Tracing T cell differentiation by genetic barcoding

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Chapter 1

Introduction and scope of this thesis

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T cells play a crucial role in preventing takeover of the body by pathogenic microorganisms, such as different viruses, bacteria, fungi and parasites. Because the body can suffer from such a wide variety of insults, the pathogen recognition potential of T cells needs to be extremely broad. Both $CD8^+$ (killer) and $CD4^+$ (helper) T cells express specific T cell receptors (TCRs) that can recognize antigenic peptides presented by major histocompatibility complex (MHC) molecules. $CD8^+$ T cells recognize antigens in the context of MHC class I, which is expressed on the surface of all nucleated cells. In contrast, $CD4^+$ T cells recognize antigens presented by MHC class II, which is only found on professional antigen-presenting cells (APCs) that consist of dendritic cells (DCs), B cells and macrophages. Due to this divergence in antigen recognition, T cells play different roles in the immune response against pathogens. The main function of $CD8^+$ T cells is to track down and kill infected host cells, whereas $CD4^+$ T cells are primarily involved in activating and directing other cells of the immune system, such as B cells, macrophages and $CD8^+$ T cells.

Generation of T cell receptor diversity

The key to the recognition of a broad array of antigens lies in the way that TCRs are generated in the thymus. TCR $\alpha\beta$ heterodimers are comprised of variable ($V\alpha$ and $V\beta$) and constant ($C\alpha$ and $C\beta$) domains. $V\beta$ domains are encoded by variable (V), diversity (D) and junctional (J) gene segments, while $V\alpha$ domains are only encoded by V and J segments. Selection and rearrangement of these gene segments into single TCR α and β chains is performed by a mechanism known as V(D)J recombination[1,2]. The TCR α locus contains ~70 V segments and 60 J segments, whereas the TCR β locus harbors about 50 V segments and two separate clusters each containing a single D segment and six or seven J segments. Each of these gene segments is flanked by a recombination signal sequence (RSS). These RSSs consist of two types and are comprised of a conserved heptamer and nonamer sequence, separated by a 12 or 23 base pair (bp) spacer. The length of this spacer determines the functionality of the RSS and efficient recombination occurs only between two gene segments flanked by a 12-bp RSS and a 23-bp RSS, known as the 12/23 rule[3]. To allow proper V(D)J recombination, all V segments in the TCRα locus are flanked by a 3' 23-bp RSS, while all J segments have a 5' 12-bp RSS. In the TCRβ locus, V and J segments have similar RSS arrangements as in the TCRα locus, whereas D segments are flanked by a 5' 12-bp RSS and a 3' 23-bp RSS, allowing proper V-to-D and D-to-J joining.

V(D)J recombination occurs in two distinct phases and is initiated by the lymphoid-specific recombination activating gene (RAG) proteins RAG1 and RAG2, which cooperate to recognize RSSs and to induce a single-strand DNA break between each RSS heptamer and the neighboring gene segment[4]. Subsequently, a 12-bp RSS-RAG complex synapses with a 23-bp RSS, leading to the generation of double-strand DNA breaks that yield two hairpin coding ends and two blunt signal ends. In the second phase of V(D)J recombination, the synapsed coding and signal ends are joined by the double-strand break repair machinery known as non-homologous end joining (NHEJ). Signal ends are usually joined by precise head-to-head fusion, giving rise to TCR excision circles (TRECs), which have been used as an experimental tool to measure thymic output[5-7]. In contrast, joining of coding ends is imprecise and characterized by the deletion and insertion of nucleotides. First, to initiate repair, hairpin coding ends are opened by the enzyme Artemis[8]. This opening can either occur exactly at the center of the hairpin or can occur off-center, in the latter case leaving a single-strand overhang. During further repair this single-strand overhang will be filled in, resulting in the insertion of palindromic P nucleotides at the coding joint[9]. Second, in addition to templated P nucleotides, nontemplated N nucleotides can also be inserted following hairpin opening, and this occurs by the activity of the enzyme terminal deoxynucleotidyl transferase (TdT)[10,11]. Alternatively, nucleotides can also be deleted from coding ends by exonuclease activity.

Together, these three mechanisms give rise to enormous nucleotide diversification at the V-J and V-D-J joints of the TCR α and β chains, and these hypervariable coding joints are known as the complementarity-determining region 3 (CDR3). Thus, whereas the selection of single V-J and V-D-J combinations out of many available gene segments already allows more than 10^6 different VαVβ pairs, the addition of junctional variability ultimately allows a theoretical diversity of ~10^{18} different TCRs to be generated[12]. Given that the CDR3 regions of the TCR form the center of the antigen-binding site, the extreme variability of this region provides a structural basis for the required broad antigen recognition potential of the total T cell repertoire.

Initiation of T cell responses

Although theoretically a T cell repertoire of ~10^{18} different TCRs could be generated, many of these TCRs will never see the light of day. In the thymus, positive and negative selection filter out all TCRs that either cannot bind self peptide-MHC complexes or that recognize self peptide-MHC complexes with high affinity. As a result, only a tiny fraction of all generated TCRs eventually enter the peripheral T cell pool, which has been estimated to have a size of ~10^7 distinct TCRs in mice and ~10^8 in humans[13,14]. More important than the number of distinct TCRs is the number of T cells that can actually recognize any given antigen. Based on titration of known quantities of TCR transgenic T cells and pull-down assays with MHC tetramers, it has been estimated that uninfected mice contain ~200 T cells specific for any single antigen[15-17]. Given that mice have ~2x10^7 $CD8^+$ T cells, this means that naïve antigen-specific $CD8^+$ T cells are present at a frequency of ~1 in 10^5 cells. In other words, when a foreign antigen enters a host, on average 10^5 naïve $CD8^+$ T cells have to be scanned by the pool of antigen-presenting DCs in order to find one T cell that can recognize the foreign antigen and respond. This seemingly impossible objective is largely overcome by the astounding rate at which naïve T cells scan antigen-presenting DCs, which has been estimated to range from 500-5000 different naïve T cells interacting with one DC per hour[18-20].

When naïve T cells encounter their cognate antigen in the spleen or lymph nodes, these T cells arrest and undergo stable interactions with the antigen-presenting DCs[21-24]. During these stable contacts, naïve T cells receive instructions to embark on a program of proliferation and differentiation that does not require continuous antigenic stimulation[25,26]. In general, T cell responses to acute infections can be divided into three distinct phases: I) during the expansion phase, activated naïve T cells proliferate rapidly, acquire effector functions, migrate to sites of infection and mediate pathogen clearance by killing infected cells[27-32]; II) during the contraction phase, ~90% of activated T cells die, leaving behind a stable pool of long-lived memory cells[33-37]; III) during the memory phase, memory cells are maintained at stable levels for many years and can provide enhanced protection upon pathogen re-exposure[38-42].

Diversity of T cell responses

During the process of differentiation, the progeny of activated T cells can adopt many distinct fates. As mentioned above, effector and memory fate can be separated based on a difference in longevity, as well as on the distinct ability of memory cells to undergo recall proliferation and differentiation into secondary effector cells. Combining both parameters of longevity and proliferative potential allows at least four different T cell subsets to be distinguish, namely naïve, effector, effector-memory (T_{EM}) and central-memory (T_{CM}) T cell populations[43]. T_{EM} cells, which mainly reside in peripheral tissues, are considered to have a shorter lifespan and decreased proliferative potential compared to T_{CM} cells that are found in secondary lymphoid organs[44-46]. In addition to memory cells, effector T cell populations also display heterogeneity. Depending on Notch ligands present on the priming APC, naïve CD4 T cells can be instructed to differentiate into either IFN-γ secreting T_H1 or IL-4 secreting T_H2 cells[47,48]. Furthermore, depending on the signals encountered, CD4 T cells can give rise to additional subsets, such as IL-17 producing T_H17 cells and *Foxp3*-expressing induced regulatory T cells[49-53]. Along similar lines, the nature of the priming APC can also induce distinct migratory potential in effector T cells[54-57].

From these data, it is clear that T cell responses are highly diverse and that depending on the conditions of infection, T cell differentiation can follow alternative paths to give rise to distinct T cell subsets.

Scope of this thesis

In this thesis, I investigated how activation of a pool of naïve antigen-specific T cells can lead to the generation of different T cell subsets that are characterized by distinct phenotypes or functions. Conventionally, cell fate is monitored at the bulk population level, by adoptively transferring T cells separated by a congenic or fluorescent marker into recipient mice. However, such strategies cannot discriminate whether all of the transferred cells display a certain behavior, or whether part of the transferred cells differentiates into one lineage and a second part into another lineage. To address such fundamental questions regarding T cell differentiation, methods are required that can follow the fate of individual cells rather than bulk populations. In such an experimental setup, each precursor cell would have to bear a unique and heritable marker to allow the progeny of different precursors to be distinguished.

To allow high-throughput assessment of differentiation at the single-cell level, we have developed two novel technologies in which individual precursor cells are marked with unique DNA sequences (barcodes) and cell fate is analyzed by barcode comparison of different daughter populations. In the first technology, termed cellular barcoding, introduction of barcodes is performed in vitro using a retroviral barcode library; after which cell fate is monitored by reintroduction of the labeled cells in vivo. The second technology, termed in vivo barcoding, allows the introduction of unique DNA sequences in vivo, via an inducible V(D)J recombination transgene; thereby overcoming the requirement to isolate cells out of their natural environment for barcode-labeling and allowing cell fate study of non-hematopoietic cells.

Given that naïve T cells only circulate through secondary lymphoid organs, pathogen entry at a peripheral tissue site requires antigen-specific T cells to gain novel migratory capacities. Tissue-selective migration of T cells is mediated by unique combinations of adhesion molecules and chemokine receptors, which are induced during T cell activation and can differ depending on the tissue origin of the priming APC. T cell activation by gut-associated DCs results in the upregulation of the integrin $\alpha4\beta7$ and the chemokine receptor CCR9, whereas skin-associated DCs induce E- and P-selectin ligands as well as CCR10[58]. During infection, inflamed intestinal epithelial cells release CCL25 (the ligand for CCR9), while inflamed keratinocytes release CCL27 (the ligand for CCR10), thereby providing a mechanism to guide effector T cells to the site of pathogen infection[59]. Indeed, it has been shown that T cells that are activated by gut- or skin-associated DCs, preferentially migrate to the corresponding effector site[54,55]. Although these studies have suggested that T cell migration can be selective when antigen is present at a local site, it remains unclear how migration patterns are influenced by the presence of multiple inflammatory sites within the same host, as can be seen during systemic infections. In **chapter 3** we use cellular barcoding to monitor T cell migration, by simultaneously challenging recipients of barcode-labeled ovalbumin (OVA)-specific OT-I T cells with a subcutaneous EL4-OVA tumor and an intranasal influenza-OVA (WSN-OVA) infection. In this setup, two local independent effector sites are generated, resulting in activation of part of the barcode-labeled OT-I T cell pool in each of the corresponding draining lymph nodes. By analyzing the barcode content of T cells that migrated to distinct effector sites, this study reveals that virually all T cell clones are endowed with the capacity to home to different effector sites, independent of their site of activation.

Following antigen-induced activation, T cells undergo massive clonal expansion, during which a starting populations of a few hundred cells can give rise to more than 10^6 antigen-specific progeny[27,28]. After resolution of the infection, a contraction phase ensues during which the number of antigen-specific T cells declines to ~10% of the number that was present at the peak of the response. The population of T cells that remains after contraction forms the memory pool that is generally stable in number

and can provide long-term protection against re-infection[43,60-62]. Although these data intuitively suggest that at the peak of the expansion phase ~90% of the antigen-specific T cell population is comprised of short-lived effector cells and ~10% are precursors of long-lived memory cells, the mechanism behind this divergence in cell fate remains poorly understood. In **chapter 4** we use cellular barcoding to examine whether short-lived effector and long-lived memory cells differentiate from the same or from distinct sets of naïve T cells. To this end, we challenge recipients of barcode-labeled OT-I T cells with *Listeria monocytogenes* expressing OVA (LM-OVA) and compare the barcode content of T cells present at the peak of the response (day 8) to T cells present in the resting memory phase (day 28). This analysis shows that effector and memory T cells are progeny of the same naïve T cells, indicating that commitment to either the short- or the long-lived fate is not predetermined in the naïve T cell and is also not determined by the nature of the priming APC or the time of T cell priming[63-66].

One study has suggested that divergence in effector and memory fate might be regulated by asymmetry during the first T cell division[41]. In this model, the prolonged interaction between the naïve T cell and the APC results in an unequal segregation of cell fate determinants between the proximal and the distal daughter T cell. Indeed, proximal and distal T cell daughters were shown to display differential expression of phenotypic and functional markers associated with effector and memory fate, such as CD62L, IL-2Rα, IL-7Rα and granzyme B[43]. In **chapter 5** we test this hypothesis of cell fate divergence during the first T cell division by labeling the first four OT-I daughter cell generations (D1-D4) that arise after LM-OVA infection with barcodes and monitoring their fate in infection-matched recipients. This study shows that virtually all early daughter cells are still multipotent and can yield both short-lived effector and long-lived memory cells, suggesting that lineage commitment occurs not during but after the first T cell division.

Since the dawn of vaccination in 1796, when Edward Jenner discovered that inoculation of cowpox could be used to induce protection against human smallpox, immunologists have aimed to generate vaccines that induce strong immune responses. With the introduction of the concept of clonal selection by Frank Burnet in 1957 (REF[67]), it became clear that 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. Yet, how these two factors regulate antigen-specific T cell responses (i.e. is a stronger immune response caused by more efficient recruitment or by a more efficient expansion) has not been understood, due to the difficulty of measuring naïve T cell recruitment. In **chapter 6** we use cellular barcoding to quantify recruitment of antigen-specific T cells in response to disparate conditions of infection and link this data to the overall magnitude of the T cell response, allowing us to calculate clonal expansion. These results show that recruitment of rare antigen-specific T cells is highly efficient and that the strength of T cell responses is primarily controlled by clonal expansion.

Although cellular barcoding is a powerful technology to study the simultaneous differentiation of many cells at the single-cell level, this strategy is based on in vitro transduction and therefore limited to the analysis of transplantable cell types. Ideally, a barcoding technology would allow tracing of cell fate without having to isolate the target precursor cells out of their natural environment. Such technology would require the induction of unique genetic tags in vivo. In the vertebrate immune system there is a clear precedent for this concept; namely the recombination machinery that generates antigen receptor diversity. In **chapter 7** we have generated a novel mouse model that allows in vivo barcoding by inducible V(D)J recombination. In this model, Cre recombinase initiates expression of the V(D)J recombinase enzymes RAG1, RAG2 and TdT, which subsequently drive nucleotide diversification of a pseudo-VDJ substrate and induce GFP expression as a marker. This study demonstrates the feasibility of in vivo barcoding, by conditionally inducing recombination in lung

and liver tissues and showing that many of these cells contain unique DNA sequences. This technology should prove useful for the analysis of lineage relationships and stem cell issues of virtually any cell type in vivo.

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