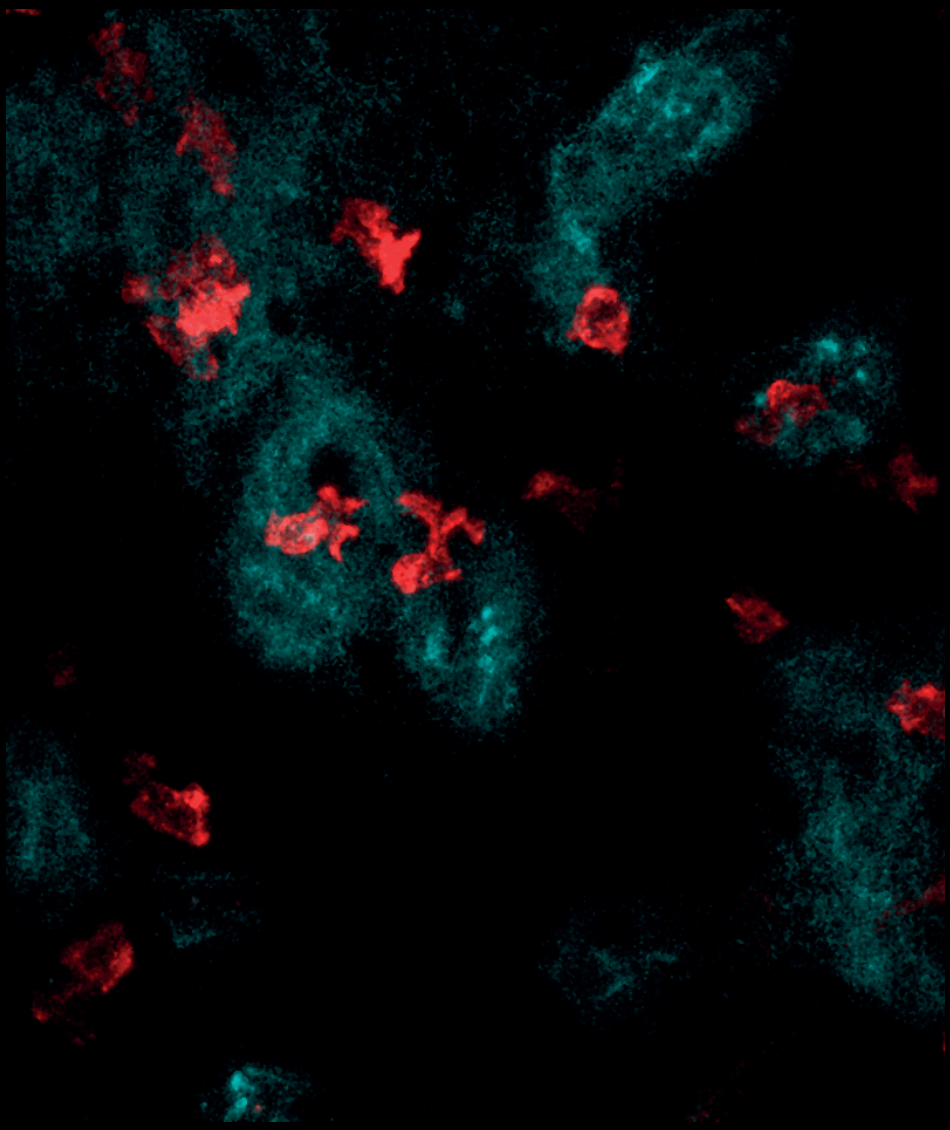

Chapter 1

Scope of the thesis

SCOPE OF THE THESIS

The body is constantly exposed to pathogens such as viruses or bacteria. These insults activate the immune system that is equipped to defend the body. The mammalian immune system is composed of an innate and adaptive branch that work together to provide host protection. The innate immune system comprises various cell types, including dendritic cells (DC), macrophages, neutrophils and natural killer cells that provide rapid and non-specific host protection by effector mechanisms such as phagocytosis and secretion of toxic molecules[1]. The adaptive immune system consists of B and T lymphocytes that provide pathogen-specific immunity. B cells become activated after recognition of soluble or membrane-bound antigens and provide humoral immunity. T lymphocytes recognize processed antigens presented on major histocompatibility (MHC) complexes. T cells expressing the co-receptor CD8 recognize antigen in the context of MHC class I molecules. $CD8^+$ T cells are also known as 'killer' cells, due to their ability to directly kill target cells via secretion of cytotoxic molecules such as granzyme B and perforin. In addition, activated $CD8^+$ T cells can modulate the function of other cells by secretion of cytokines like interferon gamma (IFNγ) and interleukin 2 (IL-2). T cells expressing the co-receptor CD4 recognize antigens presented on MHC class II molecules. $CD4^+$ T cells are also known as 'helper' T cells that can either have stimulating or inhibitory effects[1]. Activation of B and T lymphocytes following pathogen encounter leads to the formation of 'immunological memory' that provides enhanced protection against a secondary encounter with the same pathogen[2]. Consequently, the composition of the adaptive immune system reflects the various pathogens the body has encountered over time. This acquired immunity forms the basis of vaccination strategies.

T lymphocytes originate from hematopoietic stem cells in the bone marrow, but complete their development in the thymus[3]. During thymic development, rearrangement of the T cell receptor (TCR) genes takes place, creating a broad repertoire of TCRs with a more or less random antigen-specificity. Subsequently, thymic tolerance mechanisms operate to shape the T cell repertoire, such that only T cells bearing a TCR with a low affinity for self-peptide MHC complexes are retained. During thymic development, T cells also commit to either the CD4 or CD8 lineage. The resulting mature naïve T cells exit the thymus and recirculate in blood, lymph and secondary lymphoid organs (SLO).

When a pathogen successfully breaches a tissue barrier, cells of the innate immune system get activated by recognition of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Subsequently, professional antigen presenting cells (mainly DCs) that are activated in this process travel to the draining lymph node (LN) to present antigens to naïve T cells at that site. Upon cognate antigen recognition, T cells become activated, rapidly divide and leave the LN to eliminate the pathogen at infected tissue sites. Following pathogen clearance, the majority of the 'effector phase' T cells undergoes apoptosis, leaving behind a small fraction of long-lived memory T cells[4].

Traditionally, memory T cells have been subdivided into two main subsets: central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). T_{CM} constantly circulate through the blood, efferent lymph and SLO. T_{EM} primarily recirculate in the blood and peripheral tissues. However, work over the past decade has identified a third memory T cell subset that does not recirculate but stays put at previously affected sites[5]. These resident memory T cells (T_{RM}) remain present within the tissue long term, and show a distinct transcriptional and protein expression profile from their circulating counterparts[6]. T_{RM} are generally characterized by CD69 expression and in epithelial tissues also by the expression of the integrin receptor CD103, two cell surface molecules that play a role in the retention and maintenance of T cells within tissues[6-8]. T_{RM} have been described in various murine tissues such as the lung, vaginal mucosa, brain, intestine and skin[9,10]. As shown by intravital microscopy at different sites including the skin, $CD8^+$ T_{RM} continuously patrol their surroundings in order to detect newly infected cells[11,12]. In case of local reinfection, $CD8^+$ T_{RM} provide superior pathogen control as compared to their circulating counterparts[12,13]. In addition, $CD8^+$ T_{RM} have been shown to play a protective role in murine cancer models[13,14].

$CD8^+$ T_{RM} can likewise be found in nearly all human body sites[15] and these cells share a core transcriptional profile with virus-specific $CD8^+$ T_{RM} in murine tissues[6,16]. The clearance of local infections in several parts of the human body has been associated with the influx of $CD8^+$ T_{RM}[17] and the presence of tumor infiltrating T lymphocytes that express CD103 has been correlated with improved patient survival in various cancer types[13,18]. Conversely, $CD8^+$ T_{RM} that recognize self-antigens have been implicated in autoimmune diseases, such as vitiligo and psoriasis[19,20].

While we have obtained substantial knowledge on the biology of systemic memory T cell subsets in the past decades, our understanding of tissue-specific T cell immunity has been limited. In this thesis, I aim to investigate the formation and function of $CD8^+$ T_{RM} by combining genome engineering, lineage tracing and *in vivo* and *ex vivo* imaging. In the first two chapters of this thesis (chapter 2-3), I examine how genetic perturbation methods can be used to study the 'lives of T cells'. In chapter 4-5, I investigate the formation of tissue-resident T cell memory. In chapter 6-8, I focus on the mechanism by which skin-T_{RM} mediate local protection, and present an *ex vivo* imaging technology to study the dynamic behavior of these cells within murine and human skin.

Much of our knowledge on the signals involved in T cell differentiation and function stems from single gene perturbation methods, such as overexpression or knockout models. While these approaches have been valuable to determine gene function, they do not inform on the regulation of gene activity under physiological conditions. In **chapter 2**, we review how gene-expression reporters and cell signaling reporters can be applied to study T cell activation, function and differentiation in relatively unperturbed settings.

As also discussed in chapter 2, one of the potential caveats of using reporter proteins (RP) in biomedical studies is that, due to the fact that RPs are derived from other species,

RP-expressing cells are at risk of being rejected upon introduction into immunocompetent mice. In an attempt to overcome this issue, we generated a multiple reporter protein tolerant mouse model ('Tol'), as described in **chapter 3**. Tol transgenic mice contain a large chimeric open reading frame encoding commonly used RPs and modifier proteins (MP) assembled in a scrambled format. Using a vaccination strategy, we demonstrate that RP-expressing cells can engraft in Tol mice while these cells fail to persist in non-transgenic mice. In this work, we show that the Tol strain that we established can be used as a recipient for transfer experiments with cells expressing a series of different RP and MP genes, but also present a general methodology to create novel poly-tolerant mouse strains for biomedical research.

Chapter 4 of this thesis discusses the key external factors that drive formation of tissue-resident $CD8^+$ T cell memory. I highlight that, already before responding $CD8^+$ T cells enter the affected tissue, several signals provided during priming or even at the naïve T cell stage can differentially imprint $CD8^+$ T cells with the capacity to develop into tissue-resident memory T cells. In addition, the factors provided within the inflamed tissue microenvironment that promote T_{RM} differentiation *in situ* are outlined. Finally, I discuss how this knowledge may be exploited to either stimulate formation of T_{RM}, or to inhibit the survival or retention of pre-existing T_{RM} pools, for instance in autoimmune disease.

In **chapter 5**, we investigate how and when the capacity to form tissue-resident memory is instilled in antigen-specific $CD8^+$ T cells. In this work, we track the clonal output of naïve $CD8^+$ T cells after vaccination by cellular barcoding. We show that while different T cell clones have a similar capacity to disseminate in the systemic and skin compartment during the effector phase, a subset of clones is biased to form skin-T_{RM} after antigen clearance. Importantly, this clonal bias is observed regardless of the anatomical skin site involved or the time of memory formation, showing that preferential T_{RM} formation is a clone intrinsic attribute rather than a stochastic process. Together, these data demonstrate that skin-T_{RM} formation is imprinted at the clonal level prior to tissue entry, and that this capacity is fixed upon secondary antigen encounter.

Once established at previously affected sites, $CD8^+$ T_{RM} contribute to pathogen control during local reinfection. In **chapter 6**, we investigate the effector mechanisms by which $CD8^+$ T_{RM} mediate such protection. We demonstrate that upon activation by cognate antigen, $CD8^+$ T_{RM} transiently alter the skin transcriptome in a largely IFNγ-manner. This tissue-wide conditioning by $CD8^+$ T_{RM} is induced within a few hours after their activation and is characterized by the upregulation of genes involved in broad-spectrum host defense. Importantly, skin tissues that contain TCR-triggered $CD8^+$ T_{RM} show superior control of a secondary infection with an antigenically unrelated pathogen as compared to skin tissues harboring resting $CD8^+$ T_{RM}. Thus, antigen-dependent activation of a small number of T_{RM} rapidly induces a tissue-wide 'pathogen-alert' that protects against pathogens, including antigenically unrelated (opportunistic) pathogens. Together, these data provide mechanistic

insight in the protective effects of skin-T_{RM} and highlight the therapeutic potential of local T cell memory.

While T_{RM} play a significant role in human skin immunity, much of our knowledge on these cells is based on studies in mice. In addition, the majority of data on human skin-T_{RM} comes from work in which these cells are isolated from the tissue, thereby ignoring the important role of the tissue microenvironment in T cell behavior[21]. In order to overcome these issues, I developed an *ex vivo* imaging technology to study the behavior of $CD8^+$ T_{RM} and other immune cells in real-time within fresh murine and human skin tissue. In **chapter 7**, a detailed description of this approach to label and track immune cells in murine and human skin by *ex vivo* microscopy is provided.

In **chapter 8**, this *ex vivo* culture system is then exploited to study the behavior of $CD8^+$ T_{RM} in human skin. First, I show that the behavior of nanobody-labeled and unlabeled skin-T_{RM} in mouse tissue mounted in the *ex vivo* setup reflects *in vivo* migration parameters of murine skin-T_{RM}, thereby validating the experimental approach. When applying the same technology to skin biopsies, I uncover that $CD8^+$ skin-T_{RM} actively migrate through the basal layer of the epidermis below a pool of sessile Langerhans cells in human skin. In addition, the data describe how $CD8^+$ T cells migrate in the papillary dermis through collagen type I rich and poor areas. Together, these experiments establish that tissue patrol is a property of human $CD8^+$ skin-T_{RM} and provide a platform to study the dynamic behavior of $CD8^+$ T_{RM} and other resident immune cells *in situ.*

In **chapter 9**, I contextualize the results presented in this thesis, pose a series of questions on $CD8^+$ T_{RM} biology that in my view require further work, and provide suggestions on how to address these matters.

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