Assessing T cell differentiation at the single-cell level

Gerlach, C.

1

GENERAL INTRODUCTION

Thanks to our immune system we only rarely suffer from infectious diseases, although we are continuously exposed to pathogens. Many pathogens are prevented from accessing our body by the physical barriers of our skin and inner epithelia, but when a pathogen succeeds in crossing these barriers, it is attacked by the immune system from multiple angles.

The first signs of 'danger' (infection and tissue damage) are sensed by the innate immune system. Macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) cells, mast cells and eosinophils are classified as cells of the innate immune system. They express intracellular and extracellular innate immune receptors that are also called pattern recognition receptors (PRR), which allow them to recognize molecular patterns that are indicative of infection (pathogen-associated molecular patterns; PAMPs) or tissue injury (damage-associated molecular patterns; DAMPs)[1-7]. Interestingly, these mechanisms of innate immune recognition are shared between vertebrate animals, invertebrates and even plants[8-10]. The ability to recognize PAMPs and DAMPs allows the innate immune system to identify pathogens and infected, damaged, and in some cases cancerous host cells. Bacteria are recognized by their lipopolysaccharide or teichoic acid structures, which are essential cell wall components of gram-negative or gram-positive bacteria, respectively[1,2,11]. Infected host cells can be identified by the presence of DNA containing unmethylated CpG motifs, which is characteristic of bacterial DNA, or by the presence of double-stranded RNA, which is indicative of viral infection[1,2,11]. Also cells lacking the expression of molecules that are normally present on the cell surface, such as major histocompatibility complex (MHC) class I and sialylated glycoproteins and glycolipids can be identified as infected or cancerous[2]. Conditions of cellular stress and injury are sensed if endogenous factors that are normally shielded from recognition by the immune system are released or exposed upon cell death[3-7,12]. Examples of such DAMPs are heat shock proteins, uric acid and mitochondrial DNA[3,7,12].

Both PAMP and DAMP detection activates the innate immune cells to commence pathogen clearance, the inflammatory response and the adaptive immune response. Pathogen clearance is initiated by locally resident macrophages and maintained by neutrophils, macrophages, DCs and NK cells that are recruited to the site of infection or injury in response to inflammatory signals. The phagocytes engulf pathogens, abnormal cells and cell debris and subsequently destroy it. NK cells actively lyse infected or otherwise abnormal host cells through the release of cytotoxic molecules. The inflammatory response is initiated when tissue resident macrophages release cytokines and chemokines upon PAMP or DAMP binding. Inflammation functions as an alarm signal sent out to further parts of the body to recruit other innate and adaptive immune cells to the site of infection, where many of these join in the production of inflammatory mediators. As all innate immune cells of the same cell type express the same set of PRRs, virtually all cells of that particular cell type can aid in the response. The high numbers of readily available innate immune cells provide

immediate action. However, complete resolution of infection often requires additional actions of the adaptive immune system, which is activated when DCs and to minor extent macrophages that had been recruited to the site of infection migrate further to lymphoid organs where they activate B and T lymphocytes.

B and T lymphocytes (also termed B and T cells) are the cells of the adaptive immune system. These cells are predominantly activated by mature DCs and only when the DCs display pathogen-derived peptide fragments on their cell surface. In contrast to innate immune cells, lymphocytes do not recognize general patterns associated with infection or injury, but specific pathogen-derived peptide sequences. This specificity is provided by the B cell receptor (BCR) or T cell receptor (TCR) molecules[13-17]. The exact BCR and TCR sequence – and thereby lymphocyte specificity - is generated through random gene rearrangements within the BCR and TCR loci occurring during lymphocyte development. This process generates a large diversity of lymphocyte specificities which ensures that the adaptive immune system can identify also those pathogens that have evolved strategies to evade their recognition by innate receptors. As a downside to the large diversity in lymphocyte specificities, each pathogen is recognized by not more than a minor subset of B and T cells that needs to be expanded by extensive proliferation in order to generate sufficient cell numbers to clear the infection – a process that takes time. Adaptive immunity is therefore slow, but highly pathogen-specific. Furthermore, adaptive immune cells are capable of providing long-lasting protection to previously encountered infections, which is termed 'memory'[18-20] and forms the basis of most prophylactic vaccines[18,21]. Immune memory provides the host with large numbers of pathogen-specific B and T cells that are rapidly reactivated upon renewed infection with the same pathogen.

The major difference between B and T cell responses to infection is the mechanism by which the cells exert their function. B lymphocytes act through the secretion of antibodies, which are soluble proteins that can directly bind extracellular pathogens and thereby tag them for destruction by the innate immune system. T lymphocytes in contrast secrete cytokines and cytotoxic proteins. Depending on their mode of antigen recognition and particular functions, T lymphocytes are subdivided into $CD4^+$ and $CD8^+$ subsets. $CD8^+$ T lymphocytes recognize antigen presented by MHC class I molecules (and thus mainly derived from intracellular pathogens) and can directly kill infected host cells through mechanisms similar to NK cells. $CD4^+$ T cells on the contrary recognize antigen derived from phagocytosed particles, presented on MHC class II complexes. These cells mainly act through the secretion of inflammatory or suppressive cytokines and play a role in enhancing B cell and $CD8^+$ T cell responses, or alternatively, in dampening the actions of $CD8^+$ T cells to prevent excessive responses.

This thesis focuses on $CD8^+$ T cell responses to infection, which will therefore be described in more detail here. $CD8^+$ T cell responses are initiated when naïve, antigen-specific $CD8^+$ T cells encounter mature DCs that display pathogen-derived peptides bound to MHC class I molecules on their cell surface. This leads to activation of the

antigen-specific T cells, resulting in their proliferation and differentiation. Already early during the response, the pool of activated $CD8^+$ T cells displays a remarkable heterogeneity, with lymph node homing molecules (CD62L)[22,23], cytokines (IL-2)[24], cytokine receptors (CD25, CD127)[22,24-27] and other molecules (KLRG-1)[24,28-30] being differentially expressed. Nevertheless, most activated T cells acquire effector functions, which allow them to specifically lyse infected host cells. After pathogen clearance, the majority of activated T cells die by apoptosis, but a small fraction (~10%) remains alive and forms a stable pool of long-lived memory cells[19].

In this thesis I wished to investigate I) how different antigen-specific $CD8^+$ T cell clones contribute to the heterogeneity within the $CD8^+$ T cell respons, II) at what point during *in vivo* $CD8^+$ T cell differentiation fate decisions take place and III) to what extent the clonal expansion of individual antigen-specific $CD8^+$ T cells shapes the overall response magnitude. To address these issues, it is crucial to follow individual T cells over time rather than tracking the behavior of a cell population, as not necessarily all cells within a population follow the same path of differentiation.

Over the past years, several *in vitro* and *in vivo* technologies have been developed with the aim to track individual cells. **Chapter 2** describes these technologies and discusses their potential and limitations, and how informative lineage tracing experiments should be set up. This chapter also describes the 'cellular barcoding' technology that we have developed in chapter 4.

Since long, immunologists have been fascinated by the question why some antigen-specific T cells are able to persist long-term as memory cells, while others die by apoptosis when the infection has been eradicated. What determines whether a T cell adopts a short-lived or a long-lived fate and when is this fate decision taken? **Chapter 3** of this thesis discusses the current knowledge regarding the generation of memory T cells. The main focus lies on different models that have been proposed to explain when $CD8^+$ T cells commit to a short- or long-lived fate.

Most single-cell tracking methodologies are limited by the number of individual cells that can be followed over time. To address this issue, we have developed a technology termed 'cellular barcoding' that enables fate mapping of hundreds of individual T cells during infection *in vivo*. With this technology, individual T cells are provided with unique DNA sequences (barcodes) that are transferred to all progeny of the labeled T cell during cell division. In this way, all T cells that share a common precursor are marked with the same genetic tag. To determine which barcodes are present in a particular cell population, the DNA content of the cells is isolated, barcode sequences are amplified and subsequently hybridized onto a barcode-microarray for sequence identification. In **chapter 4** we describe this novel technology and apply it to determine whether T cells that have been activated in a particular lymph node preferentially migrate towards the organ that was drained by the lymph node in which the T cells were activated, or whether T cells also migrate to distant tissue sites, irrespective of their site of priming.

As discussed in chapter 3, a longstanding question in immunology is at what point during an immune response CD8$^+$ T cells commit to a short- or long-lived fate. One of the proposed models predicts that this fate is imprinted in the antigen-specific T cells as early as during T cell activation and thus before the first cell division[31]. In this model, the factor that determines T cell fate is the strength of T cell activation, as imposed by the priming dendritic cell and its surroundings. In particular at early and late times after infection, the levels of costimulatory molecules and antigenic peptides on the surface of the dendritic cells, as well as the inflammatory environments are likely to differ to such an extent that T cell activation strengths could be considerably dissimilar. In **chapter 5**, we directly tested by cellular barcoding if short- and long-lived T cell fates are *in vivo* imprinted into activated T cells before their first cell division. To achieve this, it was crucial to obtain naïve T cells that are uniquely labeled by a barcode, so that we could study if these naïve T cells developed into either short-lived effector cells or long-lived memory cells or both. Therefore, I first developed a new technology that allows the generation of naïve, barcode-labeled T cells. This technology relies on the transduction of thymocytes with the barcode sequences and is described in detail in this chapter.

An alternative model has been put forward that poses that fate commitment does not occur before, but during the first cell division through a process called asymmetric cell division[32,33]. Specifically, Reiner and colleagues have proposed that through the asymmetric partitioning of cell fate determining factors into the first two daughter cells, the daughter cell that is formed proximal to the priming dendritic cell will commit to a short-lived effector fate, while the distal daughter cell adopts a memory fate. In **chapter 6** we test this hypothesis by providing daughter cells of the 1st to 3rd generation with our unique barcode sequences and subsequent monitoring if these early daughter cells were already committed to either fate.

In addition to the functional heterogeneity that exists within the responding T cell population, CD8$^+$ T cell responses to different infections are highly variable in their overall size. The magnitude of the total response depends on the pathogen type, dose of infection and route of pathogen entry. In general, more severe infections lead to larger T cell response sizes. In **chapter 7**, we address the question how the magnitude of the overall CD8$^+$ T cell response is regulated. Principally, this could be achieved either through regulating the number of antigen-specific naïve T cells that are recruited into the response, or through controlling the expansion (a combination of proliferation and cell death) of the recruited T cell clones. As the overall magnitude of the response is the product of naïve T cell recruitment and clonal expansion, measuring two of these parameters allows calculation of the third. The cellular barcoding technology enables us to quantify naïve T cell recruitment by counting the amount of different barcodes that are found in the response. This is a direct reflection of the number of recruited naïve T cells. The overall magnitude of the response can be easily determined by flow-cytometry based counting of how many barcode-labeled

T cells are present during the response. Using these two measurements we determined to what extent changes in naïve T cell recruitment and clonal expansion regulate the overall $CD8^+$ T cell response size.

Even before $CD8^+$ T cells acquire effector functions, they start to proliferate. This expansion of the antigen-specific T cell pool ensures that high numbers of pathogen-reactive T cells are available to prevent spread of the infection and to ultimately eliminate the pathogen. While the importance of this expansion is well recognized, it remains unclear to what extent individual antigen-specific naïve T cells contribute to the overall response. Do all clones produce an equal amount of progeny, or are immune responses numerically dominated by the progeny of only a few naïve T cells? Answering these questions requires on one hand the ability to distinguish between the progeny of different naïve T cells (i.e. between different T cell families) and on the other hand the quantification of T cell family sizes. The former can be achieved by cellular barcoding, but the latter was not possible using the microarray-based barcode readout system we had originally developed, as this system only provides semi-quantitative data on barcode prevalence. In **chapter 8** we therefore first set up a new and quantitative readout system for barcode analysis, which involves deep sequencing of barcodes. Using this new method, we then quantified the size of different responding T cell families during various infection conditions.

REFERENCES

1. Medzhitov, R. & Janeway, C. A., Jr. Innate immunity: impact on the adaptive immune response. *Curr. Opin. Immunol.* 9, 4-9 (1997).
2. Medzhitov, R. & Janeway, C. A., Jr. Decoding the patterns of self and nonself by the innate immune system. *Science* 296, 298-300 (2002).
3. Chen, G. Y. & Nunez, G. Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* 10, 826-837 (2010).
4. Matzinger, P. An innate sense of danger. *Ann. N Y Acad. Sci.* 961, 341-342 (2002).
5. Matzinger, P. The danger model: a renewed sense of self. *Science* 296, 301-305 (2002).
6. Seong, S. Y. & Matzinger, P. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nat. Rev. Immunol.* 4, 469-478 (2004).
7. Rock, K. L., Latz, E., Ontiveros, F. & Kono, H. The sterile inflammatory response. *Annu. Rev. Immunol.* 28, 321-342 (2010).
8. Hoffmann, J. A., Kafatos, F. C., Janeway, C. A. & Ezekowitz, R. A. Phylogenetic perspectives in innate immunity. *Science* 284, 1313-1318 (1999).
9. Jones, J. D. & Dangl, J. L. The plant immune system. *Nature* 444, 323-329 (2006).
10. Ausubel, F. M. Are innate immune signaling pathways in plants and animals conserved? *Nat. Immunol.* 6, 973-979 (2005).
11. Janeway, C. A., Jr. & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* 20, 197-216 (2002).
12. Krysko, D. V. *et al.* Emerging role of damage-associated molecular patterns derived from mitochondria in inflammation. *Trends Immunol.* 32, 157-164 (2011).
13. Krogsgaard, M. & Davis, M. M. How T cells 'see' antigen. *Nat. Immunol.* 6, 239-245 (2005).
14. Smith-Garvin, J. E., Koretzky, G. A. & Jordan, M. S. T cell activation. *Annu. Rev. Immunol.* 27, 591-619 (2009).
15. Reth, M. & Wienands, J. Initiation and processing of signals from the B cell antigen receptor. *Annu. Rev. Immunol.* 15, 453-479 (1997).
16. Davis, M. M. *et al.* Ligand recognition by alpha beta T cell receptors. *Annu. Rev. Immunol.* 16, 523-544 (1998).

17. Williams, A. F. & Barclay, A. N. The immunoglobulin superfamily--domains for cell surface recognition. *Annu. Rev. Immunol.* 6, 381-405 (1988).
18. Ahmed, R. & Gray, D. Immunological memory and protective immunity: understanding their relation. *Science* 272, 54-60 (1996).
19. Williams, M. A. & Bevan, M. J. Effector and memory CTL differentiation. *Annu. Rev. Immunol.* 25, 171-192 (2007).
20. Yoshida, T. *et al.* Memory B and memory plasma cells. *Immunol. Rev.* 237, 117-139 (2010).
21. Sallusto, F., Lanzavecchia, A., Araki, K. & Ahmed, R. From vaccines to memory and back. *Immunity* 33, 451-463 (2010).
22. Huster, K. M. *et al.* Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. *Proc. Natl. Acad. Sci. U S A* 101, 5610-5615 (2004).
23. Obar, J. J. & Lefrancois, L. Early signals during CD8 T cell priming regulate the generation of central memory cells. *J. Immunol.* 185, 263-272 (2010).
24. Sarkar, S. *et al.* Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J. Exp. Med.* 205, 625-640 (2008).
25. Schluns, K. S., Kieper, W. C., Jameson, S. C. & Lefrancois, L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat. Immunol.* 1, 426-432 (2000).
26. Kalia, V. *et al.* Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. *Immunity* 32, 91-103 (2010).
27. Kaech, S. M. *et al.* Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* 4, 1191-1198 (2003).
28. Voehringer, D. *et al.* Viral infections induce abundant numbers of senescent CD8 T cells. *J. Immunol.* 167, 4838-4843 (2001).
29. Joshi, N. S. *et al.* Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27, 281-295 (2007).
30. McMahon, C. W. *et al.* Viral and bacterial infections induce expression of multiple NK cell receptors in responding CD8(+) T cells. *J. Immunol.* 169, 1444-1452 (2002).
31. Farber, D. L. Differential TCR signaling and the generation of memory T cells. *J. Immunol.* 160, 535-539 (1998).
32. Chang, J. T. *et al.* Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science* 315, 1687-1691 (2007).
33. Chang, J. T. *et al.* Asymmetric Proteasome Segregation as a Mechanism for Unequal Partitioning of the Transcription Factor T-bet during T Lymphocyte Division. *Immunity* 34, 492-504 (2011).