# Tracing T cell differentiation by genetic barcoding

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# Chapter 8

Summary and discussion

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Following antigen encounter, T cells enter a program of proliferation and differentiation that, depending on the signals received, can give rise to different T cell subsets characterized by distinct phenotypic and functional properties. Understanding how different T cell subsets arise requires technologies that can monitor the developmental potential of precursor cells at the single-cell level. This thesis describes the development and use of two novel genetic tagging strategies that aim to allow the analysis of cell differentiation in vivo. These strategies are based on the marking of precursor cells with unique DNA sequences (barcodes). Upon cell division, these barcodes are inherited by all daughter cells, allowing one to analyze cell fate by comparing the barcodes present in different daughter populations. The first technology developed in this thesis, termed cellular barcoding, makes use of a retroviral barcode library to provide T cells with unique genetic tags via in vitro transduction. Cellular barcoding was used to monitor the migration patterns of antigen-specific T cells homing to two local inflammatory sites (**chapter 3**) as well as to determine the kinship of antigen-specific T cells developing into short-lived effector and long-lived memory T cells (**chapters 4** and **5**). Furthermore, cellular barcoding was used to assess to what extent the overall magnitude of T cell responses is regulated by the recruitment of naïve antigen-specific T cells and the expansion of each recruited cell (**chapter 6**). The second technology developed in this thesis, termed in vivo barcoding, makes use of a transgenic mouse model in which unique DNA sequences are introduced via an inducible VDJ recombination transgene. The feasibility of in vivo barcoding was demonstrated by labeling lung and liver cells with barcodes under temporal control of tamoxifen administration (**chapter 7**). Here I will discuss how the results described in this thesis have furthered our understanding on the process of T cell differentiation and the mechanisms that drive T cell heterogeneity. Furthermore, I will outline which questions still remain unanswered and how the concept of DNA barcoding might contribute to resolving some of these issues.

**Selective vs. aselective T cell migration**

In case a pathogen enters at a local tissue site, activated T cells do not embark on a random search for the location of the infection. Rather, extensive research has indicated that during activation, antigen-specific T cells can receive instructions to upregulate specific sets of homing molecules that can selectively guide these T cells to the infected tissue[1-7]. In chapter 3 we asked how the migratory behavior of activated T cells would be affected when these cells would have a choice between multiple independent inflammatory sites. An experimental setting was generated in which recipients of barcode-labeled OT-I T cells simultaneously received two local inflammatory challenges; a subcutaneous EL4-OVA tumor and an intranasal WSN-OVA infection. In this setting, antigen-presentation is induced in two separate draining lymph nodes, resulting in priming of part of the labeled OT-I T cell pool in each lymph node. This setup allowed us to study how T cells activated in either of the two draining lymph nodes would subsequently accumulate at the two available effector sites (the subcutaneous tumor and the inflamed lung).

At an early time point during the expansion phase, the barcode repertoires recovered from both draining lymph nodes were largely distinct. Because individual barcode-labeled OT-I T cells can only be present in one of the two draining lymph nodes during activation, this result verified that our technology can identify unrelated T cell populations (i.e. naïve T cells activated in separate lymph nodes) by the presence of different barcodes. In contrast, during the peak of the expansion phase, the barcode repertoires recovered from both draining lymph nodes as well as from both effector sites were nearly identical, indicating that T cells primed in each draining lymph node accumulated with comparable efficiency at both the corresponding as well as the "opposite" effector site. Similar results were found when mice were challenged

with a subcutenous EL4-OVA tumor and an intestinal LM-OVA infection, suggesting that also when T cells are primed in gut-draining lymph nodes, their progeny have (or can still acquire) the capacity to migrate to effector sites in the skin. Together, these results indicate that when inflammatory signals are abundantly present, the dominant pattern of T cell accumulation at effector sites is aselective.

How can our findings be reconciled with studies that reported on selective T cell migration[1-7]? First of all, it is important to recognize that expression of homing markers displays a marked degree of plasticity. Several studies have documented that expression of tissue-homing molecules could be reprogrammed when activated T cells entered a distant lymph node or were cultured with DCs associated with a distinct tissue site[3,5,7]. Based on these data, it seems that regulation of T cell migration is highly dynamic and T cell homing potential appears to be matched to whichever site the cells have last seen antigen. Therefore, one explanation for our results is that progeny of T cells primed in the skin-draining lymph node first migrated to the lung-draining lymph node and at that site got reprogrammed to enter the lung effector site[8]; and vice versa. If this were true, an increase in barcode overlap between both draining lymph nodes should precede overlap between the two effector sites. This was however not the case, as barcode overlap between both effector sites and draining lymph nodes coincided. Recently, it has been shown that T cell activation in the spleen and the mediastinal lymph node also results in intermediate induction of the integrin $\alpha4\beta7$ (REF[9]), the molecule that is required for efficient homing to the intestinal mucosa. Therefore, expression of certain homing molecules might be not as specific as previously thought, providing an explanation for the aselective T cell distribution that we observed in our study. In fact, aselective migration seems a preferred strategy to combat disseminating pathogens.

Despite the fact that during the peak of the expansion phase T cell migration appears aselective, our data do not rule out a role for selective migration in settings of more limited inflammation as for instance found during early time points of infection[7]. It is tempting to speculate that the first group of T cells that leaves the draining lymph node has the capacity to migrate efficiently to the corresponding site of infection and in that way helps contain the pathogen. Recently, it has been shown that the first wave of activated T cells that exit the lymph node are typically cells that express a lower affinity TCR[10]. It will be interesting to explore whether this lower affinity population is also the first to reach an effector site and whether this correlates with the expression of specific homing markers. If this would be the case, then barcodes at early time points are expected to overlap between draining lymph nodes and corresponding effector sites, but not between draining lymph nodes and "opposite" effector sites. Given that the gut has the strongest association with preferential T cell migration[2], it seems important to test the "selective early migration" hypothesis by using a more modest gut inflammation, such as a lower bacterial dose or a local protein-adjuvant immunization.

**Effector and memory lineage decisions**

It has long been recognized that activated antigen-specific T cell populations give rise to both short-lived effector cells and long-lived memory cells[11-15]. In fact, the generation of long-lived memory cells forms the basis of any successful T cell-based vaccination strategy. Besides by their longevity, memory cells are generally characterized by their enhanced capacity to respond to secondary antigen encounter, which typically results in a faster and more dramatic expansion compared to the primary immune response. Furthermore, during this secondary expansion, activated memory cells can yield a new wave of short-lived effector cells that are destined to die following pathogen clearance, tempting some to predict the existence of memory stem cells within the memory pool that retain all the 'stem cell-like' characteristics of naïve T cells[16,17].

In chapters 4 and 5 we examined at what point during an immune response antigen-specific T cells commit to either the short-lived effector or the long-lived memory lineage. Three distinct scenarios for this lineage decision have been proposed. I) Antigen-specific T cells commit to the effector or memory fate before

the first cell division. In such a model, fate would be either predetermined in the naïve T cell or would be determined by the nature of the priming APC or the time of T cell priming[18-21]. II) Antigen-specific T cells commit during the first cell division. In this model, the first T cell division would result in an asymmetric segregation of cell fate determinants, which would lead to two daughter cells displaying phenotypic and functional markers associated with either effector or memory fate[22,23]. III) Antigen-specific T cells commit at some point after the first cell division. In this model, additional signals received by antigen-specific progeny would be required to achieve full commitment to effector or memory fate. In this model, effector or memory lineage choice could occur at any point after the first T cell division and start of the contraction phase[11,24-26].

The major aim of chapter 4 and 5 was to investigate which of these three models most accurately predicts how effector and memory fate divergence is established. In chapter 4 we used barcode-labeled naïve T cells to discriminate between model I and II/III, by asking whether effector and memory T cells are progeny of distinct or similar sets of naïve T cells. In case model I would be correct, one would expect that different barcodes end up in the effector and the memory pool. In contrast, when model II/III would be correct, one would expect the same barcodes to end up in effector and memory populations. Recipients of barcode-labeled OT-I T cells were challenged with either a systemic LM-OVA or a local WSN-OVA infection. From these mice, effector barcodes were recovered at day 8 and memory barcodes were recovered at day 28 post infection. Barcode comparison revealed complete sequence overlap between both T cell populations, indicating that effector and memory cells are progeny of the same naïve T cells in both systemic and local infections. Similar results were obtained when mice were challenged with a recombinant LM-Q4-OVA strain, which expresses a variant of the $OVA_{257-264}$ epitope that OT-I T cells bind with a lower affinity[10]. These data are incompatible with model I of effector/memory fate divergence and thereby rule out a role for the nature of the priming APC or the time of naïve T cell priming.

In chapter 5 we went one step further and tested whether we could dissect model II of effector/memory fate divergence from model III. In case model II would be correct, one would expect that the first two daughter cells (daughter generation 1, D1) would already be committed towards becoming either effector or memory cells. In case model III would be most accurate, then D1 would still be uncommitted and commitment could occur anywhere ranging from D2 to D15, assuming that 15 times is about the maximum number of cell divisions that occur during the expansion phase. To distinguish between model II and III, we employed two different approaches. First, we made use of the property that retroviruses only integrate into activated T cells[27-30]. By infecting bulk activated OT-I T cell populations at an early time point, selective barcode labeling of D1-D4 cells but not naïve T cells could be achieved. Second, we made use of CFSE labeling technology, to sort OT-I D1-D3 cells by flow cytometry and label only those cells with barcodes. By transferring the barcode-labeled daughter cells into infection-matched recipients, we were able to monitor their developmental potential into effector and memory cells. In both experimental setups, we found that barcodes between effector and memory populations were largely overlapping, indicating that early daughter cells remain to a large extent multipotent. These data argue against model II of lineage diversification and suggest that progeny of activated T cells require additional signals for full commitment to either the effector or memory lineage.

So at what point following immunization does lineage commitment of effector and memory cells occur? To answer this question, it is important to understand whether development of memory cells represents an active process that is induced by certain stimuli, or whether memory cells develop via a more passive default pathway that is completed because these cells failed to develop into short-lived effector cells. Several studies have indicated that cells with memory-like characteristics, i.e. enhanced recall proliferation, can already be identified at early time points (day 3-5) after infection[31-33]. Furthermore, memory cells can be generated in response to very brief antigen exposure[25,34], as

well as following DC vaccination in the absence of overt inflammation[35,36]. As a third point, naïve T cells responding to lymphopenic environments have been shown to undergo a slow homeostatic proliferation during which these cells progressively acquire phenotypic and functional characteristics of antigen-induced memory cells[37-39]. These homeostatic proliferation-induced memory cells can be equally effective at mediating protective immunity as antigen-induced "true" memory cells[40]. Together, these studies have suggested that memory cells develop via a default pathway following antigen-induced activation that is entered as soon as naïve T cells start dividing[14,41].

Based on this knowledge, the most pertinent experimental question appears to be via what mechanism memory precursors commit to the short-lived effector fate? Experimental evidence indicates that when inflammation increases, this favors the generation of effector cells[26,42-45]. Signaling by the proinflammatory cytokine IL-12 induces expression of T-bet while it represses eomesodermin (Eomes)[43], two transcription factors that are associated with the effector and memory lineage, respectively[26,46,47]. Similarly, sustained signaling by IL-2 selectively drives cells into the effector lineage[48,49].

But when would activated T cells receive these effector-skewing signals? The observation that cells with a terminally-differentiated phenotype can be identified at four days after infection[26,33], suggests that lineage commitment can occur during the first half of the expansion phase. It seems reasonable to assume that the time point when activated T cells are most receptive for lineage-committing signals is during their stay at the initial priming site, where the concentration of stimulatory immune cell types and soluble factors is likely to be high. Even though a very brief antigenic stimulus can drive T cells into clonal expansion[50], full activation and development of effector function has been shown to require sustained signaling by antigen, costimulation and cytokines for at least 40 hours[51]. At the end of this 40 hour activation phase, T cells will typically have divided three times, suggesting that effector commitment might be observed the earliest in the fourth daughter generation. This hypothesis is reinforced by the observation that it takes approximately 4-5 divisions before locally-primed T cells start appearing at distant lymph nodes[7,52], suggesting that the capacity to migrate to other tissue sites, a hallmark of effector cells, is first present in the fourth or fifth daughter cell generation. Currently, we are investigating this hypothesis, by systematically labeling different daughter cell generations (D1-D6) with barcodes and testing whether individual cells in these populations still develop into both effector and memory cells, or whether the progeny of more downstream daughters (D4-D6) is already committed to the short-lived fate.

**Efficiency of naïve T cell recruitment**

Given that T cell responses of increased magnitude generally also leave higher memory cell numbers[53,54], a major aim of vaccine optimization has been to induce strong T cell responses. According to the clonal selection theory, the magnitude of T cell responses is the product of both the number of recruited naïve antigen-specific T cells and the expansion of each recruited cell. Therefore, a stronger T cell response could occur as a consequence of an increase in naïve T cell recruitment and/or an increase in clonal expansion. It has been unclear how these two factors regulate the magnitude of natural pathogen-induced T cell responses. Because effective adaptive immunity depends on the recognition of a wide range of pathogens, the diversity of lymphocyte antigen receptors is large. Consequently, the frequency of T cells that can recognize any given antigen is extremely low (less than 1 in $10^5$ cells)[55-57]. As a result, the activation of a single antigen-specific T cell requires on average $10^5$ T cell-DC scanning interactions and the number of required interactions to recruit additional antigen-specific T cells increases progressively with each successive recruitment event. Based on this, it can be calculated that complete recruitment of an antigen-specific T cell population (~200 cells) requires >$10^8$ T cell-DC interactions.

Determining how recruitment and expansion regulate antigen-specific T cell responses has long been a fundamental question in immunology, which has been difficult to address due to a lack of required experimental

technology. In chapter 6, we addressed this question by cellular barcoding and specifically asked how recruitment efficiency would be affected under varying conditions of infection. In settings where we either varied pathogen dose by 100-fold, limited the duration of infection to three days or challenged mice with systemic and local infections, we found that the magnitude of T cell responses differed by ~10-15 fold. In contrast, the number of different barcodes recovered from the responding T cell populations fluctuated by only ~1.5-fold, indicating that the number of naïve T cells that gave rise to these T cell populations was markedly constant. Furthermore, by using CFSE dilution we were able to show that >95% of the naïve antigen-specific T cell repertoire is recruited upon infection, demonstrating that even under conditions of weak infection, the vast majority of the available antigen-specific repertoire participates in the immune response. Together, these data showed that naïve T cell recruitment is highly efficient, implying that the magnitude of T cell responses is primarily regulated by clonal expansion.

These results raise two important questions: I) what makes naïve T cell recruitment so efficient and II) how is the extent of clonal expansion regulated? Understanding why recruitment is so efficient first of all requires an appreciation of the astounding rate at which naïve T cells scan antigen-presenting DCs. Using intravital imaging, several studies have calculated that DCs can interact with 500-5000 different naïve T cells/hour[58-60]. Assuming that recruitment of one antigen-specific T cell requires on average $10^5$ scanning interactions, we calculated that $5.9x10^7$ T cell-DC interactions would be required to recruit 95% of the naïve antigen-specific repertoire. As each DC can interact with $>10^4$ naïve T cells/day, this suggests that a pool of <2000 antigen-presenting DCs could suffice to recruit 95% of naïve antigen-specific T cells in a three day time period. These numbers provide a theoretical basis to explain the feasibility of the experimentally-observed recruitment efficiency. Perhaps the most important variable in these calculations is whether even weak infections lead to the constant presence of ~2000 antigen-presenting DCs for a three day time period. Although experimental technology to enumerate the total number of antigen-presenting DCs during an infection is lacking, this hypothesis might be tested by injecting different numbers of in vitro antigen-loaded DCs over consecutive days. These experiments could reveal whether a few thousand antigen-presenting DCs are sufficient to activate a substantial part of the naïve repertoire. Alternatively, antigen-presenting DCs could also be depleted by using CD11c-DTR (diphtheria toxin receptor) mice, in which application of DT can induce depletion of DCs within six hours[61]. This system could accurately test the duration of the recruitment phase and therefore can reveal what percentage of the naïve repertoire is entering clonal expansion at each consecutive day after start of infection (assuming that T cell expansion is sufficient for proper barcode sampling).

Next to understanding the efficiency of recruitment at the intercellular level, it will also be interesting to dissect recruitment efficiency at the intracellular level. The most pertinent question here is what factors involved in naïve T cell activation are crucial for the observed recruitment efficiency? Two usual suspects in this regard are TCR affinity and availability of costimulatory molecules. Although very low affinity TCR-antigen interactions can induce T cell activation in vivo[10], it remains unclear what percentage of the total repertoire can become activated in these settings. Due to their rapid exit from the priming site, these low affinity T cells might play an important role as first line of defense[10], given that they are outcompeted by high affinity T cells during the expansion phase[62-64]. By infecting mice with LM-APL-OVA strains, which express mutants of the native $OVA_{257-264}$ peptide[10], it should be relatively straightforward to measure the recruitment of OT-I T cells under settings of different functional avidity. It will be interesting to combine these experiments with settings in which various costimulatory molecules, such as CD27 or CD28, are either present or absent on the T cell surface[65]. Especially CD28 appears an interesting candidate, given that in vitro, CD28 signaling has been shown to effectively lower the required amount of antigenic stimulation for cell cycle entry[66,67]. Nevertheless, it remains

to be determined whether absence of CD28 signaling would limit naïve T cell recruitment in vivo under conditions of infection, or whether in such settings lack of CD28 could be overcome by the presence of sufficient antigenic or other costimulatory input.

Given that naïve T cell recruitment is near constant, this implies that clonal expansion is the main process regulating the strength of T cell responses. The extent of clonal expansion, or the clonal burst size, reflects the net sum of all T cell proliferation and cell death. Understanding how burst size is regulated at the cellular level therefore requires the contribution of proliferation and cell death to be discerned. Dissection of these two parameters would require a cellular marker that is maintained in the T cell population upon cell division and is lost upon cell death. Currently, we are exploring the technical feasibility of such an approach, by making use of a genetic reporter system that can provide T cells with a single extrachromosomal DNA minicircle by Cre-mediated excision. As these DNA minicircles do not replicate during division, they can provide a direct measurement of the fraction of surviving T cells over time. Conceptually, this approach bears resemblance to the measurement of TRECs to quantify thymic output[68-70], with the exception that this tagging system can be induced by Cre expression, allowing temporal control over the time point from which cell survival is monitored. Furthermore, the minicircle could be designed such that it only becomes transcriptionally active upon excision, thereby allowing the fraction of cells that carry the minicircle to be quantified by flow cytometry. In theory, combining this clonal burst size reporter with the use of cellular barcoding should allow one to simultaneously measure naïve T cell recruitment as well as subsequent T cell division and cell death, thereby providing insight into how these three factors shape the magnitude of antigen-specific T cell responses.

**Cell fate analysis by in vivo barcoding**

A disadvantage of current technologies for cell fate determination at the single-cell level is that these strategies rely on the adoptive transfer of cells of interest. This implies that cells are harvested from one anatomical site (e.g. the spleen or bone marrow) and subsequently transferred into another (the blood). Furthermore, efficient engraftment of precursor populations often requires conditioning of the host (i.e. by irradiation), which can dramatically alter the environment in which cell fate is being monitored. As a third disadvantage, strategies that rely on adoptive transfer are not suitable for fate determination of non-hematopoietic cell types. To overcome these limitations, we set out to develop a novel barcoding strategy in which cells can be marked with unique identifiers by DNA recombination. In chapter 7, we demonstrated the feasibility of this approach, termed in vivo barcoding, by inducing unique genetic tags in lung and liver cells of non-hematopoietic origin. This technology, which is based on the mechanism behind antigen receptor diversification in developing lymphocytes[71-73], makes use of a transgene that allows induction of V(D)J recombination in non-lymphoid cells by Cre/lox technology. Following expression of Cre recombinase, a cassette encoding the VDJ recombinase enzymes RAG1, RAG2 and TdT is expressed. Subsequently, these three enzymes drive recombination of a pseudo-VDJ substrate, thereby leading to DNA diversification at the recombining V-D-J junctions as well as inducing GFP expression as a marker for successfully recombined cells. By temporally controlling Cre expression via tamoxifen administration, we showed that barcodes can be generated in non-hematopoietic tissues in vivo. Thus, this system allows control over the time point from which cell fate is being monitored.

How can this strategy be used to study cell fate with minimal perturbation of the host? Because introduction of barcodes depends on expression of Cre recombinase, two possible ways to follow a specific cell population can be envisioned. One strategy relies on the use of tissue-specific promoter systems to drive Cre expression in a given cell lineage. Ideally, such promoter systems activate Cre during a certain developmental stage and then switch off expression as soon as cells differentiate to a further stage. This would allow one to specifically assess the fate of precursor cells present at a defined developmental stage. Unfortunately, in most tissue-specific promoter

systems, Cre expression stays on even at later stages of differentiation, making it difficult to determine whether distinct barcodes present in two different cell populations were derived from separate precursors or from recent novel recombination events. To circumvent these issues, a second strategy relies on the temporal induction of Cre recombinase by administration of a soluble compound. In Cre-ER$^{T2}$ mice, Cre can only enter the nucleus and induce recombination in the presence of the ER-ligand tamoxifen[74]. As mentioned, in our transgenic mice, tamoxifen administration during five consecutive days resulted in the recombination of a small fraction of CD45$^-$ lung and liver cells, demonstrating the potential of this Cre system for temporal control of in vivo barcoding. An additional interesting feature of Cre-ER$^{T2}$ mice is that tamoxifen could also be administered to pregnant females, in which case recombination can be induced in developing embryos[74]. In combination with in vivo barcoding, this strategy potentially allows fate mapping of embryonic development in a mammalian system. Finally, both strategies for controlling Cre expression could also be combined in one experimental setup, resulting in a tissue-specific promoter driving expression of the Cre-ER$^{T2}$ construct[75]. Such combined system would allow both temporal and spatial control of in vivo barcoding.

As discussed more elaborately in chapter 7, combining in vivo barcoding with the above described Cre systems not only allows one the study kinship during embryonic or hematopoietic development, but also allows one to quantify stem cell contributions to different organs or tumor populations. The most important advantage of in vivo barcoding is that this strategy allows cell fate determination without having to isolate cells from their normal niche. The ever increasing mouse tool box of Cre transgenic lines (The Jackson Laboratory alone offers 165 different lines) and the increased access of more and more researchers to high-throughput sequencing technology should warrant sufficient applications for the use of in vivo barcoding. Future analysis should reveal whether the current mouse model suffices to address most experimental questions or whether the construct design should be further improved to yield greater barcode diversity or to enhance recombination efficiency in different cell lineages.

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