Tracing T cell differentiation by genetic barcoding

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Chapter 2

Mapping the life histories of T cells

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Mapping the life histories of T cells

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The behavior of T cells is not fixed in the germline, but displays highly adaptive characteristics. Understanding how different T cell subsets arise and how prior signaling input regulates subsequent T cell behavior requires us to couple a given T cell state to signals received by the cell, or by one of its ancestors, at earlier times. Here we discuss how recently developed technologies can be utilized to determine the kinship of different T cell subsets and their prior functional characteristics. Furthermore, we discuss the potential value of novel technologies that would allow assessment of migration patterns and prior signaling events.

It is becoming increasingly clear that T cells are not a homogeneous cell type, but display a spectrum of phenotypes and functions that relate to their developmental and immunological history. If we want to understand how these different T cell subsets arise and how prior signaling affects subsequent cellular function, we need to be able to couple a given T cell state to the prior input that cells have received. This is not a straightforward task. First, the functional activity of T cells can be influenced by signals received months or perhaps even years ago. Second, T cells are highly migratory, making it challenging to couple the input that a given cell receives to fate or functional activity of its progeny at later time points and at different locations. With the aim to address these issues, a series of technologies have been developed over the past years that allow the fate and history of individual cells to be monitored. Here we discuss the strengths and limitations of these technologies in the analysis of kinship and prior functional activity of different T cell subsets as well as other immune cell types. Furthermore, we describe the potential value of novel technologies that could aid in visualizing T cell migration patterns and prior signaling input received by activated T cells. This emerging toolbox should be of value to obtain a better understanding of T cell homeostasis and differentiation.

Understanding family ties

Depending on the nature of encountered signals, naïve T cells can give rise to several distinct subsets that differ greatly in surface phenotype and functional properties[1-4]. Furthermore, T cells can be instructed to either boost or suppress an ongoing immune response[5]. How can the origin of these different T cell subsets be determined? A relatively straightforward way to determine the fate of T cells is the adoptive transfer of cells that can be followed by a congenic[6-8] or fluorescent[9,10] marker (TABLE 1). Such markers can allow multiple T cell populations to be monitored simultaneously in the same host. For example, the joint transfer of recently-generated and long-term memory CD8 T cells (1-month vs. 12-months old) isolated from different congenic strains of mice has been used to demonstrate that memory T cell recall capacity increases progressively over time[11]. Via a similar approach, the combined transfer of naïve CD4 T cells from young and aged donors showed that naïve T cells become progressively long-lived with age[12].

As a variation on this theme, the kinetics with which immune cell populations equilibrate over different immunological sites can also be dissected with the discriminatory potential of congenic markers in parabiotic mice. In this setup, mice are surgically joined to share a common circulation, thereby allowing the

distribution of different cell types over various tissues to be followed through time[13]. Joining of immune to uninfected congenic mice has for instance shown that circulating memory T cells rapidly populate both lymphoid and non-lymphoid tissues, illustrating that T cells present in the blood contribute to the maintenance of tissue-resident memory populations[14]. In contrast, Langerhans cells in skin of parabionts showed no cross engraftment of partner-derived cells, indicating that in the steady-state these cells are likely maintained by local precursors[15].

Although congenic markers provide a valuable tool to follow the behavior of a bulk population of cells that have been transferred into a different host environment, there is one important limitation to the conclusions that can be drawn from these studies. Namely, it is difficult to distinguish whether all of the transferred cells display a certain behavior, or whether some of the cells differentiate into lineage A and others into lineage B (Fig. 1A). To address such fundamental questions regarding T cell differentiation, methods are required in which the fate of individual cells rather than a bulk population of cells can be traced. Over the past few years, three different experimental strategies have been developed that can be utilized to follow cell fate at the single-cell level. The value and technological issues of each of these strategies will be discussed.

Monitoring cell fate by continuous observation. Traditionally, microscopy techniques have been used to gain insight into the static distribution of hematopoietic cells[16]. However, with the advent of dynamic imaging techniques, such as confocal single-photon and multi-photon microscopy, it has become possible to study the interactions of individual cells during the development of immune responses in physiological environments as well as in real time[17-22]. By using intravital microscopy, the interaction of single naïve T cells with antigen-presenting dendritic cells (DCs) in lymph nodes could be visualized for up to a few hours[23-26]. These studies revealed that naïve T cell priming occurs in distinct phases, with initial transient interactions between T cells and DCs being followed by stable contacts that eventually lead to T cell division. The ability to track individual T cell-DC interactions also allowed these contacts to be quantified[27-29]. These studies highlighted that DCs are able to interact with 500-5,000 different naïve T cells per hour, thereby illustrating how it can be achieved that rare antigen-specific naïve T cells are recruited with high efficiency upon infection[30].

Intravital microscopy provides the very significant advantage that cells can be visualized in their physiological environments, which includes interaction with other fluorescently labeled cell types. However, the information obtained by this technology is mostly restricted to cell behavior over a period of a few hours. Specifically, photodamage induced by the excitation source can affect cell viability during prolonged imaging. As a second and more fundamental limitation, T cells that leave a particular site (e.g. the lymph node) are rapidly lost from analysis. As a result, intravital imaging

Table 1. Strategies for monitoring family ties of T cell subsets

Strategy	Level of resolution	Advantages	Limitations
AT using congenic markers	Bulk	Straightforward to perform	No data on potential of individual cells
TCR sequencing	Clonal	Tracking endogenous repertoire	TCR sharing by different cells
Intravital imaging	Single cell	Real-time analysis at physiological sites	Temporally and spatially restricted
AT of single cell	Single cell	Unambiguous readout of developmental potential	Difficult to demonstrate rare alternative fates
Cellular barcoding	Single cell	High-throughput identification of cell fate	Kinship analysis in retrospect
Brainbow mice	Single cell	Direct visualization of descend and function	Long-term stability of different colors unclear

AT, adoptive transfer; TCR, T cell receptor.

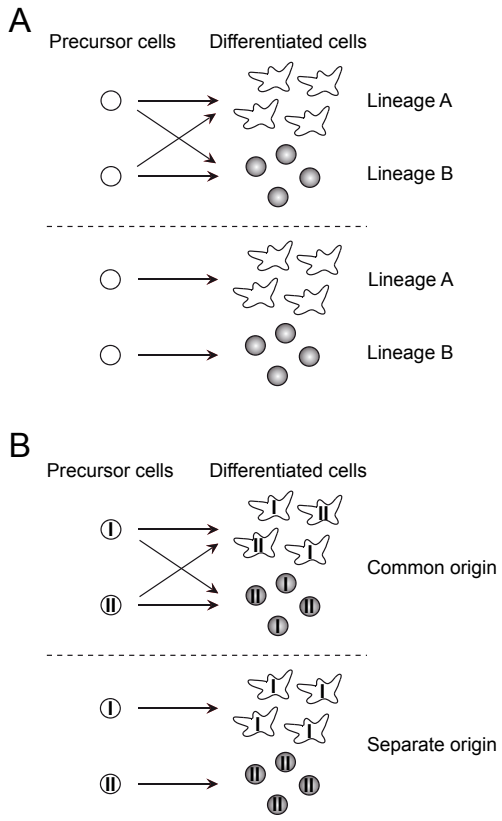


Figure 1. Identifying kinship by comparing barcodes. (**A**) The two differentiated lineages depicted here could be derived from either common (top panel) or separate (bottom panel) precursors. Analysis of cell fate at the bulk population level, e.g. by the use of congenic markers, cannot distinguish between these two scenarios. (**B**) When each precursor cell is labeled with a unique genetic tag that is passed on to all progeny, here depicted by marker I and II, the origin of the two differentiated cell populations can be revealed. In case the differentiated populations have a common origin, genetic tags present in these populations will be overlapping (top panel, marker I and II are found in both populations). In case the differentiated populations have a separate origin, genetic tags present in these populations will be distinct (bottom panel, either marker I or II is found in each population).

is at present primarily used for short-term monitoring of T cell activation and function at a given site.

One powerful alternative is formed by the recent development of methodology for the long-term imaging of cell differentiation in vitro[31,32]. This can be achieved by plating individual cells or small numbers of cells in separate wells containing conditioned culture medium and following these cells by time-lapse microscopy. This type of bio-imaging setup can allow the continuous monitoring of single cells and their progeny for up to one week. By tracing the fate of individually-plated mouse embryonic stem cell-derived mesoderm cells, the group of Schroeder demonstrated that adherent endothelial cells can directly give rise to non-adherent hematopoietic cells, which suggests that during embryonic development the first hematopoietic stem cells (HSCs) may derive from endothelial precursors[33]. Using a similar approach to image the differentiation of individual HSCs in conditioned culture medium, the same group demonstrated that cytokines can instruct hematopoietic lineage choice[34].

A different study by the group of Hodgkin made use of long-term in vitro imaging to monitor the fate of individual CpG-stimulated B cells[35]. This study found that all progeny of single founder B cells underwent a similar number of cell divisions, whereas the number of divisions between individual founders differed greatly, which leads to the fascinating model that the proliferative potential of each cell is a heritable property. Furthermore, this study found a strong correlation between the size of the founder B cell at the time of first division and the maximum division number of its progeny. One hypothesis to explain these results would be that cell-cycle promoting factors that are produced by the founder prior to the first cell division are subsequently diluted in all progeny through consecutive divisions, until a level is reached that is no longer permissive for cell cycle entry[35]. Although these results provide evidence for a potential cell-intrinsic mechanism to regulate the magnitude of adaptive immune responses, it remains to be tested whether the progeny of B cells that are activated in vivo display similar synchronized properties.

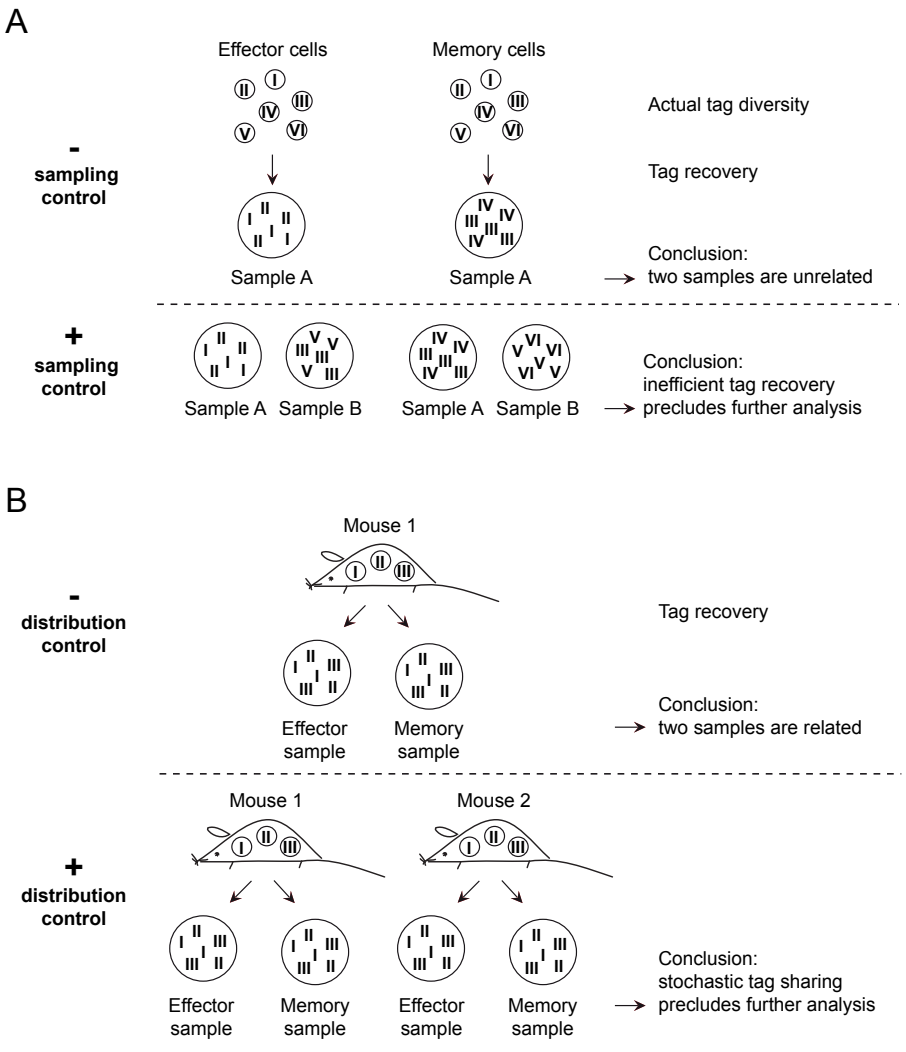
Monitoring cell fate by single-cell transfer. As indicated above, approaches that aim to trace cell fate by continuous observation are restricted to relatively short periods of time and are complicated by cell migration. An alternative strategy for fate monitoring of individual cells in vivo is to adoptively transfer a single cell that can be distinguished from all host cells by a congenic marker. This strategy allows one to unambiguously assess the developmental potential of this single cell within its physiological

environment. In a pioneering study, transfer of a single CD34$^-$ c-Kit$^+$ Sca-1$^+$ lineage$^-$ cell was shown to provide long-term reconstitution of the hematopoietic system in 20% of recipients, indicating that this cell population was highly enriched for HSCs[36]. Further purification of the HSC-containing population based on dye efflux capacity has shown that long-term multilineage differentiation after single-cell transfer can reach close to one hundred percent efficiency,

Box 1. Key controls in genetic tracing studies

In studies that use genetic tags to analyze kinship between different cell populations, two essential controls have to be performed before meaningful conclusions can be drawn about the relatedness of the populations under investigation. The first control is a tag sampling control, which tests how well the entire repertoire of genetic tags that is present in the population of interest is recovered. To assess total tag coverage, each sample can be split into two halves prior to analysis (sample A and B). Overlap in tags between these A and B samples (which are by definition related) will indicate the maximum tag overlap that can be obtained by any biological comparison (e.g. tags derived from effector and memory T cells). In case no sampling controls are performed, one cannot distinguish whether two cell populations are unrelated or whether tag recovery from both populations was inefficient (see Figure part **A**).

The second control is a tag distribution control, which tests to what extent individual precursors share similar tags due to stochastic mechanisms. This can for instance occur when different T cells share the same TCR sequence or are labeled by the same genetic tag due to limitations in library size. To assess background tag overlap between cell populations, it is therefore important to compare tags recovered from two samples that are by definition unrelated, such as labeled cells present in different mice. Overlap between the tags recovered from these unrelated samples will indicate the maximum tag difference that can be obtained by any biological comparison. In case no tag distribution controls are performed, one cannot distinguish whether two cell populations are related or whether they share tags due to stochastic events (see Figure part **B**). Together, these tag sampling and distribution controls set the experimental window in which kinship of two separate cell populations can be measured.



indicating that HSCs display a markedly high capacity for bone marrow homing and engraftment[37]. As an important caveat to these studies, adoptive transfer was in these cases performed into irradiated recipients, and the altered cytokine and cellular environment in these mice could influence cell fate. On a more general note, lineage tracing studies in which cell differentiation is studied in an altered host environment can report on the potential of cells but do not necessarily report on natural cell fate.

More recently, the concept of single-cell transfer was brought to the analysis of T cell differentiation by the group of Busch[38]. By transferring a single congenically marked antigen-specific CD8 T cell into a *Listeria monocytogenes*-infected recipient, it was shown that one naïve T cell can give rise to diverse effector and memory T cell subsets. Recently, the same group has demonstrated that the descendents of one naïve CD8 T cell can, after vaccination, provide protection against an otherwise lethal bacterial challenge (D. Busch, personal communication). These results highlight that all T cell types required for effective immunity against infection can in theory be provided by a single activated antigen-specific T cell.

As a downside to the single-cell transfer system, the successful engraftment of viable single cells might be an issue for more fragile cells (e.g. activated T cells). Furthermore, while single-cell transfer allows one to reveal common cell fates, the fact that each experiment tests the fate of only a single cell makes it difficult to exclude (or demonstrate) more rare alternative fates.

Monitoring cell fate by unique labeling of many cells. The limitations of single-cell adoptive transfer raise the question of how this in vivo cell tracking approach can be extended to high-throughput analysis. Ideally, one would like to study the behavior of the progeny of a population of cells, while still being able to determine which ancestor gave rise to which daughter cell. In such an experimental setup, each ancestor would have to bear a unique and heritable marker to allow the progeny of different ancestors to be distinguished. Three such approaches have been developed so far.

A first strategy for fate analysis of endogenous T cell populations makes use of the natural sequence variation that occurs in rearranged T cell receptor (TCR)[39] and B cell receptor (BCR) genes. TCR sequence analysis was used to monitor the evolution of TCR repertoires during antigen-driven responses[40-50], to analyze the kinship of different memory T cell subsets[51-55], and to examine the conversion of conventional CD4 T cells into *Foxp3*-expressing regulatory T cells upon self-antigen encounter in the periphery[56]. BCR sequence analysis was used to study the clonal origin of early antibody producing and germinal center B cells[57].

A major drawback of TCR sequencing-based approaches for the monitoring of cell fate is that multiple T cells can -and in most cases will- exist within the naïve T cell pool that share a given TCR sequence, making it difficult to determine the fate of individual T cell clones. This problem is particularly prominent when analyzing TCRβ chain sequences, as thymocytes undergo a strong proliferative burst following beta-selection. In addition, a given TCR sequence can also occur multiple times because this sequence is formed by a frequent recombination event (known as public TCRs), or because of homeostatic proliferation. If multiple founder T cells within the naïve T cell pool share the same TCR sequence, a difference in TCR sequence between two T cell populations is still informative and indicates a separate ancestry. However, sharing of TCR sequences no longer provides evidence for a shared population of founder cells. Given that developing B cells also undergo a strong proliferative burst after BCR heavy chain rearrangement, similar concerns apply to the interpretation of BCR sequencing data. On a more general note, in cases where a given tag used for lineage tracing, such as a TCR or BCR sequence, occurs multiple times within a precursor population, kinship of two cell populations can only be demonstrated if a correction is made for the overlap in these tags that occurs by chance (BOX 1).

To allow lineage tracing without the limitations of TCR or BCR based analyses and to allow kinship studies outside of the lymphocyte lineage, strategies are required that allow the experimental introduction of unique

markers. In early work in this field, irradiation-induced damage and retroviral insertion sites have been used to mark cells in an essentially random manner (TABLE 2). More recently, two different approaches have been developed that allow unique labeling of many individual cells. One approach is based on the introduction of unique DNA sequences and the other on unique fluorescent labeling.

In the first approach, a retroviral library containing thousands of unique DNA sequences (termed barcodes) was developed and coupled to a microarray-based detection platform[58]. Barcode-labeling of founder populations of interest is achieved by infection with this barcode library under conditions that favor one integration per cell. In this way, each individual cell is labeled by a unique hereditable marker. After transfer of a pool of uniquely labeled cells into recipient mice, analysis of the barcode content within cell populations that emerge in vivo can be used to dissect the origin of many individual cells in a single experiment. To enable lineage tracing within the T cell lineage, which requires barcode-labeling of naïve T cells, thymocytes are infected with the barcode library and allowed to differentiate into barcode-labeled naïve T cells after intra-thymic injection[59]. This cellular barcoding technology can be used to address two types of biological questions concerning T cell responses. First, the technology can be used to determine whether cell populations that differ in location or functional activity arise from common or separate precursors (Fig. 1, 'comparing barcodes'). Second, the technology can be used to determine the number of precursors that produce a given cell population (Fig. 2, 'counting barcodes').

With respect to the first application, cellular barcoding has been employed to monitor T cell migration patterns in the context of multiple inflammatory sites. In an experimental setup in which the same mouse received two localized antigenic challenges simultaneously, it was found that although distinct naïve T cells were primed in both draining lymph nodes, following lymph node exit their progeny had the capacity to migrate to both effector sites[58]. These data highlight that, independent of the site of priming, individual T cell clones retain the capacity to migrate to multiple tissues, as has been shown at the bulk T cell population level by others[60,61]. In a different study, cellular barcoding has been used to determine whether effector and memory CD8 T cells are progeny of the same or different naïve T cells[59]. Under conditions of either local or systemic infection, it was found that each naïve T cell gives rise to both effector and memory T cells, indicating that the progeny of single naïve T cells can take on multiple fates. Furthermore, this shared ancestry of effector and memory T cells was observed for both low and high affinity naïve T cells.

A second type of biological question that can be addressed by cellular barcoding concerns the clonal diversity of cell populations (Fig. 2). Because DNA barcodes are passed on to all progeny, the number of different barcodes present in an antigen-specific T cell population directly correlates with the number of naïve T cells that yielded this population. Based on this concept, cellular barcoding was used to test to whether the magnitude of antigen-specific T cell responses is regulated by the number of naïve T cells that are recruited into the response or the clonal burst (i.e. the number of progeny) of each recruited cell[30]. Under disparate conditions of infection, with different pathogens and doses, it was found that recruitment of naïve antigen-specific T cells is highly constant and in fact

Table 2. Early studies using genetic tagging for lineage tracing

Strategy	Detection system	Conceptual advance	REFS
Radiation-induced chromosome aberrations	Karyotype analysis	First to demonstrate multilineage potential of single precursors	92-94
Retroviral integration site analysis	Southern blotting	Stable introduction of unique clonal markers	95-97
Retroviral oligonucleotide marking	PCR and sequencing	Tag libraries of high complexity	98,99

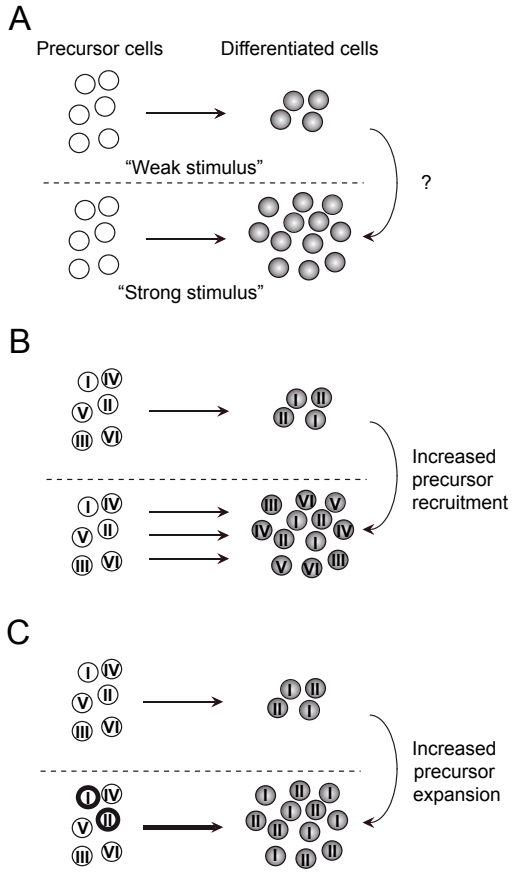


Figure 2. Measuring clonal diversity by counting barcodes. (**A**) In this example, varying stimulation of precursor cells gives rise to either a small (top panel) or a large (bottom panel) population of differentiated cells. Analysis at the bulk population level cannot reveal whether the increased population size upon stronger stimulation is the result of an increase in precursor recruitment or an increase in precursor expansion. (**B**) When each precursor is labeled with a unique genetic tag that is passed on to all progeny, here depicted by marker I to VI, the clonal diversity of the two differentiated populations can be revealed. In case the population increase upon stronger stimulation is the result of increased precursor recruitment, the number of different genetic tags present in the larger differentiated population will increase proportionally (all six markers are found). (**C**) In case the population increase upon stronger stimulation is the result of increased precursor expansion, the number of different genetic tags present in the larger differentiated population will remain constant (only marker I and II are found).

close to complete. These findings indicate that recruitment of rare antigen-specific T cells is highly efficient for T cell responses of varying magnitude. From these data it can be concluded that the overall magnitude of T cell responses is primarily regulated by clonal burst size.

While cellular barcoding provides a powerful technology for the analysis of T cell fate, the unique identifiers that the labeled cells carry can only be revealed by DNA isolation. Consequently, it is impossible to determine the kinship of the same cells and their progeny at multiple timepoints. A potential solution to this issue would lie in the use of cellular tags that can be analyzed in a non-invasive manner, either by antibody staining or by intrinsic fluorescence. In an impressive study, the group of Lichtman developed and utilized a mouse strain (termed *Brainbow*) in which Cre/lox recombination is used to drive stochastic expression of three fluorescent proteins[62]. By using multiple tandem integrations of the *Brainbow* construct and limiting expression to the developing brain, this study showed that individual neurons could be distinguished by expression of one out of close to one hundred different color variations. Thus far the *Brainbow* system has not been used to determine precursor – progeny relationships either within neuronal tissue, or in other tissues. In order to follow cell populations in time, this would require long-term stability of the different color variations, something that is at this point unclear. Furthermore, it remains to be determined how large the spectral overlap between the different color variations is, which would decide whether the analysis could be extrapolated to a high-throughput platform, such as flow cytometry. However, as the *Brainbow* system can in theory be utilized in any cell type in which Cre recombinase can be selectively expressed, this strategy may be valuable to directly visualize the progeny of individual naïve T cells and to monitor their interactions with other cell types of the immune system.

Revealing prior functional states

Following antigen encounter, activated T cells undergo dramatic changes in their gene expression program, resulting in the acquisition of novel functional attributes[63-65]. Furthermore, when antigen subsides (and also when antigen becomes persistent), T cell populations emerge that lack some of the functions present in effector cells and display again a different set of properties[66,67]. How do these prior functional states influence subsequent T cell fate? For instance, do T cells that express granzymes or

perforin during an ongoing immune response preferentially die or form memory upon antigen clearance, or is this specific functional characteristic irrelevant for long-term survival? Furthermore, is the ability of differentiated T helper cells to express a certain profile of effector cytokines stably maintained over long periods, or does a reset take place[68]?

The key requirement to answer these questions is that (transient) expression of a specific functional property is translated into a stable and heritable marker that can be measured later. Towards this aim, several research groups have generated reporter mice in which expression of a gene of interest is coupled to upregulation of a fluorescent label. One way to generate these reporter mice is by inserting a bicistronic fluorescent reporter cassette into the genomic locus of the gene of interest. In such knock-in mice, reporter expression is transient as fluorescent marking is only displayed as long as the gene of interest is expressed. Using this approach, two reporter mice were generated in which IL-4 and IFN-γ expression can be monitored by upregulation of GFP and YFP fluorescence, respectively[69,70].

Because marker expression of knock-in reporter mice is transient, these mice do not allow longitudinal fate mapping of T cell populations. Several strategies have therefore been employed to provide differentiating cells with a more stable marking. One way to prolong marker expression is to stabilize reporter transcripts by inclusion of exogenous untranslated regulatory sequences. Stabilizing a bacterial artificial chromosome (BAC)-IFN-γ/Thy1.1 reporter by a 3' untranslated SV40 intron/polyadenylation sequence allowed identification of IFN-γ-positive T cells for a prolonged period following termination of IFN-γ protein expression[71]. Using this system, the group of Weaver tracked the fate of IFN-γ-positive T cells following LCMV infection. Both reporter-positive CD4 and CD8 T cells were observed directly ex vivo in the memory phase following resolution of the infection, indicating that T cells that expressed IFN-γ during the effector phase have the capacity for surviving contraction and entering into the memory pool.

As the function of stabilized reporter constructs depends on the half-life of the measured transcript, these systems are generally restricted to short-term monitoring of gene expression. Allowing gene expression to induce an irreversible genetic marking would circumvent this limitation. In general, this approach requires two transgenic mouse lines: one in which expression of the gene of interest is linked to expression of Cre recombinase and another in which a reporter protein is expressed following Cre-mediated excision of a transcriptional stop cassette. By linking a truncated human granzyme B promoter driving Cre to a placental alkaline phosphatase (PLAP) reporter, it was shown that CD8 T cells that had expressed granzyme B during primary LCMV infection also had the capacity to develop into long-lived memory T cells[72]. However, as an exogenous granzyme B promoter might not accurately reflect endogenous granzyme B expression, a later study by the group of Fearon generated a BAC transgenic line, in which a tamoxifen-inducible Cre cassette was inserted into the granzyme B gene[73]. To allow granzyme B reporter expression, this BAC transgenic line was crossed to a YFP reporter strain. Also in this experimental setup, which has the distinct advantage that it measures granzyme B expression using the natural promoter and chromosomal context, it was found that CD8 T cells that had expressed granzyme B during primary influenza infection gave rise to functional memory T cells.

Although these results intuitively suggest that effector cells are precursors of memory cells, these data should be interpreted with caution for several reasons. First, what these reporter constructs measure is transcriptional activity of the gene of interest and not expression or activity of the protein itself. In that regard, it cannot be definitively concluded that reporter-positive cells are functional effector cells, because additional layers of regulation exist that can prevent protein expression until later stages of cell differentiation, such as post-transcriptional regulation by microRNAs[74]. Second, reporter systems do not have absolute tagging efficiency and therefore typically mark only a fraction of gene expressing cells. As a result, it cannot be discerned whether memory cells only descend from effector cells that expressed the gene

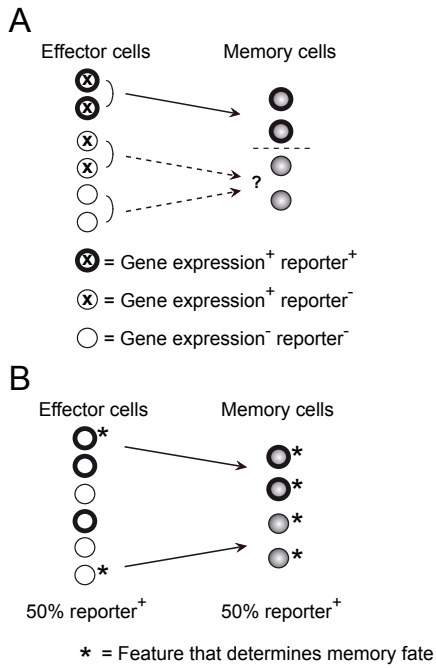


Figure 3. Pitfalls of gene expression reporters in kinship analysis. (**A**) In this example, a reporter for gene expression associated with effector function (e.g. granzyme B or perforin expression) is used to assess kinship of effector and memory cells. Because of inefficiency in the reporter system, only half of all effector cells that expressed the gene of interest also upregulated reporter expression. As a result, the origin of the reporter-positive memory cells (top two memory cells) can be traced back to effector cells that expressed the gene of interest. However, the origin of the reporter-negative memory cells (bottom two memory cells) cannot be traced back, as these cells could be the progeny of reporter-negative effector cells that did or did not express the gene of interest. (**B**) Similarity in the fraction of reporter-positive effector and memory cells does not automatically show that the effector population is homogeneous and that all reporter-positive effector cells give rise to memory cells. Namely, it could be that memory selection is determined by an entirely different feature that is not measured. As a result, the presence of reporter-positive memory cells primarily indicates the existence of cells with an effector property that also can give rise to memory cells.

of interest, or whether memory cells can also descend from effectors that did not express that gene (Fig. 3A). Third, in case the fraction of effector and memory cells that is reporter-positive is constant, this does not show that the fate of the effector population is homogeneous and that all reporter-positive effector cells give rise to memory cells, as it remains unclear whether the gene linked to the reporter is the factor that determines the formation of memory. Instead, it primarily indicates the presence of cells with an effector property of which some can also give rise to memory cells and the actual mechanism behind memory determination could be an entirely different feature that is not measured (Fig. 3B).

Mapping migration and prior signaling

T cells travel through highly dynamic environments and interact with many other hematopoietic as well as non-hematopoietic cells. During an immune response, many of these interactions have the potential to affect T cell proliferation, differentiation and survival[75,76]. Measuring the cellular interactions that T cells undergo during an immune response and understanding how these interactions affect subsequent behavior represent great challenges for immunologists in the coming decades. In the final part of this review, we will discuss how novel technologies might aid in visualizing T cell migration patterns, as well as revealing the diverse signaling input received by T cells at different sites.

Tracking T cell migration. Although it is well established that antigen-specific T cell responses are initiated in secondary lymphoid organs, it remains largely unclear where activated T cells migrate to as soon as these priming sites are abandoned. For instance, did bone marrow-resident memory T cells at one point reside at the site of infection[77-79]? Furthermore, do T cells present at an effector site yield further progeny when leaving that site via the afferent lymph vessels? Part of this obscurity stems from the technical difficulty to keep track of migrating T cell populations. Any experimental system that aims to follow T cell migration patterns in vivo will depend on the ability to induce stable markers at the moment T cells are present at a given site and to record the same markers at a later time point and at a different site (Fig. 4).

A promising approach for the monitoring of T cell migration builds on the use of light to induce conformational changes in photoreceptive proteins. One group of studies made use of the protein Kaede, which is green fluorescent after synthesis but can be photoconverted to red fluorescence by exposure to violet

light[80]. In transgenic mice expressing Kaede, photoconversion at a specific site allows the subsequent migration of these red fluorescence-labeled cells to be followed. By photoconverting inguinal lymph node cells and tracking their migration, the group of Kanagawa found that one day after exposure the photoconverted cells had disseminated to spleen as well as other lymph nodes, indicating that in the steady-state lymph node cells are highly migratory[81].

As photoconverted Kaede is rapidly diluted by cell proliferation, this system is less suitable for longitudinal monitoring of activated T cells. Ideally, strategies based on light-switching would give rise to irreversible genetic marking of cells present at a defined site. Conceptually, this marking strategy resembles reporter systems that are used to reveal prior T cell function (see above), with the important difference that the signal that drives marking is not based on endogenous transcriptional activity, but rather requires the triggering of a synthetic signaling pathway. The emerging field of synthetic biology, which aims at engineering custom-configured signaling pathways, is providing some interesting entry points for the generation of cell migration reporters[82]. Many signal transduction proteins consist of two types of domains: an output domain that harbors catalytic activity and an input protein interaction domain that links the protein to upstream regulators and downstream targets. Because these domains are often structurally autonomous, they can perform their function in a context-independent fashion. In theory, by coupling a light-sensitive input module to a DNA binding output module, a light-activated transcription factor could be conceived. By fusing the photoactive LOV2 domain of *Avena sativa* phototropin 1 to the *Escherichia coli* trp repressor, the group of Sosnick showed that DNA binding of the trp repressor transcription factor became selectively inducible by blue light photoexcitation[83]. As many LOV domains respond to photoexcitation with a conformational change, it should be feasible to design photoactive switches in which LOV domains are coupled to diverse output domains, ultimately aimed at giving rise to stable fluorescent cell marking.

Revealing prior cell signaling events. As activated T cells journey throughout the body, they can receive a multitude of signals from many different cell types, each of which can potentially direct subsequent T cell function. Mapping these diverse signals will require experimental strategies that can couple a given signaling event to the expression of detectable markers. Generally, two types of signaling events can be monitored.

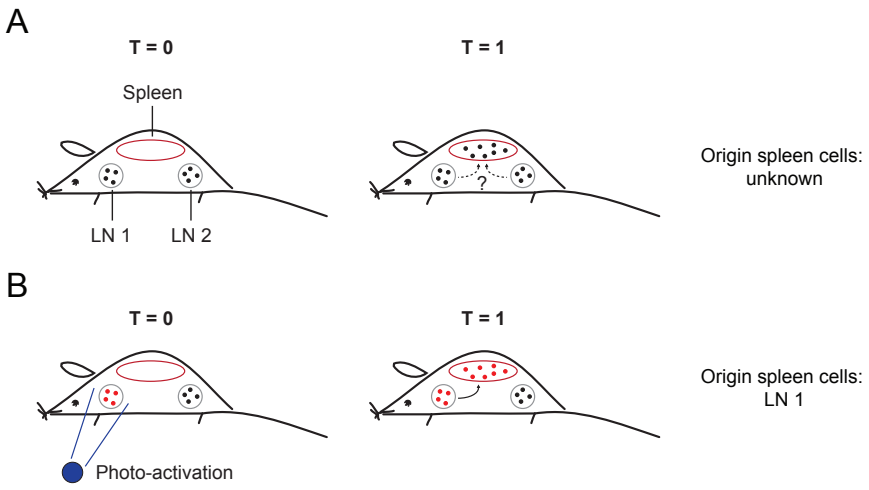


Figure 4. Tracking T cell migration by light-activated markers. (**A**) At T=0 both lymph nodes contain a phenotypically-identical T cell population (e.g. recently primed antigen-specific T cells). At T=1, some of these T cells have migrated to the spleen. In this scenario it is impossible to determine whether the splenic T cells are derived from lymph node 1, 2 or both. (**B**) In this scenario, photo-activation of cells in lymph node 1 is used to specifically mark these T cells with a unique label (e.g. a red fluorescent protein induced via light-activated transcription). Because at T=1 all splenic T cells harbor the red fluorescent marker, the origin of these T cells can be traced back to cells originating from LN 1.

In one case signaling output (e.g. activation of a cytokine promotor) is being measured. This can be achieved by placing a fluorescent protein or Cre recombinase under control of this promoter (as mentioned above)[84,85]. In the other case, signaling input (e.g. triggering of a cytokine or costimulatory receptor) is being measured. As the transcriptional program initiated by this receptor triggering is not always known, reporter function depends on the use of synthetic signal transduction pathways. In such pathways, receptor signaling input would have to be coupled to novel signaling output driving marker expression; such that a cell will for example express a fluorescent protein after a defined cell surface receptor has been triggered.

What kind of strategies could be pursued to build such synthetic signaling pathways aimed at monitoring signaling input? One study that aimed at designing new signaling pathways made use of the property that activation of Notch-family receptors triggers release of their cytoplasmic tail. When this cytoplasmic tail was replaced by a heterologous transcription factor, a novel transcriptional program could be initiated by Notch signaling[86]. Using a similar strategy, it might be feasible to visualize Notch signaling in T cells during antigen-specific responses by linking its signaling to expression of a detectable marker[87]. A different study by the group of Lim generated a construct in which Rho guanine-nucleotide exchange factor (Rho-GEF) activity is controlled by flanking the Rho-GEF with a PDZ domain (a peptide-binding motif) at one end and its cognate target peptide at the other[88]. The resulting intramolecular PDZ-peptide interaction inhibited the intervening Rho-GEF activity. As the peptide was modified to contain a protein kinase A (PKA) target sequence, PKA signaling could disrupt the PDZ-peptide binding and hence activate the GEF[88]. Given that PDZ-peptide interactions have also been used to inhibit the function of other proteins[89], it may be worthwhile to pursue the generation of novel autoinhibition constructs in which PKA activity or other signaling input that can disrupt PDZ-peptide binding can be monitored by the activation of reporter proteins. A third strategy aimed at engineering new signaling circuits makes use of hybrid adaptor and scaffold proteins. One study artificially connected the epidermal growth factor (EGF) receptor pathway to the Fas death receptor pathway, by constructing a hybrid adaptor protein[90]. As a result, mitogenic EGF receptor signaling now led to caspase activation and cell death. In another example, engineering of the mitogen-activated protein (MAP) kinase scaffold Ste5 allowed recruitment of unique non-native kinases to the scaffold, resulting in a synthetic MAP kinase pathway with altered input-output properties[91]. Together, these studies highlight how synthetic signaling pathways might be used to link the multitude of signals received by activated T cells during the course of an immune response to the expression of detectable markers.

Concluding remarks

The immune system is arguably one of the most complex organ systems present. Rather than being confined to one specific location, T cells travel around the body and receive many receptor-mediated signals at different locations and points in time that impact subsequent T cell behavior. At this point, we are just beginning to understand the total 'package' of signals that T cells encounter during antigen-induced responses and how these different signals influence functional states both of that cell and of its downstream progeny. In this review, we have focused on existing technologies and discussed some potential strategies that allow us to 'look back in time' and thereby examine the cellular origins and past cellular experiences of different T cell subsets. Technologies such as single-cell and genetic tracing have already proven valuable to further our understanding of how cell fate heterogeneity can arise. However, as neither of these technologies can directly reveal the signals that cells have received, novel strategies will be required to help explain how the sum of signaling input dictates eventual T cell function and destiny. Ultimately, by connecting the dots between a cell's history and its current function, we should be better able to predict T cell behavior in both disease as well as therapeutic settings.

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