanistic understanding of fate diversification is particularly relevant in those cases where lasting immune protection requires T cells with the same antigen specificity to execute highly distinct functions. One such case is the parallel development of classical T helper subsets that migrate toward inflammatory regions and Tfh cells that remain in secondary lymphoid organs and orchestrate the B cell response (35–38). Another one is the development of long- and short-lived T cells recognizing the same epitope, which guarantees both the transient nature of acute adaptive immune responses as well as the long-term preservation of useful TCR specificities. With recent data suggesting that certain Tfh cells serve as an equivalent to central memory T cells (39, 40), these two diversification processes appear closely related.

In summary, technological advances in phenotypic and functional characterization of T cells at the single T cell level have identified diverse effector and memory T cell subsets. There is also strong evidence that distinct patterns of diversification are required to adapt the quality of protective immunity to different types of pathogens. However, until recently, the mechanisms underlying T cell fate diversification were largely unknown.

FUNDAMENTAL MECHANISMS FOR GENERATION OF T CELL DIVERSITY

Diversification of cellular progeny is a key feature of T cell immunity. It provides specialized T cell subsets that can respond distinctively within shared environments. These distinct cellular responses to shared environmental stimuli may include propensity to die, capacity to proliferate, and ability to perform directed migration or specific effector functions. For the differential survival capacity of memory and short-lived T cells, the selective expression of receptors recognizing homeostatic cytokines such as IL-7 (41, 42) and IL-15 (43–45) is of major importance. Interestingly, distinct immunological stimuli provided through defined vaccinations or infections generate characteristic ratios of T cells expressing or lacking these receptors (46). When T cell responses are subjected to more detailed multivariate analysis on the phenotypic, functional, and transcriptional level, more complex patterns of diversification emerge (47). Although these patterns exceed a dichotomous categorization into long- and short-lived T cells, they, too, are closely determined by the type of immunological stimulus encountered (28, 48). Thus, defined inputs through infection or vaccination generate robust immunological outputs, i.e., predictable patterns of T cell fate diversification. The impact of different factors such as inflammatory cytokines or antigen availability on the shape and size of a fully developed immune response has been studied in great detail (49). But only now are we beginning to resolve how these stimuli are integrated during the earliest phase of the response by individual naive T cells and how, starting out from these smallest units, clonal expansion and generation of diverse T cell fates is regulated.

The concept of clonal selection is compatible with robust immunity emerging from an individual epitope-specific T cell. However, we know today that most immune responses to defined peptide epitopes emerge from considerably more than just a single T cell. This redundancy was first recognized through sequencing studies that suggested a significantly less diverse TCR repertoire than expected (50, 51), which was later confirmed by titration of adoptively transferred TCR-transgenic T cells (52) and direct MHC-multimer-based enrichment of epitope-specific T cells from the naive T cell repertoire of mice (53, 54). Together, these studies show that for $CD4^+$ and $CD8^+$ T cells, between 20 to 200 and 80 to 1,200 naive T cells specific for a given epitope can be found per mouse (55). Finally, it has recently been shown that during systemic infection nearly all of the naive T cells specific to a defined epitope of a bacterial or viral pathogen are effectively recruited into the response (56). Thus, phenotypically and functionally diverse T cell immune responses emerge from multiple naive T cells specific to the same epitope. Relying on these observations, various nonmutually exclusive ways of generating response diversity can be envisioned.

First, distinct fates might emerge only from distinct naive T cells, with one activated T cell giving rise to daughters of only one fate. Alternatively, an individual naive T cell might be capable of generating a diverse T cell family that encompasses all relevant phenotypes characteristic for a functional T cell response (**Figure 1**). These two concepts have been named the "one cell, one fate" and the "one cell, multiple fates" hypotheses (57). In either case, adoption of a certain T cell fate could be determined by cell-intrinsic and/or cell-extrinsic factors. An important intrinsic factor

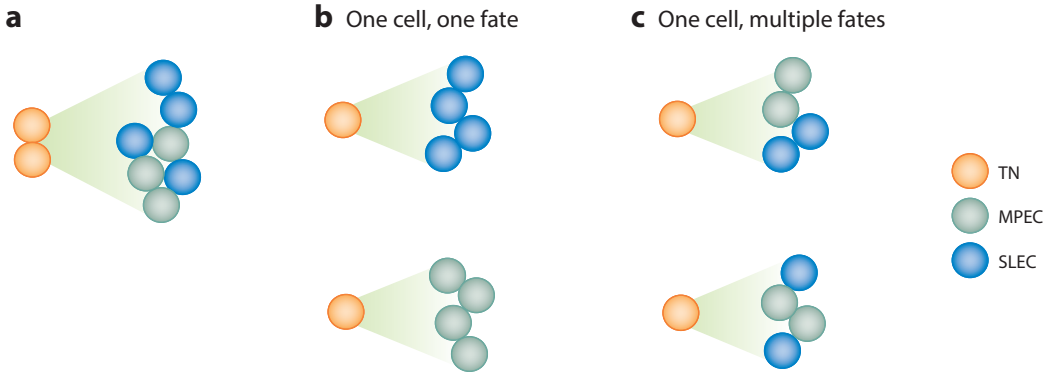

Figure 1

Output of individual T cells. During a T cell response, the majority of naive T cells (TNs) specific for a defined epitope are recruited and induced to proliferate and differentiate. The combined progeny of these naive precursors form a phenotypically and functionally diverse population of effector phase T cells: Some of these cells have a high propensity (are destined) to be long lived and are designated memory precursor effector cells (MPECs), whereas others are destined to die during the contraction phase and are designated short-lived effector cells (SLECs). (*a*) When analyzing T cell responses derived from multiple naive precursors, it is impossible to discern whether individual precursors are (*b*) restricted to generating daughter cells of only one fate (the one cell, one fate hypothesis) or (*c*) capable of generating a diverse progeny (the one cell, multiple fates hypothesis). In vivo fate mapping of single T cells has shown that an individual TN can generate a diverse progeny encompassing long- and short-lived T cells.

that has been shown to influence proliferation and differentiation of TCR transgenic populations of $CD8^+$ (58) and $CD4^+$ T cells (59) is the affinity of their TCR for a given peptide-MHC complex. Whereas TCR affinity is structurally fixed for T cells that carry the same TCR, other intrinsic factors, such as the amount of signaling molecules present in a given T cell, have been shown to vary, potentially creating diverse antigen responsiveness among the members of a clonal population (60). Such cell-intrinsic variation in the abundance of signaling molecules or key transcription factors has been implied as a causative factor in the generation of diverse cell types during embryonic development or during stem cell–based tissue maintenance (61–63). However, it would seem an evolutionarily inferior solution if a T cell response (which, after all, is a dynamic response tailored to a specific infection) were governed exclusively by T cell–intrinsic signals. In fact, many lines of evidence indicate that T cell response size and subset composition are modulated by various extrinsic signals (49). These extrinsic signals can be envisioned as shaping the phenotypic and functional composition of a T cell response by two nonexclusive mechanisms: instruction of activated T cells to adopt a certain phenotype through differentiation, and selection of a certain signal-receptive T cell phenotype to survive, proliferate, or die. Thus, under the one cell, one fate hypothesis, distinct T cell fates could develop owing to distinct instruction provided by an antigen-presenting cell (APC), or owing to selection of individual precursors that possess distinct signal receptiveness. In a one cell, multiple fates scenario, diversification could be induced either through extrinsic signals differentially instructing the expanded progeny of a single cell to adopt distinct fates or through cell-intrinsic processes autonomously generating diverse fates to be subsequently selected by extrinsic signals.

One cannot conclusively test any of these hypotheses by observing the outcome of infection or vaccination induced T cell bursts on the population level. However, such population data do show that on an immune systemic level, expansion and diversification are robustly regulated processes that lead to reproducible response patterns. The mechanistic question remaining is how

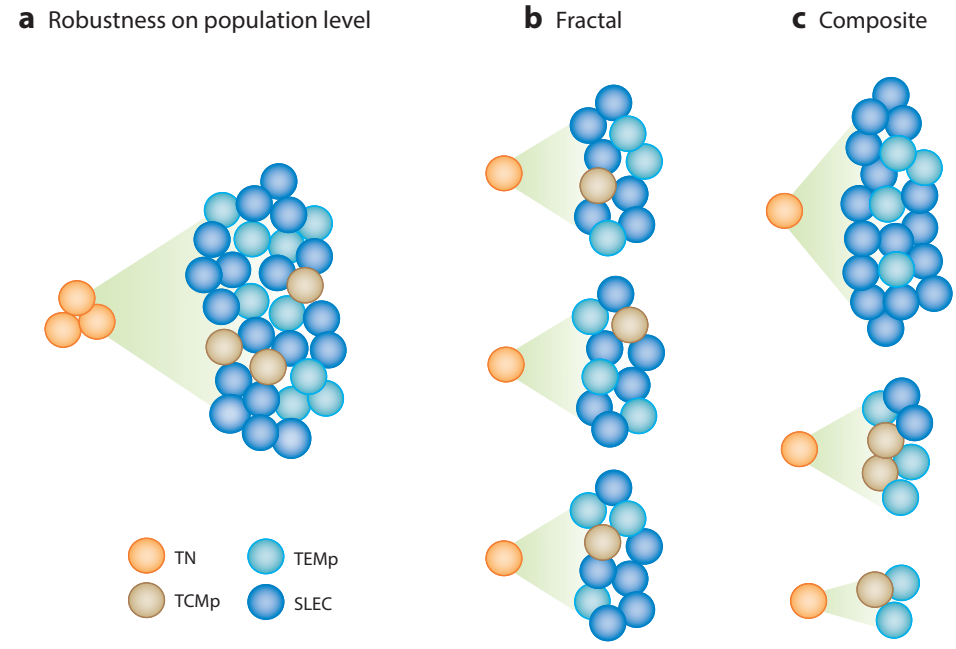

Figure 2

Robustness of T cell responses through population averaging. During the expansion phase of an epitope-specific T cell response, proliferating T cells subdivide into short-lived effector cells (SLECs) and memory precursor effector cells (MPECs). MPECs may be further subdivided into central memory precursors (TCMps) and effector memory precursors (TEMps). In response to a defined infection or vaccination (defined with respect to type, dose, route of entry, etc.), the quantity (i.e., *n*-fold expansion) and quality (i.e., phenotypic and functional composition) of an epitope-specific T cell immune response are robust (i.e., predictable) at the organismal level. (*a*) In this schematic example 3 naive T cells (TNs) generate 30 effector T cells; thus, the overall expansion is tenfold. Qualitatively, the resulting T cell population consists of 10% TCMps, 30% TEMps, and 60% SLECs. Such a robust response pattern could be generated either (*b*) in a fractal manner—meaning that the output derived from a single TN resembles a scaled version of the response derived from the entire epitope-specific population 1 TN generates 10 effector T cells, with 10% TCMps, 30% TEMps, and 60% SLECs)—or (*c*) in a composite manner—where population responses are composed of distinctively acting T cell families whose size and phenotypic composition cannot be inferred by simple downscaling of population-level characteristics (1 TN generates between 3 and 20 effector T cells, with 0–33% TCMps, 20–66% TEMps, and 0–80% SLECs). Studies mapping progeny derived from single $CD8^+$ and $CD4^+$ TNs in vivo have supported a composite structure of T cell immune responses in which robustness is not guaranteed by a single responding T cell but is achieved through population averaging.

this robust regulation is realized at the cellular level. Starting out from individual precursors, regulated diversity could be generated in a fractal manner—meaning that each response derived from a single T cell resembles a scaled version of the response derived from the entire epitope-specific population. Or, it could be generated in a composite manner, where population responses comprise distinctively acting T cell families whose size and phenotypic composition cannot be inferred by simple downscaling of observations made at the population level (**Figure 2**). This issue also touches on the significance of single-cell research in general. If analysis of regulatory processes at the single-cell level only provides scaled versions of population data, the net worth of such data is at best modest. If, however, single T cell behavior cannot be casually inferred from population

data, investigation of single T cell–derived responses will be essential to uncover the precise cellular wiring that transforms an infectious input into an adequate immunological response.

TECHNOLOGIES TO ANALYZE KINSHIP AND FATE

To answer the questions posed in the previous section, kinship between a given naive T cell and its progeny must be established. Especially in the T cell field, defined T cell receptor sequences have often been interpreted as cell-specific barcodes that can be used for fate mapping experiments. However, such experiments usually refer only to one subunit of heterodimeric receptors (e.g., the TCRβ chain), leaving substantial uncertainty regarding the true diversity of the analyzed population. Furthermore, precursor frequencies in the naive repertoire of lymphocytes expressing identical TCR sequences (on the nucleotide level) are often expanded during their development as well as through homeostatic proliferation in the periphery to cell numbers significantly larger than one (7). Thus, kinship cannot be traced unambiguously just by using natural genetic signatures provided by TCR rearrangement, unless exploiting adoptive transfer of cell populations coupled to appropriate repeat-sampling controls that test for repeat occurrence of the same (TCR) barcode (64). As such, studies that are primarily based on this strategy, as is the case for a 2015 study suggesting a shared origin of recirculating and tissue resident memory T cells (65), must be interpreted with some caution. Therefore, additional single-cell identifiers and/ or appropriate lineage tracing controls have to be used to establish kinship on a single-cell level.

The introduction of fluorescence microscopy, and especially two-photon microscopy, has revolutionized the field of in vivo single-cell analyses, allowing the visualization of fluorescently labeled single T cells and analysis of their migration, activation, and expansion. This approach has provided important insights into the early phases of T cell immune responses. For instance, it helped in deciphering how antigen affinity, availability of costimulation, and inflammatory cytokines modulate T cell–dendritic cell (DC) interactions and how migratory behavior of naive and memory T cells is differentially orchestrated within secondary lymphoid organs (10, 66). However, owing to phototoxicity, this approach cannot be used to monitor T cells for more than a few hours, which is only just enough to observe a single T cell division. More importantly, following encounter of antigen, T cells and their progeny migrate actively and regularly escape the field of view, making continuous tracking over extended periods and throughout multiple division events virtually impossible. The Bousso group (67) has tried to combine in situ microscopy with subsequent single-cell sorting and short-term cytokine-driven in vitro expansion to at least partially overcome this limitation and gain first insights into how early events during T cell priming can affect the subsequent fate of a single cell's progeny.

An elegant genetic approach that has been used for in vivo fate mapping is the so-called brainbow technology (68). Here, Cre-mediated excision or inversion of *loxp*-flanked genes encoding different fluorescent proteins enables the tagging of individual cells and their progeny with distinct fluorescent labels (69). Clevers and colleagues have inserted the original *Brainbow 2.0* construct into the *Rosa26* locus together with a strong CAGG promoter and a Cre-flanked neomycin roadblock (70, 71). This *Confetti* construct, which allows Cre-mediated cell type–specific removal of the roadblock and random expression of four distinct colors, has been successfully used to map the fate of individual stem cells in the intestinal crypt (70, 71). Inheritable multicolor labeling can also be achieved through the use of viral vectors, further increasing the flexibility and applicability of this approach (72). These technologies have proven especially useful when progeny cells stay in close proximity to each other within a given tissue (e.g., the gut epithelium or liver tissue). Although first attempts have been made (73), its applicability for mapping kinship among vigorously proliferating and migrating T cells awaits further investigation.

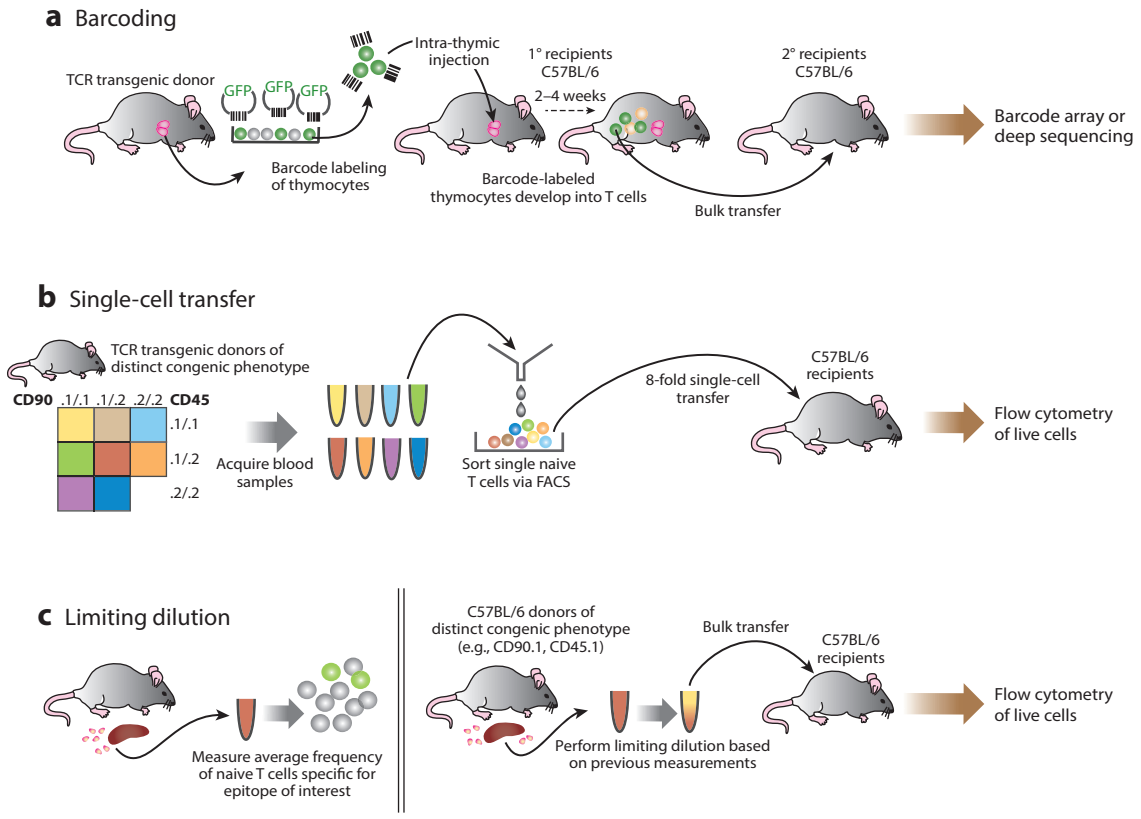

Figure 3

Approaches for in vivo mapping of T cell fate. (*a*) Barcoding: Thymocytes are harvested from a T cell receptor (TCR)-transgenic donor and transduced in vitro with a viral barcode library at a concentration yielding approximately 5–10% transduction efficiency. At this rate of transduction, multiple barcode integrations per thymocyte are very unlikely. Viral constructs also encode for green fluorescent protein (GFP), which allows enrichment of successfully transduced thymocytes via fluorescence-activated cell sorting (FACS). Two to four weeks after reintroduction of transduced thymocytes via intrathymic injection into first-degree recipients, mature naive barcoded T cells are harvested from peripheral lymphatic organs and transferred in bulk (with other spleen cells) into secondary recipients. Abundance of single-cell-derived T cell families is evaluated quantitatively by deep sequencing. Fate mapping controls are included to ensure analysis measures the output of single cells. (*b*) Single–T cell transfer: Naive T cells are harvested from peripheral blood of a set of TCR-transgenic donors that express eight distinct congenic phenotypes (generated by breeding codominantly expressed congenic markers CD90.1/.2 and CD45.1/.2 in a congenic matrix). Eight individual epitope-specific T cells of distinct congenic phenotype are assembled by flow-cytometric single-cell sorting and transferred into C57BL/6 recipients. Detection of single-cell-derived T cell families is based on congenic marker expression. Phenotypic and functional analysis is directly performed via flow cytometry of living cells. (*c*) Limiting dilution: Adoptive transfer of what are likely to be single epitope-specific endogenous T cells is performed by first measuring the frequency of naive T cells specific to a certain epitope. Following the assumption that this frequency is constant in MHC-identical individuals, limiting dilution is performed based on these previous measurements. Transfers are performed in bulk, and detection of progeny is based on congenic markers.

To combine the power and flexibility of viral vector–mediated cell labeling with precise detection methods, genetic cell barcoding approaches have been developed (74, 75) (**Figure 3**). Here, T cell populations are transduced with a library (e.g., containing thousands of unique sequences) of integrating vectors (retrovirus based) that transduce single cells with a unique heritable identifier (barcode). Barcode-labeled cells can be TCR-transgenic T cells, and provided barcode labeling

is carried out at the thymocyte state, truly naive barcoded T cells can be generated in vivo. Following T cell activation and expansion, barcode regions can be amplified by PCR, followed by subsequent identification of individual markers by microarray hybridization (56, 74, 75) or next generation sequencing (76). The major advantage of this technology is the capability to analyze large numbers of barcoded T cell families simultaneously. Extensive titration and quality control experiments have demonstrated that this experimental system can be calibrated to a level where the overwhelming majority of barcode labels are restricted to single T cells (64). A disadvantage of the barcoding approach is that identification of the barcode requires indirect procedures (microarray, sequencing) that do not allow direct antibody costaining, cell isolation, or functional analyses of progeny derived from the same precursor.

To overcome some experimental hurdles of genetic cellular barcoding, adoptive transfer of single T cells that carry congenic markers distinct from the recipient has been developed (77, 78) (**Figure 3**). Originally, this approach was performed by picking single $CD8^+$ TCR-transgenic naive T cells via a syringe under microscopic control and then adoptively transferring them to immunocompetent recipients (77). Subsequent in vivo antigen challenge can expand single cell–derived T cell families to sizes that are readily detectable by rare-event flow cytometry (acquisition of approximately 10–50 million events). A major advantage of this challenging technology is that T cell families can be directly visualized via surface expression of the congenic marker, allowing further phenotypic as well as functional analysis and, if required, the sorting and retransfer of single T cells into secondary recipients (79). A major drawback is that only one transferred T cell can be analyzed per recipient. To increase the number of T cell families successfully recovered and to be able to analyze multiple T cell families within the same recipient, a matrix of coexpressed congenic markers (based on CD90.1/.2 and CD45.1/.2) has been bred onto TCR-transgenic donor mice. This currently allows the transfer of up to eight T cells that can be distinguished from each other as well as from recipient cells by their distinct congenic marker expression, thus substantially improving the power of adoptive single-cell transfer experiments by making even rare outcomes of single T cell–derived responses readily detectable (78).

The transfer of single congenic T cells or of T cells individualized via genetic barcoding is the most convincing experimental setup for in vivo fate mapping of T cell families. In addition, adoptive transfer of very low numbers of polyclonal T cell populations has been used with the aim of reaching single-cell resolution (80, 81) (**Figure 3**). Although it is mathematically plausible that limiting dilution does result in a certain frequency of single-cell transfers, with this procedure it is difficult to know whether this really has been accomplished.

Adoptive transfer of single T cells with heritable genetic barcodes or congenic surface markers is a powerful technology to study kinship and fate of expanding and differentiating T cells. However, both methods are restricted to the analysis of defined time points and provide single-cell resolution of fate only with respect to the founding ancestor (82, 83). Because of this, these technologies are not suited to fully resolve the branching process of a growing T cell family in vivo. However, recent developments have made it feasible to continuously image genealogical trees derived from single precursor cells (such as pluripotent hematopoietic stem cells) in vitro (84–86). First attempts for long-term imaging of lymphocyte fate decisions in vitro have been made (87, 88), and it is important to resolve to what extent these data can be translated to in vivo scenarios.

INSIGHTS INTO PRIMARY T CELL IMMUNE RESPONSES FROM IN VIVO LINEAGE TRACING

Population analyses clearly show that, on the one hand, acquisition of different effector functions and the transcriptional changes underlying them already are apparent early during the expansion

phase of a primary T cell immune response (89, 90). On the other hand, key memory features, such as the capacity for renewed expansion upon secondary antigen encounter, appear only later during the contraction phase (11). Furthermore, studies using genetic reporter systems that lead to heritable labeling of $CD8^+$ and $CD4^+$ T cells expressing the effector molecules granzyme-B and IFN-γ, respectively, have shown that expression of these effector molecules at some point during ontogeny is not obstructive to the development of memory T cells that have the potential to expand during recall responses (26, 91, 92). The discovery that in $CD8^+$ T cells the expression of IFN-γ and of the IL-15 receptor (IL-15R), which is important for maintenance of memory T cells, is induced by the same transcription factors, T-bet and Eomes (93), further supports the notion that effector and memory fates are closely coupled. From these data, it has been concluded that activated T cells can acquire effector functions and differentiate into short-lived or memory T cells. But when during the highly dynamic expansion of an epitope-specific T cell population is this decision made? The acquisition of memory function toward the tail end of the primary response does not have to coincide with the ontological decision to commit to a long- or short-lived fate. It is, however, the timing of this ontological decision and its consequences for further cell proliferation that need to be defined in order to better understand and effectively manipulate the development of memory T cells. In this section we showcase insights into this decision-making process that were gained through in vivo fate mapping of single T cells and discuss how these insights have influenced our models of T cell fate diversification and memory T cell ontogeny.

One Cell, One Fate or One Cell, Multiple Fates

A very basic question concerning memory T cell development is whether long- and short-lived T cells can be derived from the same T cell or must be generated by distinct ones. Lineage development of a T cell could already be determined before its priming or through an instructive priming signal, to which all daughter cells adhere. There is indeed evidence showing that extensive self-peptide–MHC signaling can alter responsiveness to cognate antigen (94–97). Furthermore, TCR repertoire analyses in distinct memory and effector T cell subsets have shown somewhat discordant repertoires but also identified clonotypes shared between subsets (98, 99). Considering the technical limitations of TCR-based fate mapping approaches mentioned above, these findings cannot be interpreted conclusively.

The first study that genuinely investigated the fate of individual T cells and their progeny in vivo was based on the adoptive transfer of single $CD44^{low}$ and thereby phenotypically naive $CD8^+$ T cells expressing a TCR transgene specific to the SIINFEKL epitope of chicken ovalbumin (OVA) and the congenic marker CD45.1 (77). Directly after adoptive transfer of individual OTI $CD45.1^+$ T cells, immunocompetent C57BL/6 recipients were infected with *Listeria monocytogenes* expressing OVA (*L.m.*-OVA). This bacterial pathogen selectively targets $CD8\alpha^+$ DCs (100, 101), which are highly efficient in activating $CD8^+$ T cells (102, 103). Analysis at peak expansion and memory time points revealed detectable single-cell-derived T cell populations in secondary lymphoid and peripheral organs, showing phenotypic (according to surface markers CD62L and CD127) and functional (according to secretion of IL-2, TNF-α, and IFN-γ) diversity. In addition, single T cell–derived families detected at peak expansion were adoptively transferred to naive secondary recipients and, after a resting period of five weeks in the absence of antigen, were successfully reexpanded into phenotypically and functionally diverse secondary populations. In a subsequent study, a barcode library was introduced via retroviral vectors into OTI thymocytes, which subsequently were transplanted into first-degree hosts by intrathymic injection (75). Developing mature naive T cells, whose thymic precursors had been successfully barcode labeled, were then transferred into secondary C57BL/6 recipients, in which in vivo fate mapping was

conducted. These experiments showed that barcodes detected at peak expansion in OTI T cells responding to infection with *L.m.*-OVA substantially overlap with barcodes detected at memory time points or after recall expansion in the same hosts. This was also observed after infection with *L. monocytogenes* expressing altered peptide ligands that bind to the OTI TCR with lower affinity. Together, these studies show that the fate of individual T cells and their progeny can be successfully mapped in vivo and that individual T cells are in principle capable of generating a diverse offspring encompassing phenotypically and functionally distinct effector and memory T cells. Thus, diverse T cell fates can arise within the progeny of a single cell, lending support to the one cell, multiple fates hypothesis (**Figure 1**). But when during the expansion of a single-cell-derived T cell family are the relevant decisions to diversify induced?

Early, Late, or Continuous Fate Determination

The traditional linear differentiation model of memory T cell development proposes that the ontological decision to diversify into short- and long-lived T cells happens at the onset of contraction, after population expansion has reached its maximum (104, 105). This idea has been challenged by various recent discoveries. For example, it has been shown by adoptive transfer studies that within an activated effector T cell population, memory precursor effector cells (MPECs) with a high propensity to become functional memory T cells can be discerned from short-lived effector cells (SLECs) well before onset of contraction (89). In $CD8^+$ T cells, the cell surface expression of markers such as CD127, KLRG1, or CD27 segregate MPECs from SLECs as early as day four after infection with various pathogens (41, 42, 89, 106). Although less is known about CD4 memory precursors, studies suggest that a similar subdivision can be made using markers such as PSGL-1, Ly6c, CXCR5, and PD1 (40, 107). It has further been shown that inflammatory cytokines, mainly IL-12 and type I interferons, selectively drive SLEC expansion by prolonging their responsiveness to IL-2 (108). Depletion of inflammatory cytokines, however, will lead to diminished SLEC numbers, reduced contraction, and (at the population level) accelerated acquisition of recall capacity (109, 110). Abrogation of antigen exposure between days 3 and 5 after priming will also decrease the fraction of SLECs present at peak expansion (90). These and other observations support the decreasing-potential model of memory T cell differentiation, which proposes continuous integration of antigen stimulation as well as costimulatory and inflammatory signals by the expanding T cell population. Conceptually, this model relies on the initial priming event chiefly for inducing proliferation, whereas fate diversity is subsequently generated through instructive signals that shape the growing T cell population by the differentiation of long-lived precursors into various shorter-lived descendants (**Figure 4**) (11, 104, 111). While proposing a similar developmental order, with long-lived memory precursors differentiating into shorter-lived descendants, the progressive-differentiation model puts substantially more emphasis on the fate-determining role of the initial priming event. Here, signal strength—an integrated measure of antigen, costimulation, and inflammatory cytokines available on the surface or in the vicinity of the priming DC—is thought to substantially influence the proliferation and differentiation of a primed T cell and its progeny (**Figure 4**) (112, 113). Recent in vivo imaging studies have lent some support for this idea by showing that the expression of IFN-γ strongly differs among individual premitotic $CD8^+$ T cells having been exposed to peptide pulsed DCs (67). To fully decipher the influence of the initial priming event and further modulatory signals on a response derived from a single T cell, one would have to monitor all the division, differentiation, and signaling events that the members of the expanding T cell family encounter. Although this is still out of reach, single–T cell fate mapping approaches have contributed key insights that put renewed focus on the earliest events of a T cell immune response.

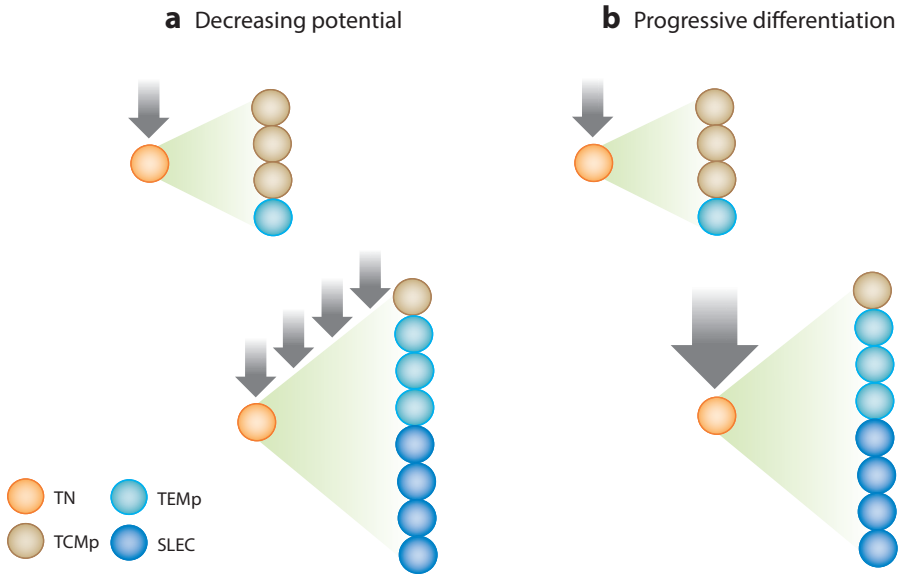

Figure 4

Timing of fate decisions. Both the decreasing potential model and the progressive differentiation model of memory T cell development suggest that T cells receiving strong cumulative signals (via antigen, costimulatory molecules, and inflammatory cytokines) yield progeny that is more likely to have a short-lived fate. However, the two models differ in how signals are suggested to accumulate: (*a*) In the decreasing potential model, signal accumulation is suggested to occur continuously during the expansion phase. T cell families whose members have received more signals over time will contain more short-lived effector cells (SLECs) than those that have received fewer signals. (*b*) According to the progressive differentiation model, signal integration occurs primarily during initial T cell priming and before substantial proliferation has occurred. T cell families derived from a naive T cell (TN) that received a strong signal at priming will contain more SLECs than T cell families derived from a TN that received a weak signal at priming. Abbreviations: TCMp, central memory T cell precursor; TEMp, effector memory T cell precursor.

Stochastic Events and Population Robustness

Single–T cell fate mapping is particularly suited for investigating the ontological aspects of memory development, owing to its capacity to provide a continuing link between the priming of individual cells and their response patterns days and months later. Two studies using TCR-transgenic OTI T cells, with either a multiplexed adoptive transfer approach utilizing multiple congenic marker combinations to track up to eight single-cell-derived responses in vivo (78) or viral barcoding combined with quantification of barcode frequency via deep sequencing (76), generated complementary results. Both studies showed that responses derived from single $CD8^+$ T cells are characterized by immense variation in proliferative output. Despite harboring the same TCR specificity, single naive OTI T cells generated between 200 and 200,000 daughter cells at peak expansion, with a median burst size of roughly 4,000 T cells, after infection with *L.m.*-OVA. Whereas median burst size increased or decreased depending on the type and dose of infection, variability of individual family sizes remained consistently high in responses against various OVA-expressing pathogens, as well as *L. monocytogenes* expressing lower-affinity peptide ligands of SIINFEKL (76, 78). In addition, substantial covariation between a T cell family's size and its surface marker phenotype (76, 78), cytokine secretion capacity, and transcription factor profile (78) was found. High proliferative

output at peak expansion was connected with high numbers of SLECs, whereas smaller families contained a higher fraction of $CD27^+$ $KLRG1^-$ $T\text{-}bet^{lo}$ $Eomes^{hi}$ MPECs. MPECs could be further subdivided into $CD62L^-$ $IL2^-$ effector memory precursors (TEMps) and $CD62L^+$ $IL2^+$ central memory precursors (TCMps)—the latter being particularly enriched in small families (76, 78). Thus, immune responses derived from single T cells are subjected to substantial stochastic variation. This variation, however, occurs within a developmental framework in which strong proliferative expansion appears tied to terminal differentiation and loss of memory capacity. How this variation is induced is unknown. Even adoptive transfer of latecomer $CD8^+$ T cells three days after onset of infection did not substantially reduce response variability (76, 78), thereby arguing against the timing of single-cell recruitment as a key aspect. An interesting observation made in these studies is that whereas a T cell family's size is generally a good predictor of its differentiation state, T cell families similar in size can differ strongly in the fraction of progeny that express, for instance, CD27. This observation appears most consistent with a model in which early stochastic variation or factors encountered during or shortly after priming determine cell fate many generations later, as homogeneous differentiation of large single-cell-derived T cell families is statistically highly improbable, unless this differentiation is imprinted or sister cells are forced to encounter similar signals (see below) (76, 78). Thus, these single–T cell fate mapping studies suggest that early events create substantial variation in subsequent family size and phenotype. We propose that occurrence of these events on a single–T cell level should be regarded as stochastic in the sense that it cannot be predicted (before onset of the immune response) which event or succession of events a single T cell will encounter. As in drawing colored balls from an urn (a classical thought experiment in probability theory), the result of a single draw is highly variable, but as the number of draws increases, the result becomes more predictable and is more closely related to the color distribution within the urn. Thus, we do not imply that the immune system acts randomly; rather, it robustly integrates a stochastic pattern of extrinsic infection-associated signals and/or of T cell–intrinsic factors through population averaging. Given that key fate-determining events seem to occur before T cell families have substantially expanded, a sufficient number of naive T cells specific to a given epitope must be provided to guarantee successful pattern integration. Using adoptive transfer of what can be presumed to be single-epitope-specific $CD4^+$ (80) and $CD8^+$ T cells (81), two other studies showed that strong quantitative and qualitative variability of single-cell-derived responses is also present within the endogenous polyclonal TCR repertoire. Interestingly, one of these studies has provided evidence that for $CD4^+$ T cells, TCR affinity plays an important role in modulating the subset composition within a T cell family (80). How substantial the effect of TCR affinity is, relative to effects determined by the antigen and costimulatory makeup of the priming APC and the inflammatory signals provided by the microenvironment, remains to be determined. Taken together, these studies indicate that the robustness of immune response quantity (i.e., burst size) and quality (i.e., functional diversity) at the organismal level requires recruitment and activation of multiple naive T cells specific for the same epitope. Thus, robustness of T cell responses is generated in a composite manner (**Figure 2**). In line with this, Jenkins and colleagues (80) demonstrated that responses originating from smaller epitope-specific $CD4^+$ endogenous T cell populations were substantially more variable in size and phenotypic composition than those originating from large ones. This finding is particularly interesting in the context of TCR repertoire attrition observed in aged individuals (114) and in the context of the (presumably) low frequency of T cells specific for tumor-associated self-antigens (115). Specifically, it suggests that a failure to generate effective epitope-specific T cell immunity in such settings may not only be due to complete absence of a certain epitope specificities within the TCR repertoire (116) but may also be due to an insufficient capacity of a dwindling number of naive precursors to average out fate-determining stochastic variations.

STEM CELL PROPERTIES IN THE MEMORY T CELL POOL

Slow and Fast Cyclers

The size of a $CD8^+$ T cell family at peak expansion correlates inversely with the prevalence of family members expressing memory markers (e.g., CD62L, IL-2, and Eomes) and positively with the fraction of family members expressing markers of terminal differentiation (e.g., KLRG-1 and T-bet) (76, 78). Mechanistically, these observations are consistent either with division driving differentiation or with differentiation driving division; that is, either a T cell that has divided is more likely to differentiate from a TCMp into a shorter-lived T cell, or a shorter-lived T cell will divide more than the TCMp it has differentiated from (**Figure 5**). The first hypothesis is supported by data from the B cell field. An elegant in vitro study showed that the number of divisions that an individual B cell goes through under homogeneous priming conditions varies stochastically and closely determines the ensuing progeny's fate in that isotype switching and plasmablast differentiation become more likely in later generations (87). For $CD8^+$ T cells a mathematical model based on such division-linked differentiation could replicate the successive loss of CD62L expression observed in expanding T cell populations in vivo (117, 118). However, other in vivo experiments suggest that proliferating $CD8^+$ T cell populations segregate into quicker- and slower-cycling subsets early during the expansion phase. This process is thought to be driven by continued expression of the high-affinity IL2-R α-chain on rapidly cycling cells (119). These cells also lose CD62L expression and capacity for IL-2 production and do not progress into the memory phase (119, 120). In addition, the transcription factor Foxo1, which induces Eomes, suppresses T-bet, and supports TCM development (121, 122), is known to prevent cell

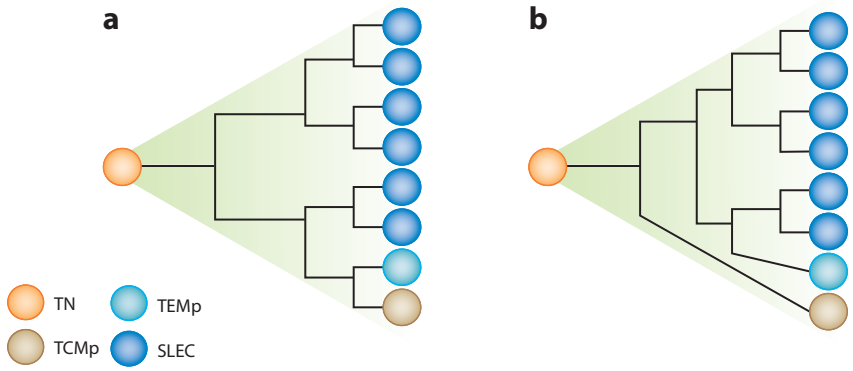

Figure 5

Branching points in T cell differentiation. Single–T cell fate mapping shows that the size of a T cell family and the degree to which its members acquire a short-lived fate are positively correlated. Two nonmutually exclusive mechanisms could underlie this observation: (*a*) The hypothesis of "division driving differentiation" proposes that the likelihood of differentiation into a shorter-lived subset increases with each generation of division. According to this hypothesis, the genealogical tree grows symmetrically unless additional stochastic components are assumed. Central memory T cell precursors (TCMps), effector memory T cell precursors (TEMps), and short-lived effector cells (SLECs) have gone through the same number of divisions and thereby display a shared replicative history. (*b*) The hypothesis of "differentiation driving division" proposes that the likelihood of dividing increases with differentiation into a shorter-lived subset. According to this hypothesis, the genealogical tree grows asymmetrically. TCMps, TEMps, and SLECs have gone through increasing numbers of divisions and thereby display distinct replicative histories.

cycle progression (123). Interestingly, a computational model derived from in vivo fate mapping data of single $CD8^+$ T cells generates highly adequate simulations of primary and recall immune responses based on a progressive differentiation framework. According to this framework, naive T cells first differentiate into slow-cycling TCMps and then via more rapidly cycling TEMps into fast-cycling SLECs (78). Another recent study, in which intracellular dye dilution was combined with continuous in vitro imaging of up to three T cell divisions, suggests that such fate decisions occur with some delay. It showed that T cells sorted from the first- or third-division peak and then further monitored in vitro continue to divide at similar speeds, whereas cells taken from the eighth-division peak (generations between three and eight were not examined) heritably segregate into slow and fast cyclers (88). Although the in vivo relevance of these data remains to be determined, one might speculate that T cell expansion is divided into two phases, phase one being governed by quick and homogeneous proliferation of all developing daughters and phase two then showing slow proliferation (or frequent cell cycle dropout) of memory precursors and cytokine-driven rapid proliferation (or continued cell cycling) of shorter-lived T cells. However, the immense variability observed in responses derived from single T cells is not easily compatible with fate decisions occurring only after eight divisions. Specifically, if fate determination were to occur so late, why would the 256 daughters (i.e., 2^8 daughters) of a single T cell behave so differently from the 256 daughters of another? One could propose that the members of an expanding T cell family are confined for an extended period to a certain microanatomical region, preventing an averaging out of fate-determining signals and thus yielding relatively homogeneous behaviors of cells within an individual T cell family. Alternatively, extrinsic signals or cell-intrinsic signal fluctuations could determine T cell fate before substantial proliferation occurs but only lead to palpable consequences multiple generations later. Recently, first pieces of evidence for the latter hypothesis have been provided at the population level by a study showing that early IL-12 signaling leads to late effects on SLEC expansion through the extended expression of IL-2Rα (108). Further testing of these hypotheses at the single-cell level in vivo will be an exciting task. In summary, multiple lines of evidence suggest that $CD62L^+$ memory precursors present at peak expansion have a shorter replicative history than their terminally differentiated relatives. Given that TCM cells are thought to be of prime importance for recall expansion and lasting immunity, curbing their proliferation during the acute response phase could be a means of reducing their long-term risk for replicative senescence.

The Role of Asymmetric Cell Division in Determining T Cell Fate

Single–T cell fate mapping studies support the idea that early stochastic events are of major importance for determining the fate of a T cell's progeny. They suggest that response robustness is not reached at the level of an individual responding cell but at the level of a responding population of naive epitope-specific T cells. The critical question is whether variation in responses derived from single T cells goes so far as to include a fraction of failed responses (e.g., T cell families not containing memory cells) or whether certain deterministic mechanisms hardwired into the biological machinery of every T cell regularly prevent such failure (124, 125). Asymmetric cell division could provide such a deterministic mechanism that guarantees for generation of long- and short-lived daughters at the immediate onset of T cell immune responses. This concept, which was first introduced into T cell biology through a seminal paper by Reiner and colleagues (126), proposes that if signaling molecules and certain components of the intracellular machinery such as the proteasome (127) are focused close or opposite to the immunological synapse formed during T cell–APC interaction, the subsequent division will generate two distinct daughter cells: a proximal cell outfitted with effector function and destined for a short-lived fate, and a distal

sister destined to provide memory. Further T cell–APC encounters, which could lead to renewed asymmetric partitioning of cellular content and signaling machinery, have been suggested to introduce further diversification. Asymmetric cell division has also been proposed to occur after priming of TCM (128) and is thought to be preferentially induced by high-affinity TCR binding (129). Current in vivo fate mapping studies have not examined asymmetric cell division directly. However, computational modeling has shown that strict asymmetric division is not compatible with the observed outcomes in one of these studies (130). Importantly, early segregation of memory and effector fates, as supported by work investigating the single-cell expression profile of activated $CD8^+$ T cells shortly after onset of division (131), is not by itself evidence for asymmetric cell division. Fate segregation could be realized without asymmetric cell division—through either a cell-intrinsic mechanism, such as division-linked differentiation, or a mechanism based on extrinsic signals that differentially instruct the members of an expanding progeny to adopt long- or short-lived fates. In addition, the complete loss of $CD62L^+$ daughters in approximately 20% of single-cell-derived T cell families, as well as the finding that 20% of single-cell-derived T cell families detectable in peripheral blood at peak expansion could not be recovered at memory time points (despite whole-organ analysis of spleen, lymph nodes, and bone marrow) (79), argues against a mechanism that stably guarantees every recruited T cell to generate memory. Interestingly, similar observations have been made for stem cell–based homeostatic maintenance of the intestinal epithelium (70). Here it was shown that clonal diversity of fluorescently labeled crypt stem cells substantially decreased over time, with some clones disappearing and others dominating the crypt. Whether such neutral drift dynamics (71) also govern the T cell response will be a fascinating issue to explore, especially in the context of chronic infection. To better understand the role of asymmetric cell division for T cell fate determination, it would be of interest to interfere with the cellular machinery required for it. Previously, such efforts—potentially due to the redundancy of the asymmetric division machinery—influenced neither B nor T cell differentiation (132). Another approach to unambiguously investigate the fate-determining impact of asymmetric division would be to distinctively and heritably label the proximal and distal cells derived from an asymmetric division and map the long-term fate of their respective offspring in vivo. An in vitro study that investigated the fate of the first two daughters derived from a DC-primed $CD8^+$ T cell found that under limiting culture conditions, development of phenotypically distinct subfamilies derived from daughter one versus daughter two was an exception rather than the rule (133). Performance of such sibling studies in an in vivo setting, as was performed for hematopoietic stem cells, is an interesting challenge (134).

Searching for a T Memory Stem Cell

A proliferative hierarchy such as the one described above is reminiscent of precursor-progeny dynamics described in other tissues with high cellular turnover (135). In the intestinal epithelium, the skin, or the hematopoietic system, slowly cycling so-called tissue stem cells replenish the shorter-lived and more rapidly cycling cellular layers (136). They do so by self-renewing division and multipotent differentiation into a diverse offspring. Kept in a dynamic balance, these two key features of a stem cell guarantee the homeostatic maintenance of the tissue (137). By analogy, a T cell immune response could be described as the outgrowth of an epitope-specific tissue. After thymic involution, this tissue must be intermittently reexpanded (in the case of acute reinfection) or even persistently maintained (in the case of chronic infection), independent of relevant cellular influx from the thymus (138, 139). Thus, it is tempting to speculate that this maintenance is orchestrated in a stem cell–like fashion by a subset of T cells that can self-renew and generate a functionally diverse offspring of short-lived daughter cells.

The operational stem cell criteria of multipotency and self-renewal can only be tested by evaluating the cellular output, i.e., the clonogenic potential, of a single putative stem cell (140). Such assays were first realized in the hematopoietic field by Till and McCulloch, who used limiting dilution transfer of bone marrow aliquots into lethally irradiated recipients to test for the presence of self-renewing hematopoietic stem cells (141, 142). More recent genuine single-cell transfers from a phenotypically defined compartment of hematopoietic precursors suspected to contain tissue stem cells indeed demonstrated successful reconstitution of all hematopoietic lineages (143, 144). Single-cell-based clonogenic assays have since then become a mainstay in stem cell research (145), and demonstration of stem cell capacity is now generally assumed to require serial clonogenic assays: This is because self-renewal of a single putative stem cell is only formally demonstrated when a single daughter of this putative stem cell displays similar multipotency as its mother in generating a phenotypically and functionally diverse offspring.

With regard to lymphocyte biology, the stem cell hypothesis of T and B cell memory, as proposed by Fearon and colleagues (146), has received substantial attention. Stem cell–like genetic profiles were found in certain memory T cells, and various putative memory stem cell subsets were identified (147). While the term stem cell–like has been used quite extensively for certain $CD4^+$ and $CD8^+$ T cell memory subsets, only a few studies have performed the clonogenic assays that are required to corroborate the T cell stemness they propose.

In a recent study, single congenic $CD62L^+$ TCMs were subjected to repetitive adoptive transfer and infection-driven reexpansion in the acute murine *L. monocytogenes* infection model (**Figure 6**) (79). This study found that single TCMs show similar expansion and diversification capacity as

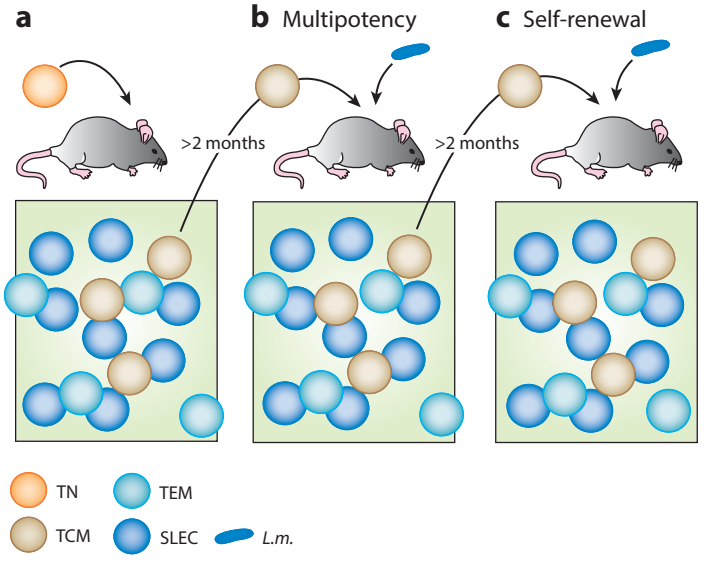

Figure 6

Stem cell properties within the memory T cell compartment. Individual $CD8^+$ T cells within the central memory T cell (TCM) compartment of mice fulfill the operational criteria for defining tissue stem cells: (*a*) Single naive T cells (TNs) transferred into immunocompetent C57BL/6 recipients and exposed to bacterial infection can generate a diverse progeny. (*b*) Multipotency of TCMs: Single primary TCMs can mount a diverse secondary immune response. (*c*) Self-renewal of TCMs: Single secondary TCMs derived from single primary TCMs can mount a diverse tertiary immune response. At this stage of the experiment, self-renewal of single primary TCMs is shown. Note that a stringent assessment of memory T cell self-renewal requires at least two successive single-cell transfers.

single naive T cells and clearly surpass TEMs in terms of multipotency and self-renewal capacity. Whereas even 100 TEMs could not be retransferred and reexpanded more than once, single TCMs could be subjected to this procedure two times consecutively and in a third ten-cell transfer retained full capacity for reconstituting protective immunity to severely immunocompromised hosts. Given that secondary transfers of hematopoietic stem cells have been conducted only by transplantation of multicellular bone marrow aliquots (148), this is to our knowledge the first description of a serial adoptive single-cell transfer of any mammalian cell type. These observations together with the finding that single TCMs could generate phenotypically and functionally diverse offspring show for the first time that cells derived from the TCM compartment fulfill all key requirements for defining a tissue stem cell. Importantly, the stochastic response variability observed for single naive T cells appears to be equally present in T cell responses derived from single TCMs (79). This argues that robust stem cell–like maintenance of T cell memory is guaranteed by population averaging rather than through single-cell-based deterministic mechanisms. Apart from TCMs, a newly defined naive-like subset of memory T cells detected at only very low frequencies and identified by expression of CXCR3 and CD122 but lack of memory marker CD44 in mice (149, 150) and expression of CD95 but lack of memory marker CD45RO in humans (151) has shown superior expansion capacity compared to TCMs in certain settings of graft-versus-host (GVH) disease and cancer immunity (149, 151). Accordingly, these cells have been termed T memory stem cells (TSCMs). As serial single-cell transfers show, they must share this status with the much more abundant TCMs, at least in mice. Furthermore, despite the name TSCM implying stemness, a stringent analysis of this subset's multipotency and self-renewal capacity by clonogenic assays is lacking. Until these data are provided, it remains in question whether this subset is truly a self-renewing, physiological part of T cell memory or a powerful but transient cellular state in the differentiation path from naive to stably maintained TCMs.

CALL FOR NEW TECHNOLOGIES

In vivo single-cell fate mapping by genetic barcoding or single-cell transfer has provided fascinating new insight into the dynamics and regulation of T cell responses. However, further technological advances are much needed. For example, at this moment it is not possible to directly correlate distinct characteristics or signals that an individual T cell acquired during the expansion and differentiation process with its subsequent fate. This is due to the fact that we obtain only snapshots for distinct time points during the immune response. Continuous monitoring of single or low numbers of defined cells throughout the entire body of a mammalian organism would be ideal, but the high migratory capacity of T cells makes the development of such approaches a considerable challenge. Within the field of stem cell research, in vitro bioimaging approaches for long-term single-cell observations via multiparameter real-time microscopy have been applied to obtain first continuous tracing data and have provided fascinating novel insights into fate decisions and differentiation processes (84–86). These techniques might also be applicable to the analysis of T cell fate. Although in vitro systems are reductionist models compared to the physiological complexity in vivo, such analyses can help to obtain basic parameters on the dynamics of T cell expansion and differentiation (e.g., cell division rates, exact timing of subset generation and acquisition of distinct effector functions or cell death). Furthermore, in vitro settings facilitate manipulation of defined factors and/or the implementation of genetic reporters to gain deeper mechanistic insights (86).

The integration of stable heritable reporters via conditional Cre expression could become a powerful tool in combination with barcoding and adoptive single-cell transfer technologies (64, 145, 152). This could allow one to address questions regarding the influence of defined prior

signals on subsequent T cell fates. However, suitable reporter signals need to be strong and durable enough to guarantee a high and constant yield of cell labeling, and experimental variability would severely complicate the interpretation of such experiments.

It might also be possible to combine single-cell fate mapping with in situ microscopy and subsequent in situ single-cell labeling. The rapidly growing field of optogenetics is especially likely to provide interesting tools to realize such experiments (153). This would allow the monitoring of individual T cells during a crucial time period in situ (e.g., during interaction with a priming DC) while following their progeny's subsequent fate throughout different tissues.

Genetic barcoding and single-cell transfer for fate mapping experiments require adoptive transfer of individually traceable T cells. Depending on subtype, time after in vivo activation, or tissue type, T cells might tolerate this experimental procedure, which is often combined with tissue extraction and subsequent cell labeling and isolation, to a variable degree. One way to cope with this situation is to introduce single-cell labels without the need for adoptive transfer, as was performed to investigate the contribution of individual HSCs to homeostatic maintenance of the unperturbed hematopoietic compartment (154). A second way is to perform cell isolation and reintroduction as gently as possible. During isolation of cell populations, surface-bound antibodies, used for T cell lineage and/or subset identification, can interfere with in vivo functionality or maintenance of transferred T cells. T cell isolation procedures that keep processing times as short as possible and minimize stress on cell specimens will therefore become increasingly important. Reversible cell labeling that allows complete removal of the surface-bound label from the purified cell population of interest might be of specific value in this context. This can be achieved by multimerized low-affinity Fab fragments. In its multimeric state, such a reagent can stably bind to surface-expressed antigens. As soon as the multimeric complex is disrupted, which can be efficiently achieved by addition of suitable binding competitors, monomerized low-affinity Fab fragments dissociate from the cell surface and can be washed away (155). In this way, highly purified minimally manipulated T cells can be generated.

For in vivo single-cell fate mapping experiments, it would be highly valuable to further add individual barcodes to daughter cells during expansion and differentiation within a developing family tree. Cumulative genetic diversification of barcodes would then allow the reconstruction of kinship not only of ancestor and offspring, but also of close or more distant relatives within the overall T cell family. Such dynamic barcoding could also serve as a "division logbook," providing a per cell measure for past replicative activity. By analogy, this could identify differentially growing branches of the family tree (83). Suitable structures for the generation of such division barcodes might be microsatellite sequences (156) or gene segments that can be randomly rearranged (like VDJ segments from the TCR or immunoglobulin locus) by expression of recombination-activating gene (RAG) proteins made inducible by cell cycle–controlling factors (83).

Overall, we have significant technical hurdles to overcome, but exciting new technological options could bring us closer to fully understanding the kinships and fate decisions shaping the genealogical trees that sprout and grow and maybe sometimes wither during a T cell–mediated immune response.

BIOMEDICAL USE

Single-cell in vivo fate mapping has uncovered unexpected details about the generation and maintenance of highly diverse antigen-specific T cell responses. These findings have direct clinical implications.

For example, the identification of a progressive differentiation pathway (78) with adult tissue stem cells residing within the $CD8^+$ central memory compartment (79) is relevant with respect

to the design of T cell–based vaccines. Upon infection with a slowly replicating intracellular pathogen, $CD8^+$ TCMs should have enough time to generate large numbers of protective TEMs and SLECs (157, 158). Therefore, vaccines against this group of pathogens should mainly address efficient generation of pathogen-specific $CD8^+$ TCMs. Given the self-renewal capacity of TCMs, long-term maintenance of protective immunity should in these cases not require regular boost vaccinations. On the other hand, T cell immunity against quickly replicating pathogens is potentially more dependent on large numbers of preexisting TEMs and SLECs (157, 159). Epitope-specific TEMs can persist over long periods, but because of their lack of self-renewal, their maintenance is more fragile, especially when they have to compete for space and survival factors with other TEMs generated during heterologous infections (160). Thus, in order to maintain sufficient numbers of TEMs specific to a certain pathogen, frequent boost vaccinations might be valuable.

These novel concepts could also have therapeutic relevance for the treatment of T cell–mediated pathologies, such as some autoimmune diseases. Available T cell–targeting therapies act by neutralizing effector cytokines (e.g., anti-TNF-α) (161) or by preferentially depleting effector T cell subsets (e.g., anti-CD52/alemtuzumab) (162). Although these are highly effective for symptomatic treatment, disease activity often recurs upon interruption of treatment. This might be explained by autoreactive T cell clones residing within the TCM compartment from which new effector cells are rapidly regenerated. Therefore, strategies aiming at additional depletion of antigen-specific TCMs or inhibition of their reactivation (e.g., by antigen-specific regulatory T cells) may be useful for achieving more sustained therapeutic results.

Adoptive T cell therapies based on primary (unmodified) or genetically engineered T cell products have demonstrated impressive clinical results in the treatment of some chronic infections (163–165) as well as cancers (1–3, 166). Studies show that not the overall dose of T cells applied but rather their long-term persistence within the recipient is crucial for therapeutic success (1). This emphasizes the importance of identifying and utilizing self-renewing T cell subsets for adoptive T cell therapy. Basic research shows that the progeny of an individual T cell can provide immunity against an otherwise lethal infection (79, 165). These observations emphasize that establishing classical dose-response correlations in such dynamic cell therapeutic settings will be a demanding task and will require intricate knowledge not only of T cell numbers but also of T cell subsets used. Because similar rules apply for potentially harmful (i.e., autoreactive or alloreactive) T cells, when generating more defined T cell products it will be important not only to know which types of T cells they contain, but also to remove harmful subsets from them. An interesting application in this context could be the prophylactic management of infectious complications after allogenic stem cell transplantation by selective transfer of donor-derived TCMs. Depending on the serostatus of the donor, these TCMs would be well suited for quickly reestablishing host immunity against a variety of pathogens (e.g., cytomegalovirus, Epstein-Barr virus, adenovirus, *Aspergillus*) that are especially relevant in the posttransplantation phase. Given that T cell alloreactivity that mediates potentially life-threatening GVH disease is believed to mainly be derived from the donor's naive T cell compartment (167), selective transfer of donor-derived TCMs could also reduce the risk of GVH disease.

Recent data indicate that basic characteristics of long-lived memory T cells can be maintained during short-term in vitro stimulation combined with introduction of recombinant antigen receptors by genetic engineering approaches (1, 3). Therapies for treatment-resistant B cell leukemia utilizing engineered T cells expressing CD19-specific chimeric antigen receptors have had spectacular clinical responses—in many cases complete tumor remission (168–170). Some of the patients with effective responses received only low doses of adoptively transferred T cells, supporting the concept that the quality of transferred T cells might be more important for clinical efficacy than the overall quantity (1, 3). These observations suggest that for genetically

engineered T cell products, preselection or enrichment of defined subsets with high regenerative potential might be a powerful strategy to further improve therapeutic efficacy.

CONCLUSION

The clonal selection theory (8) is the basis for our understanding of adaptive immunity. Accordingly, single–T cell fate mapping has shown that individual recruited T cells are capable of generating diverse T cell families encompassing both long- and short-lived T cells (75, 77). It also has brought to light that this developmental capacity is not only present in naive T cells but also maintained in a subset of memory T cells, whose individual members are capable of stem cell–like self-renewal, are multipotent, and can reconstitute protective immunity to immunocompromised hosts (79). These features of naive T cells and certain memory T cells are likely the basis for the exciting clinical results that have been generated in adoptive cancer immunotherapy (1–3). However, individual naive T cells and memory T cells do not always realize their immense developmental potential. Single T cell–derived proliferation and differentiation is executed with massive variation, and a sufficient number of naive precursors must be recruited to provide a robust relationship between infectious input and immunological response (76, 78, 80, 81). In this light, the substantial redundancy of the TCR repertoire (7) and the high efficiency of recruiting T cells specific to a certain epitope into an immune response (56) could be seen as an evolutionary adaptation to balance stochastic response variation at the single–T cell level by population averaging. A better understanding of how to modulate T cell fate decisions during the earliest phases of an immune response might help to optimally guide an expanding T cell family through the fragile phase of first divisions and fate decisions and in the long run enable generation of robust immunity even from the rarest of precursors.

DISCLOSURE STATEMENT

D.H.B. has been shareholder of STAGE Cell Therapeutics (now Juno Therapeutics GmbH), a company developing reversible cell labeling technologies for clinical cell purification.

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