Assessing T cell differentiation at the single-cell level

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MAPPING THE LIFE HISTORIES OF T CELLS

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The behaviour of T cells is not fixed in the germ line, but is highly adaptable depending on experiences encountered during a T cell's life. To understand how different T cell subsets arise and how prior signalling input regulates subsequent T cell behaviour, approaches are required that couple a given T cell state to signals received by the cell, or by one of its ancestors, at earlier times. Here we describe recently developed technologies that have been used to determine the kinship of different T cell subsets and their prior functional characteristics. Furthermore, we discuss the potential value of new technologies that would allow assessment of T cell migration patterns and prior signalling events.

INTRODUCTION

In the naive T cell repertoire, antigen-specific T cells are exceedingly rare. Prior to antigen encounter, the frequency of antigen-specific T cells is estimated to be 1 in 10^5 cells, which is ~200 T cells in the total lymphoid system of a mouse[1, 2, 3]. Following activation these rare antigen-specific T cells expand rapidly, dividing once every 4–6 hours[4, 5, 6, 7]. The extent of this T cell expansion depends on the pathogen type and the severity of infection, but it can in some cases yield more than 10^4 progeny per activated T cell[8, 9, 10, 11]. The fate of the resulting T cell pool is not uniform. First, following antigen clearance, ~90% of activated T cells die. Second, those cells that do survive can reside either in the lymphoid compartment or in peripheral organs and may, for example, differ in their capacity for renewed proliferation[12, 13, 14, 15]. The development of different memory T cell populations is just one example of the range of phenotypes and functions that T cells can adopt. To understand how these different T cell subsets arise and how prior signalling affects subsequent cellular function, we need to be able to link a current T cell state to the prior input that the cell has 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 earlier. Second, T cells are highly migratory, making it challenging to couple the input that a given cell receives to the fate or functional activity of its progeny at later time points and at different locations. To this end, a series of technologies have been developed over the past few 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 both kinship and prior functional activity of different T cell subsets, as well as of other immune cell types. Furthermore, we describe the potential value of new technologies that could aid the visualization of T cell migration patterns and prior signalling input.

UNDERSTANDING FAMILY TIES

Kinship analysis at the population level. Depending on the nature of encountered signals, naive T cells can give rise to several distinct subsets that differ greatly in phenotypical and functional properties[15, 16, 17, 18, 19]. How can the origin of these different T cell subsets be determined? A relatively straightforward way to determine the fate of T cells at the population level is to adoptively transfer donor cells that can be distinguished from recipient cells by the expression of a congenic[20, 21, 22] or fluorescent[23, 24] marker (Table 1). Such markers allow multiple T cell populations to be monitored simultaneously in the same host. For example, the transfer of both recently generated and long-term memory $CD8^+$ T cells isolated from different congenic strains of mice has been used to show that memory T cell recall capacity increases progressively over time[25]. As a variation on this theme, the kinetics with which immune cell populations equilibrate across different immunological sites has been determined using congenic markers in parabiotic mice[26, 27, 28].

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

Strategy	Level of resolution	Advantages	Limitations
Adoptive transfer using congenic markers	Bulk	Straightforward to perform	No data on potential of individual cells
TCR sequencing	Oligoclonal	Tracks endogenous repertoire	TCR sharing by different cells
Intravital imaging	Single cell	Real-time analysis at physiological sites	Temporally and spatially restricted
Adoptive transfer of single cells	Single cell	Unambiguous read-out of developmental potential	Difficult to demonstrate rare alternative fates
Cellular barcoding	Single cell	High-throughput identification of cell fate	Invasive method of tag detection
Brainbow	Single cell	Direct visualization of descent	Stable inheritance of different colours by progeny has not been established

TCR, T cell receptor.

Although the use of congenic or fluorescent markers provides a valuable tool to examine the behaviour of a bulk population of cells that have been transferred into a different host, there is one crucial limitation to the conclusions that can be drawn from this experimental approach. Specifically, it is impossible to distinguish whether all of the transferred cells have a particular behaviour or whether some of the cells differentiate into cell type A, whereas others differentiate into cell type B (Fig. 1a). As a simple example, following the adoptive transfer of a population of haematopoietic progenitor cells that yield both T cells and granulocytes, it is impossible to determine whether the transferred cells consisted of multipotent progenitors or a mixture of lymphoid and myeloid precursor cells. To address such fundamental questions regarding cell differentiation, methods are required that enable the fate of individual cells rather than a bulk population of cells to be traced. Over the past few years, three different experimental strategies have been developed that can be used to follow cell fate at the single-cell level. The benefits and limitations of each of these strategies are discussed below.

Monitoring cell fate by continuous observation. Traditionally, microscopy techniques have been used to gain insights into the static distribution of haematopoietic cells[29]. However, with the advent of dynamic imaging techniques, such as intravital multiphoton microscopy, it has become possible to study the interactions of individual cells during the development of immune responses in a physiological setting as well as over time[30,

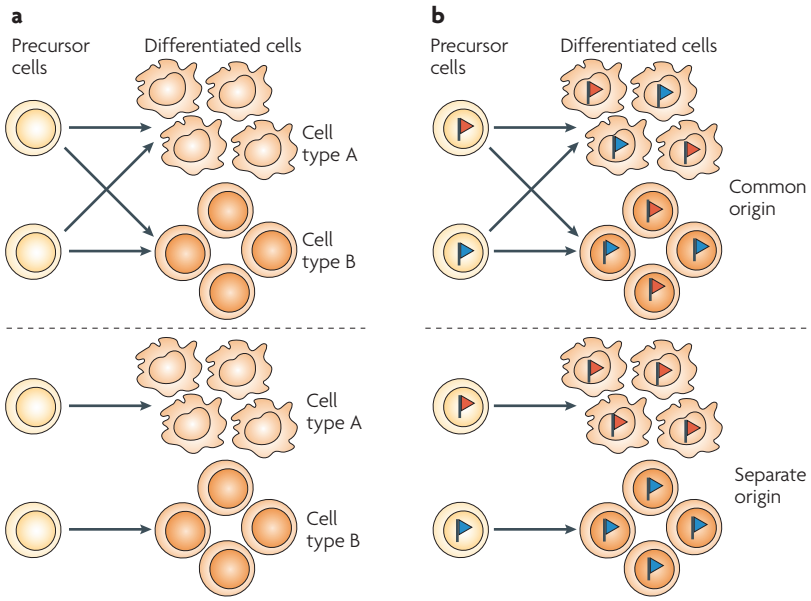


Figure 1: Identifying kinship by comparing barcodes. a | The two cell types depicted here could be derived from either common (top panel) or separate (bottom panel) precursors. Analysis of cell fate at the population level (for example, by the use of congenic markers) cannot distinguish between these two scenarios. **b** | When each precursor cell is labelled with a unique genetic tag that is passed on to all progeny (shown as a red or blue flag) the origin of the two cell populations can be revealed. If the two cell populations have a common origin, genetic tags present in these populations will be overlapping (top panel, both red and blue flags are found in both populations). If the two cell populations have a separate origin, genetic tags present in these populations will be distinct (bottom panel, only one type of flag is found in each population). Note that in those cases in which individual precursor cells have a bias to produce a certain type of progeny, the tags carried by these precursor cells will show a proportional enrichment in the descendant population of daughter cells.

[31, 32, 33, 34, 35]. Intravital microscopy has allowed the interaction between single naive T cells and antigen-presenting dendritic cells (DCs) in lymph nodes to be visualized[36, 37, 38, 39] and quantified[40, 41, 42]. These studies revealed that naive T cell priming occurs in distinct phases, with initial transient interactions between T cells and DCs followed by stable contacts that eventually lead to T cell division.

An important benefit of intravital microscopy is that it allows cells to be visualized in their physiological environment. However, the information obtained by using this technology is mostly restricted to cell behaviour over a period of a few hours, as 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 (such as the lymph node) are invariably lost from analysis. As a result, intravital microscopy is currently used mainly for short-term monitoring of T cell activation and

function at a given site. One powerful alternative is provided by the recent development of methodology for the long-term imaging of cell differentiation in vitro[43, 44]. This type of bio-imaging set-up can allow the continuous monitoring of single cells and their progeny for periods of up to one week. By tracing the fate of individually plated mouse embryonic stem cell-derived mesoderm cells, Schroeder and colleagues recently showed that adherent endothelial cells can directly give rise to non-adherent haematopoietic cells[45], suggesting that during embryonic development the first haematopoietic stem cells (HSCs) may be derived from endothelial cell precursors[46, 47]. Using a similar approach to image the differentiation of individual HSCs in conditioned culture medium, the same group showed that cytokines can instruct haematopoietic lineage selection[48].

The use of long-term in vitro imaging to monitor the fate of lymphocytes has so far been restricted to B cells stimulated by CpG-containing DNA[49]. An intriguing study by Hodgkin and colleagues showed that all progeny of single founder B cells underwent a similar number of cell divisions, whereas the number of divisions of progeny from different founder cells varied greatly. This observation has led the authors to propose that the proliferative potential of B cells is a (transiently) heritable property. In addition, this study found a strong correlation between the size of the founder B cell at the time of its first division and the number of divisions that its progeny underwent. One hypothesis to explain these results is that cell-cycle promoting factors that are produced by the founder cell before the first cell division are subsequently diluted in all progeny during consecutive divisions[49].

The fact that individual founder cells in this system have a distinct behaviour in an essentially homogeneous environment provides indirect evidence for the possibility that lymphocyte fate may, in some cases, be controlled stochastically. In line with a role for stochastic processes in the regulation of cell fate, naturally occurring fluctuations in the levels of apoptosis regulators have been shown to account for cell-to-cell variability in the timing and probability of receptor-mediated death[50]. Interestingly, such fluctuations in protein levels can be transmitted from mother to daughter, resulting in a transient inheritance of cell state, and the rate at which this correlation in protein levels between mother and daughter is lost varies among different proteins[51]. Together, these results highlight the possibility that, in concert with the very large number of well-defined external triggers that mediate lymphocyte activation and differentiation, stochastic processes may contribute to the generation of distinct lymphocyte subsets, and this is an area that deserves greater attention[52, 53].

Monitoring cell fate by single-cell transfer. As mentioned above, approaches that aim to track cell fate in vivo by continuous observation are restricted to relatively short periods of time and are complicated by cell migration. An alternative strategy for monitoring the fate of individual cells in vivo is to adoptively transfer a single donor 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 in its physiological environment. In a pioneering study by Nakauchi and colleagues,

transfer of a single $CD34^{-}KIT^{+}SCA1^{+}lineage^{-}$ cell was shown to provide long-term reconstitution of the haematopoietic system in 20% of recipient mice, indicating that this cell population was highly enriched for HSCs[54]. A more recent study has identified expression of the signalling lymphocytic activation molecule (SLAM) family member CD150 as an additional marker for distinguishing self-renewing HSCs, and use of this marker allowed long-term multilineage reconstitution by 50% of cells following single-cell transfer[55]. An important caveat to these studies is the fact that adoptive transfer was carried out in irradiated recipients, and the altered cytokine and cellular environment in these mice could influence cell fate. More generally, lineage-tracing studies in which cell differentiation is studied in an altered host environment provide valuable insights into the potential of a cell, but do not necessarily inform us of physiological cell fate.

More recently, the concept of single-cell transfer was applied to the analysis of T cell differentiation by Busch and colleagues[56]. By transferring a single congenically marked antigen-specific $CD8^{+}$ T cell into recipient mice that were subsequently infected with Listeria monocytogenes, it was shown that one naive T cell can give rise to diverse effector and memory T cell subsets. More recent work from the same group suggests that the descendants of one naive $CD8^{+}$ T cell can, after vaccination, provide protection against an otherwise lethal bacterial challenge (D. Busch, personal communication). These results suggest that all $CD8^{+}$ T cell types required for effective immunity can, in theory, be provided by a single activated antigen-specific T cell.

As a limitation to single-cell adoptive transfers, the successful engraftment of viable single cells can be difficult for more 'fragile' cell populations (such as activated T cells). Furthermore, although single-cell transfer studies can readily reveal common cell fates, the fact that each experiment tests the fate of only a single cell makes it difficult to exclude (or demonstrate) rarer alternative fates.

Monitoring cell fate by unique labelling of many cells. The limitations of single-cell adoptive transfer raise the question of how in vivo cell tracking can be extended to high-throughput analysis. Ideally, one would like to study the behaviour of the progeny of a population of cells while still being able to determine which ancestor gave rise to which daughter cells. In such an experimental set-up, 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 with the aim of achieving this goal.

One strategy for fate analysis of endogenous T cell populations makes use of the natural sequence variation that occurs in rearranged T cell receptor (TCR) and B cell receptor (BCR) genes[57]. TCR sequence analysis has been used to monitor the evolution of TCR repertoires during antigen-driven responses[2, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67], to analyse the kinship of different memory T cell subsets[68, 69, 70, 71, 72] and to examine the conversion of conventional $CD4^{+}$ T cells into forkhead box P3 (FOXP3)-expressing regulatory T cells[73]. BCR sequence analysis has been used to study the clonal origin of early antibody producing and germinal centre B cells[74].

A major drawback of TCR sequencing-based approaches for the monitoring of cell fate is that in the naive T cell pool multiple T cells can — and in most cases will — share a given TCR sequence, making it difficult to unambiguously determine the fate of individual naive T cells. This problem is particularly evident when analysing TCR β-chain sequences, as thymocytes undergo a strong proliferative burst following β-selection. In addition, a given TCR sequence can occur multiple times when formed by a frequent recombination event or because of homeostatic proliferation. To what extent does the presence of multiple T cells with the same TCR limit the conclusions that can be drawn from clonal analyses? In cases in which multiple founder T cells in the naive T cell pool share the same TCR sequence, a difference in TCR sequence between two cell populations of interest is still informative and indicates a separate ancestry. By contrast, the 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 all cases in which a given tag used for lineage tracing (such as a TCR sequence or a designed genetic tag, see later) occurs multiple times within a precursor population, the kinship of two cell populations can only be convincingly demonstrated when a correction is made for the overlap in 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 beyond the lymphocyte lineage, strategies have been developed that are based on 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 labelling of many individual cells. One approach is based on the introduction of a highly diverse collection of DNA sequences and the second is based on the induction of a diverse set of fluorescent labels.

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[75]. The labelling of founder populations of interest with a unique heritable barcode was then achieved by infection with this retroviral library. In the case of T cells, this labelling can either be performed at the peripheral T cell stage or at the T cell precursor (thymocyte) stage, the latter to circumvent the need for T cell activation[76]. After transfer of a pool of labelled cells into recipient mice, analysis of the barcode content in cell populations that emerge in vivo can be used to dissect the fate of many individual cells in a single experiment. Such cellular barcoding technology can be used to address two types of biological question regarding 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 from separate precursors (Fig. 1). Second, the technology can be used to determine the number of precursors that produce a given cell population (Fig. 2).

BOX 1

In studies that use genetic tags (such as T cell receptor (TCR) sequences, barcodes or fluorescent colour codes) to analyse kinship of cell populations, two essential controls have to be carried out before meaningful conclusions can be drawn about the relatedness of the cell populations under investigation. The first control is a tag sampling control (see the figure, part **a**), which tests how well the entire repertoire of genetic tags that is present in the populations of interest is recovered. Only when the reproducibility of tag recovery from a labelled cell population has been assessed does it become meaningful to compare these tags to a different cell population. To assess the efficiency of tag detection, each sample is split into two equal halves before analysis (sample A and sample B). Overlap in tags recovered from these A and B samples, which are by definition related, will indicate the maximum tag overlap that can be obtained in any biological comparison (for example, effector and memory T cells). If no sampling controls are carried out, one cannot distinguish whether two cell populations are unrelated or whether tag recovery from both populations was inefficient (part **a**).

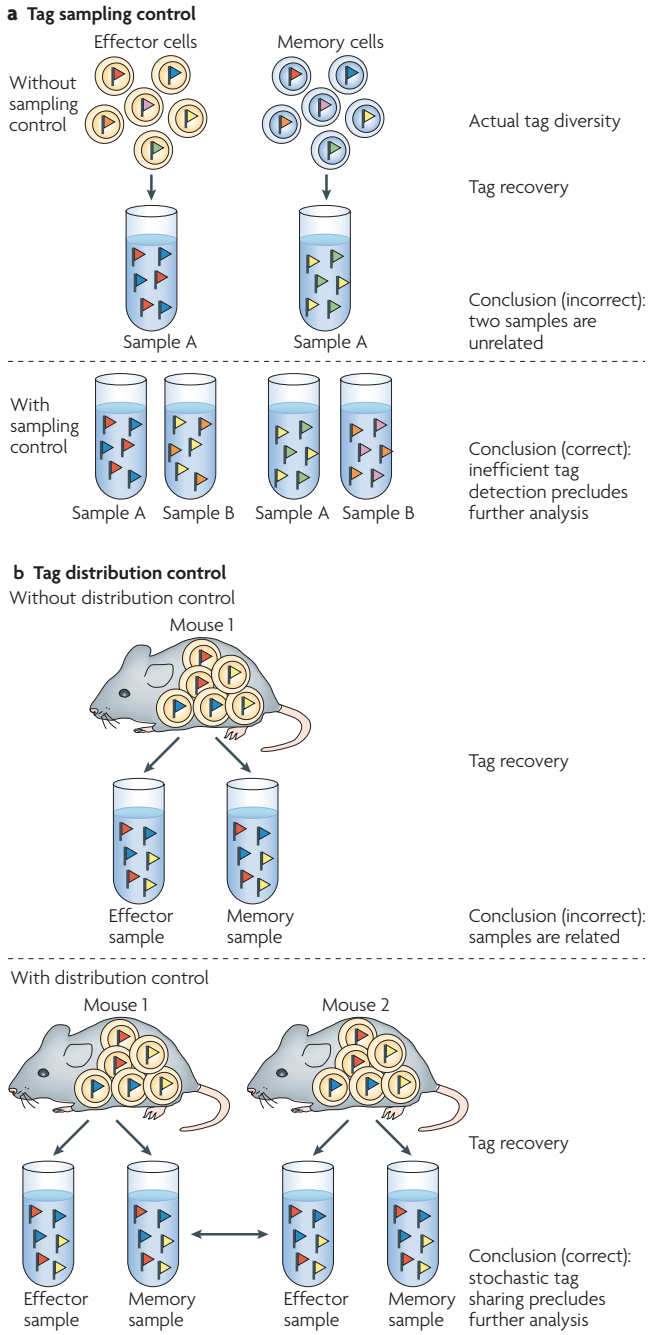
The second control is a tag distribution control (part b), which tests to what extent individual precursor cells share similar tags by chance (rather than by kinship). For example, this can occur when different T cells share the same TCR sequence or when multiple T cells are labelled with the same genetic tag. To assess background tag overlap between cell populations, tags recovered from two samples that are by definition unrelated (for example, labelled cells present in different mice) can be compared. When samples from two different mice share the same tags, an overlap in tags between samples from the same mouse must take this background overlap into account. If no tag distribution controls are performed, one cannot distinguish whether two cell populations are related or whether they share tags based on chance (part b). Together, these two controls set the experimental window in which kinship of different cell populations can be measured.

Table 2: Early studies using genetic tagging for lineage tracking.

Strategy	Detection system	Conceptual advance	Refs
Radiation-induced chromosome aberrations	Karyotype analysis	First to demonstrate multi-lineage potential of single precursors	109-111
Retroviral integration site analysis	Southern blotting	Stable introduction of unique clonal markers	112-114
Retroviral oligonucleotide marking	PCR and sequencing	Tag libraries of high complexity	115, 116

TCR, T cell receptor.

BOX 1 FIGURE


a Tag sampling control
Effector cells
Memory cells
Without sampling control
Actual tag diversity
Tag recovery
Sample A
Sample A
Conclusion (incorrect): two samples are unrelated
With sampling control
Sample A
Sample B
Sample A
Sample B
Conclusion (correct): inefficient tag detection precludes further analysis
b Tag distribution control
Without distribution control
Mouse 1
Tag recovery
Effector sample
Memory sample
Conclusion (incorrect): samples are related
With distribution control
Mouse 1
Mouse 2
Tag recovery
Effector sample
Memory sample
Effector sample
Memory sample
Conclusion (correct): stochastic tag sharing precludes further analysis

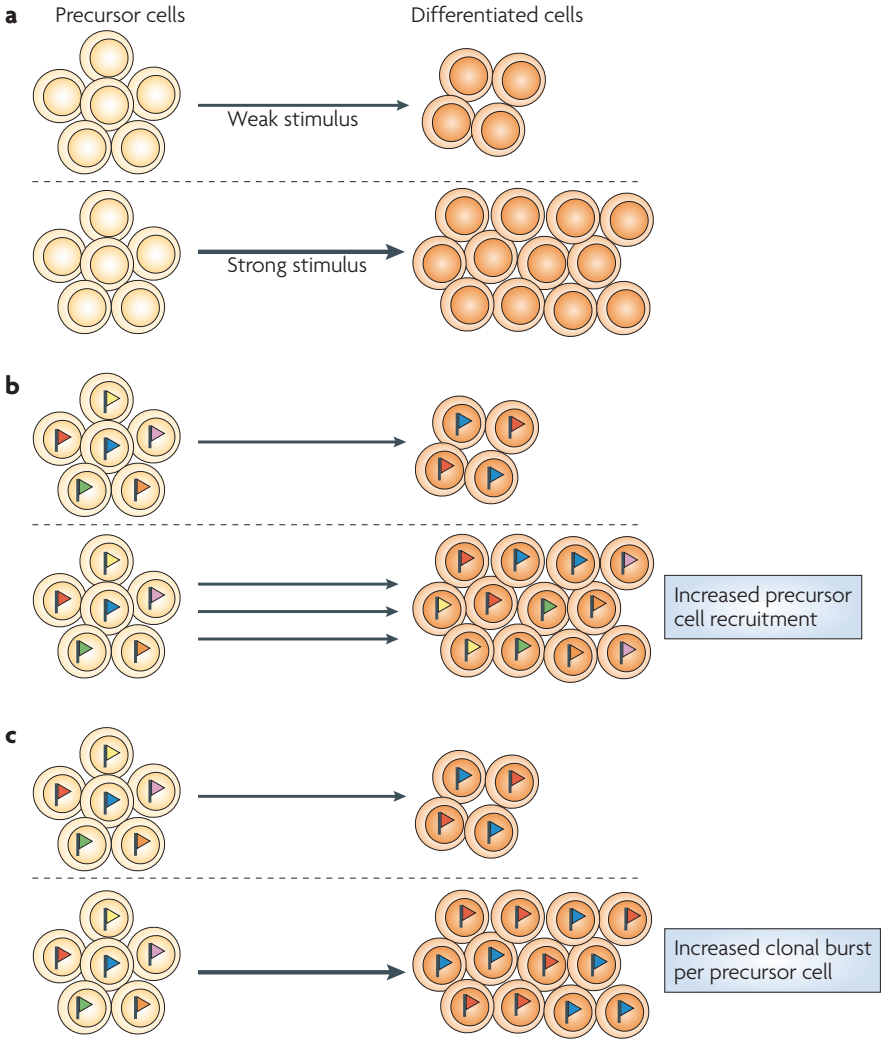


Figure 2: Measuring clonal diversity by counting barcodes. a | In this example, varying stimulation of precursor cells (for example, naive T cells, haematopoietic stem cells or natural killer cells) gives rise to either a small (top panel) or a large (bottom panel) population of differentiated cells. Analysis at the cell population level cannot reveal whether the increased population size following stronger stimulation is the result of increased precursor cell recruitment or an increased clonal burst per precursor cell. **b** | When each precursor cell is labelled with a unique genetic tag that is passed on to all progeny (shown as coloured flags) the clonal diversity of the two cell populations can be revealed. If the population increase following stronger stimulation is the result of increased precursor cell recruitment, the number of different genetic tags present in the differentiated cells will increase proportionally with response magnitude (all six flags are found). **c** | If the population increase following stronger stimulation is the result of an increased clonal burst per precursor cell, the number of different genetic tags present in the differentiated cells will remain constant (only red and blue flags are found).

With respect to the first application, cellular barcoding has been used to monitor T cell migration patterns to multiple inflammatory sites. In an experimental set-up in which the same mouse simultaneously received two localized antigenic challenges, it was found that although distinct T cell families were present in both draining lymph nodes shortly after priming, following lymph node exit their progeny had the capacity to migrate to both effector sites[75]. These data suggest that, independent of the site of priming, individual T cell clones retain the capacity to migrate to multiple tissues[77, 78]. In a different study, cellular barcoding was used to determine whether effector and memory $CD8^+$ T cells are progeny of the same or of different naive T cells[76]. Under conditions of either local or systemic infection, it was found that each naive T cell gives rise to both effector and memory T cells, indicating that the progeny of a single naive T cell can take on multiple fates. Furthermore, this shared ancestry of effector and memory T cells was observed for both low- and high-affinity T cells.

How quantitative are the data that can be obtained using this genetic tagging technology? The analyses carried out so far have used microarrays to read out barcode abundance and should therefore be considered semi-quantitative. However, with the advent of second-generation sequencing approaches, it should now be possible to quantify the contribution of individual precursors with high precision. Importantly, for such quantitative analyses to be meaningful it will be essential that the relative abundance of different tags following recovery and amplification faithfully represents their distribution in the cell population of interest; an issue that can be evaluated by a straightforward sampling control (Box 1). In particular, the development of a quantitative technology for high-throughput fate mapping should be valuable for analysing situations in which individual cells are neither fully committed nor completely bipotent but have a bias towards producing cell type A or B.

A second biological question that can be addressed by cellular barcoding (or other genetic tagging strategies) concerns the clonal diversity of cell populations (Fig. 2). Because each precursor contains a unique marker, the number of different barcodes present in a marked cell population directly correlates with the number of founder cells that yielded this population. Based on this concept, cellular barcoding has been used to test whether the magnitude of antigen-specific T cell responses is determined by the number of naive T cells that are recruited into the response or by the clonal burst (that is, the number of progeny) of each recruited cell[79]. Under different conditions of infection, with various pathogens and doses, it was found that recruitment of naive antigen-specific T cells is markedly constant and is in fact close to complete. These findings indicate that recruitment of rare antigen-specific T cells is highly efficient for T cell responses of varying magnitude, and from these data it can be concluded that the overall magnitude of T cell responses is mainly regulated by clonal burst size.

Although cellular barcoding provides a powerful technology for the analysis of T cell fate, the unique identifiers that labelled cells carry can only be revealed by DNA isolation. Consequently, it is impossible to determine the identity of a cell and to follow its fate afterwards. A potential solution to this issue might be in the use of

cellular tags that can be analysed in a non-invasive manner. In an impressive study, Lichtman and co-workers developed and used a mouse strain (termed Brainbow) in which a Cre–lox approach is used to drive stochastic expression of three fluorescent proteins[80]. By using multiple tandem integrations of the Brainbow construct and limiting expression to the developing brain, the authors showed that individual neurons could be distinguished by expression of 1 of almost 100 different colour variations. So far, the Brainbow system has not been used to determine precursor–progeny relationships either in neuronal tissue or in other tissues. To allow such tracking of cell populations, the 'hue' of cells will have to remain stable over time and across cell generations, and to our knowledge this has not been established to date. Furthermore, for many types of research questions it will be important to develop technology to distinguish different hues by flow cytometry rather than microscopy. If both of these hurdles can be overcome, the potential of this approach is considerable.

REVEALING PRIOR FUNCTIONAL STATES

Following antigen encounter, activated T cells undergo dramatic changes in their gene expression programme, resulting in the acquisition of novel functional characteristics[81, 82, 83]. Furthermore, following clearance of antigen (and also when antigen becomes persistent), T cell populations can emerge that lack some of the functions present in effector T cells and that have a different set of properties[84, 85]. How do 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 gain a memory phenotype after antigen clearance, or is this specific functional characteristic irrelevant for long-term survival? Likewise, is the ability of differentiated T helper cells to express a particular profile of effector cytokines stably maintained over long periods or can their phenotype be reset[86]?

The key requirement for answering these questions is that (transient) expression of a specific functional property is translated into a stable and heritable marker that can be measured at a later point in time. To this end, several research groups have generated reporter mice in which expression of a gene of interest is coupled to expression of a fluorescent label. One way to generate these reporter mice is by insertion of a bicistronic fluorescent reporter cassette into the locus of the gene of interest[87, 88]. In such knock-in mice, reporter expression is transient, as the fluorescent mark is only produced as long as the gene of interest is expressed and, consequently, these systems are unlikely to allow longitudinal fate mapping of T cell populations. Several strategies have therefore been developed to provide differentiating cells with a more stable mark. One way to prolong marker expression is to stabilize reporter transcripts by inclusion of exogenous untranslated regulatory sequences. Stabilization of an interferon-γ (IFNγ)-Thy1.1 reporter by inclusion of a 3' untranslated SV40 intron/polyadenylation sequence has allowed the identification of Thy1.1-positive T cells for a prolonged period following termination of IFNγ protein expression (and for more

than 40 days post-infection)[89]. The finding that expression of the Thy1.1 marker proved to be so stable in this system may indicate that regulation of IFNγ expression in memory T cells primarily occurs at the post-transcriptional stage. More importantly, the fact that reporter-positive $CD4^+$ and $CD8^+$ T cells could give rise to functional memory cells indicates that T cells that express IFNγ during the effector phase can survive the contraction phase of the immune response.

The detection of stabilized reporter constructs depends on the half-life of the measured transcript. As the half-life of most mRNAs is in the order of hours, these systems are expected to be restricted to the monitoring of prior gene expression for periods of days at most. Allowing gene expression to induce an irreversible genetic mark circumvents this limitation. By using Cre expression, driven by a truncated human granzyme B promoter to activate a placental alkaline phosphatase (PLAP) reporter by recombination, it was shown that $CD8^+$ T cells that expressed granzyme B during primary lymphocytic choriomeningitis virus (LCMV) infection also had the capacity to develop into long-lived memory T cells[90]. In a more recent study by Fearon and colleagues, a bacterial artificial chromosome (BAC) transgenic mouse line was generated, in which a conditional Cre cassette was inserted into the gene encoding granzyme B. Subsequently, this BAC transgenic was crossed with a yellow fluorescent protein (YFP) reporter strain[91]. In this experimental set-up, which has the distinct advantage that it measures granzyme B expression using the natural promoter and surrounding sequences, it was also found that $CD8^+$ T cells that expressed granzyme B during primary influenza virus infection gave rise to functional memory T cells.

Although these results convincingly show that effector T cells (as defined by specific promoter activity) can be precursors of memory T cells, it is important to realize what is and what is not measured in these types of experiment that use reporters of prior functional states (here discussed in the context of effector and memory T cells, but the underlying principles are generally applicable). First, these reporter constructs measure transcriptional activity of the gene of interest and not expression or activity of the protein itself. Thus, it cannot be definitively concluded that reporter-positive cells are functional effector T cells, as there are additional layers of regulation that can prevent protein expression until later stages of cell differentiation[92]. Second, the current reporter systems do not have a 100% tagging efficiency and typically mark only a fraction of gene-expressing cells. As a result, it is difficult to determine whether memory T cells only come from effector T cells that expressed the gene of interest or whether memory T cells can also come from T cells that did not express that gene (Fig. 3a). Third, although reporter constructs can indicate that at least some T cells with an effector property can give rise to memory T cells, they cannot provide information on whether the effector population is homogeneous in this respect. Specifically, the ability to form memory T cells could be determined by an unknown marker that is displayed by only a portion of the effector T cell population and which is not being measured (Fig. 3b). In such cases the most accurate conclusion would not be that effector T cells form memory T cells but that effector T cells contain a subset of cells that form the precursors of memory T cells.

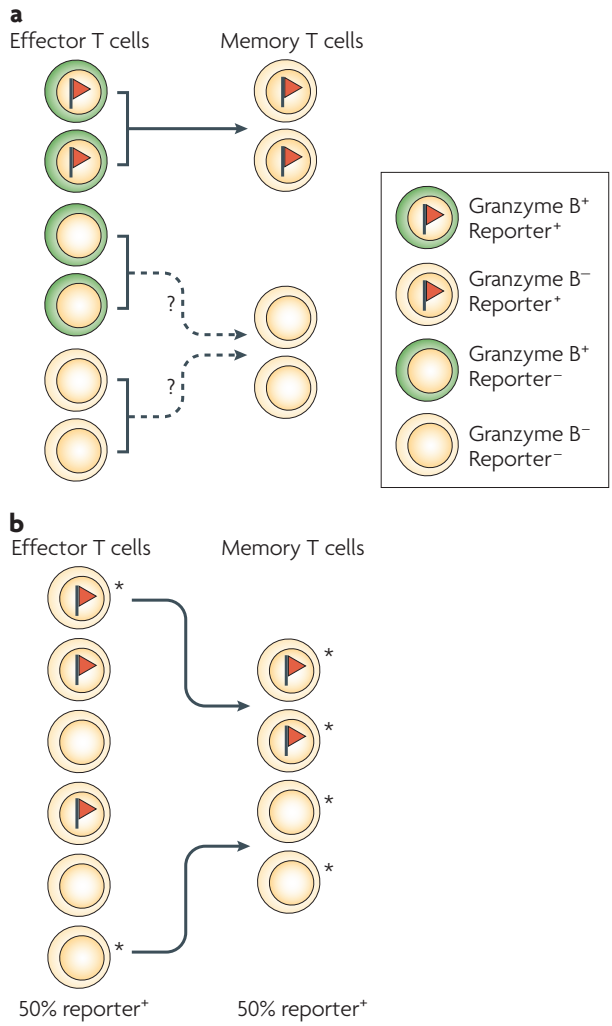


Figure 3: Pitfalls of gene expression reporters in kinship analysis. **a** | In this example, a reporter for gene expression associated with effector function (for example, granzyme B or perforin expression) is used to assess kinship of effector and memory T cells. Because of suboptimal tagging efficiency, only half of all effector T cells that express the gene of interest also express the reporter. Although the origin of the reporter-positive memory cells (top two memory T cells) can be traced back to effector T cells that expressed the gene of interest, the origin of the reporter-negative memory T cells (bottom two memory T cells) remains unclear, as these cells can be the progeny of cells that did or did not express the gene of interest. **b** | Similarity in the fraction of reporter-positive effector and memory T cells does not show that the effector T cell population is homogeneous and that all reporter-positive effector T cells can give rise to memory T cells. Specifically, the ability to form memory T cells could be determined by an unknown feature (*) that is displayed by only a portion of the effector T cells. Thus, although the presence of reporter-positive memory T cells indicates that at least some cells with an effector phenotype can give rise to memory T cells, it does not show that all effector T cells are equipotent in this respect.

MAPPING MIGRATION AND PRIOR SIGNALLING

T cells travel through highly dynamic environments and interact with many other haematopoietic as well as non-haematopoietic cells. During an immune response, many of these interactions have the potential to affect T cell proliferation, differentiation and survival[93, 94]. Measuring the cellular interactions that T cells encounter during an immune response and analysing how these affect subsequent cell behaviour are important goals to further our understanding of T cell function. In the final part of this Review, we discuss how new technologies can aid in the visualization of T cell migration patterns and reveal the diverse signalling 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 after these priming sites are abandoned. For example, did bone marrow-resident memory T cells at one point reside at the site of infection[95, 96, 97]? Similarly, do T cells that are present at an effector site yield further progeny when leaving that site through the afferent lymph vessels? Part of this obscurity stems from the technical difficulty in tracking migrating T cell populations. Any experimental system that aims to follow T cell migration patterns in vivo is therefore likely to 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 later time points and at different locations (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 used the protein Kaede, which fluoresces green after synthesis but can be photoconverted to red fluorescence by exposure to violet light[98]. In transgenic mice expressing Kaede, photoconversion at a specific site allows the subsequent migration patterns of these cells to be followed. By photoconverting inguinal lymph node cells and tracking their migration, it has been shown that 1 day after exposure to violet light, the photoconverted cells had disseminated to the spleen and to other lymph nodes, indicating that in the steady-state, lymph node cells are highly migratory[99].

As photoconverted Kaede is rapidly diluted by cell proliferation, this system is less suitable for longitudinal monitoring of cell migration patterns and, ideally, strategies based on light-induced switching would give rise to the irreversible genetic marking of cells present at a defined site. Conceptually, this strategy resembles the reporter systems that are used to reveal prior T cell function, but with the important difference that the signal that drives cell marking uses a synthetic, rather than an endogenous, signalling pathway. The emerging field of synthetic biology has already yielded customized signalling pathways that form interesting entry points for the generation of such cell migration reporters[100]. Many signal transduction proteins consist of two types of domain: one that displays catalytic activity and one that links the protein to upstream regulators and downstream targets. Because these domains are often structurally autonomous, they can carry out their function in a context-independent

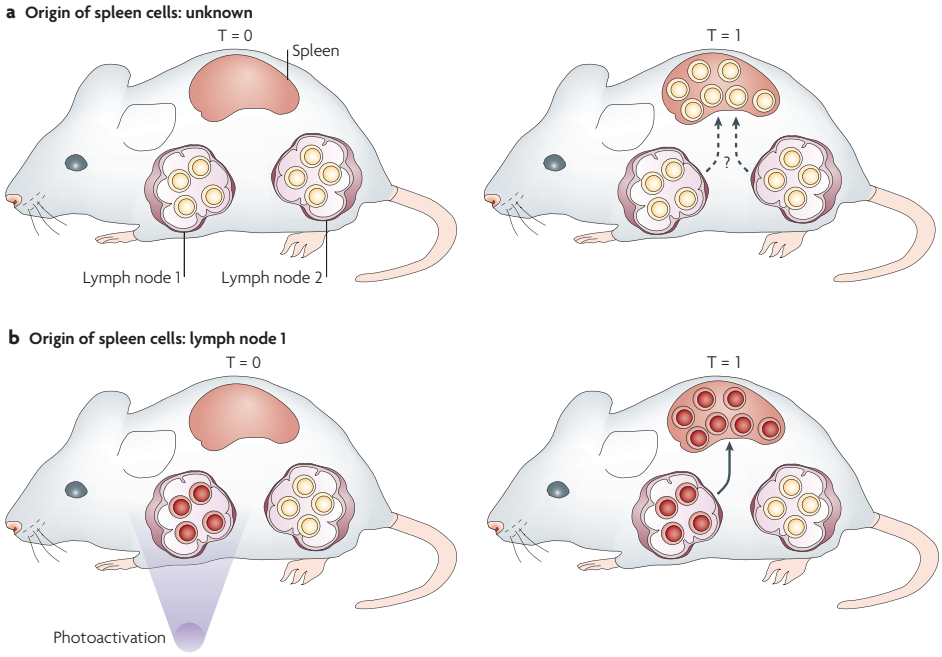


Figure 4: Tracking T cell migration by light-activated markers. a | At baseline (T = 0) both lymph nodes contain a phenotypically identical T cell population (for example, recently primed antigen-specific T cells). At T = 1, some of these T cells have migrated to the spleen. In this scenario, it is impossible to determine whether the splenic T cells are derived from lymph node 1, 2 or both. **b** | In this case, photoactivation of cells in lymph node 1 is used to specifically mark these cells with a unique label (for example, a red fluorescent protein induced by light-activated transcription). At T = 1 all splenic T cells harbour the red fluorescent marker, and because of this the origin of these T cells can be traced back to cells originating from lymph node 1.

manner. As an example, coupling of a light-sensitive input module to a DNA-binding output module might yield a light-activated transcription factor. In fact, by fusing the photoactive LOV2 domain of Avena sativa phototropin 1 to the Escherichia coli trp repressor, Sosnick and colleagues showed the feasibility of designing transcription factors for which DNA binding is made responsive to blue light excitation[101]. Although at this point there are no studies available that used light to induce stable cell marking, the fact that many LOV domains respond to photoexcitation with a conformational change suggests that it may be possible to design (sets of) photoactive switches that use a synthetic light reporter to enable stable fluorescent cell marking.

Revealing prior cell signalling events. As activated T cells migrate 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 signalling event to the

expression of detectable markers. Triggering of a given cell surface receptor may be monitored either upstream or downstream of the endogenous signalling cascade (Fig. 5). The monitoring of downstream targets of signalling cascades (for example, activation of a cytokine promoter) is now relatively well established and can be achieved by placing a fluorescent protein or Cre recombinase under the control of this promoter (as described above)[102, 103]. However, in many cases a given cell surface receptor (for example, a T cell co-stimulatory receptor) does not have unique downstream targets, making downstream signalling reporters unsuitable for selectively reporting on the triggering of that specific receptor. In such cases, receptor triggering will have to be measured upstream in the signalling cascade, and this will require the use of synthetic signal transduction pathways. In such pathways, receptor signalling input is coupled to a new signalling output aimed at inducing marker expression, such that a cell will, for example, express a fluorescent protein after a defined cell surface receptor has been triggered.

What kinds of strategies could be pursued to build such synthetic signalling pathways aimed at monitoring signalling input? As an initial strategy, Lim and colleagues generated a system in which Rho guanine-nucleotide exchange factor (Rho–GEF) activity is controlled by flanking the Rho–GEF with a PSD95, DLGA and ZO1 homology (PDZ) domain (a peptide-binding motif) at one end and its cognate

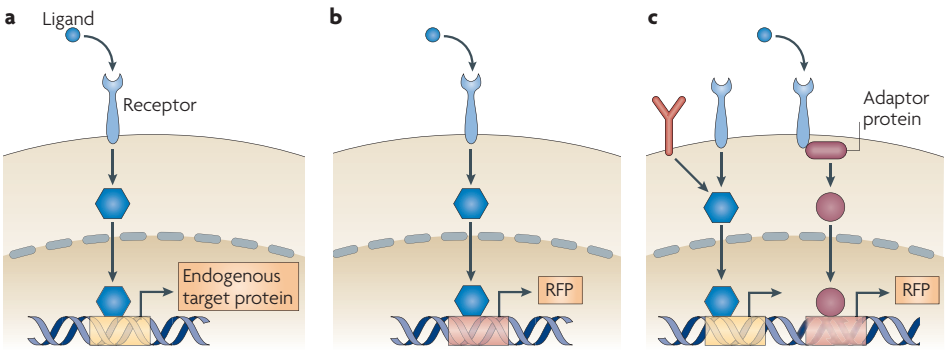


Figure 5: Strategies for revealing prior cell signalling. a | Triggering of a cell surface receptor initiates an intracellular signalling cascade, eventually leading to transcription of a target gene. To detect such a signalling event at a later point in time, the use of a reporter that converts this transient signal into a permanent mark is required. **b** | If a given cell surface receptor has a unique downstream target, receptor triggering can be monitored by placing a recombinase gene under the control of the activated promoter (not shown). Recombinase activity will lead to permanent expression of a fluorescent protein. **c** | If a given cell surface receptor does not have a unique downstream target, receptor triggering should be monitored upstream in the signalling cascade; this is likely to require the use of synthetic signal transduction pathways. Here, the cell surface receptor has been coupled to a hybrid adaptor protein, which following signal transduction will give rise to a transient burst of recombinase expression and permanent expression of a fluorescent protein. For the sake of simplicity, the recombination step in b and c is not depicted. RFP, red fluorescent protein.

target peptide at the other[104]. 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, signalling through PKA could disrupt the PDZ–peptide binding, hence making this modified GEF a reporter of PKA activity[104]. Given that PDZ–peptide interactions have also been used to inhibit the function of other proteins[105], it may be worthwhile to pursue the generation of novel autoinhibition constructs, with which specific protein kinase activity can be monitored by the activation of reporter proteins.

A second group of strategies that measure receptor triggering are based on the action of hybrid receptors or hybrid adaptor proteins. An early study that aimed to directly monitor receptor activation made use of the fact that activation of Notch family receptors triggers the release of their cytoplasmic tail. When this cytoplasmic tail was replaced by a heterologous transcription factor, a new transcriptional programme could be initiated by Notch signalling[106]. Using a similar strategy, it might be feasible to visualize Notch signalling in T cells during antigen-specific responses by linking its signalling to expression of a detectable marker[107]. As a second example, one study artificially connected the epidermal growth factor (EGF) receptor pathway to the FAS (also known as CD95)-mediated cell death receptor pathway by constructing a hybrid adaptor protein[108]. As a result, mitogenic EGF receptor signalling led to caspase activation and cell death. Although inducing cells to die will clearly not be a very efficient strategy to track cells that underwent receptor triggering, the general concept used here can also be applied to induce more innocuous tags. Together, these studies highlight how in the coming years synthetic signalling 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 T cell-based immune system is arguably one of the most complex organ systems. Rather than being confined to one specific location, T cells migrate around the body and receive many receptor-mediated signals at different locations and points in time that affect subsequent cell behaviour. Currently, 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 the 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 examine the cellular origins and past cellular experiences of different T cell subsets. Technologies such as single-cell and genetic tracking have already proven to be valuable for furthering our understanding of how cell-fate heterogeneity can arise. However, as neither of these technologies can directly reveal the signals that cells have received, new strategies will be required to help to explain how the sum of the signalling input dictates eventual T cell function and destiny. Ultimately, by connecting

a T cell's past experience to its current function, we should be better able to predict T cell behaviour in disease as well as therapeutic settings.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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