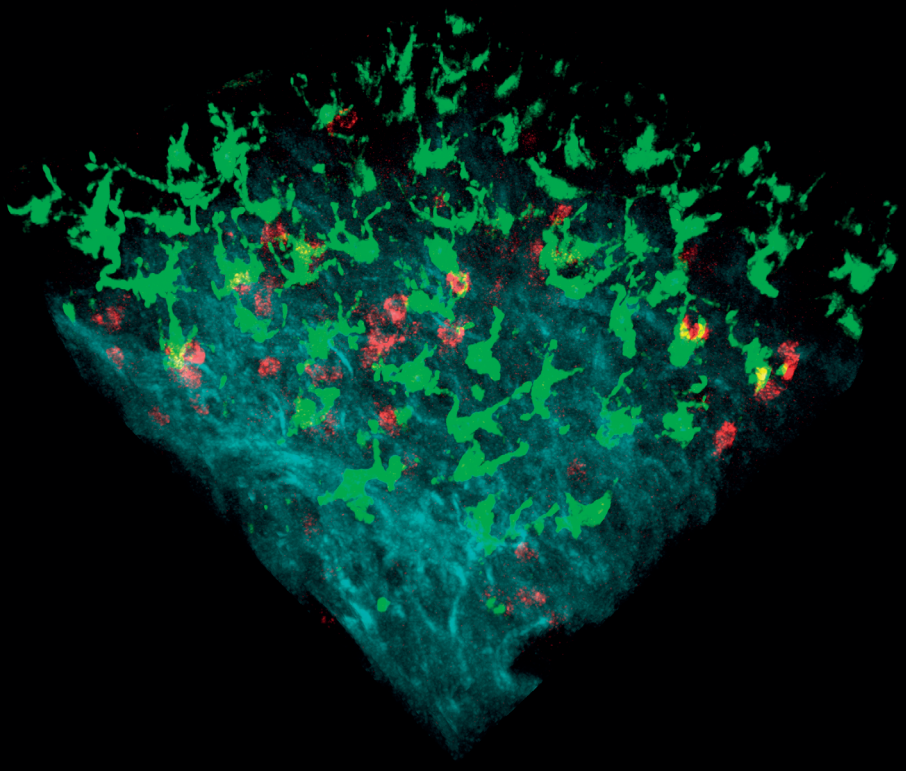

Chapter 9

General discussion

GENERAL DISCUSSION

Since the relatively recent discovery of tissue-resident memory $CD8^+$ T cells (T_{RM}) about a decade ago, the field of T_{RM} biology has rapidly evolved from basic immunological research in murine infection models to the first therapeutic applications in humans today. In this thesis, I aim to increase the fundamental understanding of $CD8^+$ T_{RM} biology, in order to ultimately contribute to the design of improved therapies. To this end, I study the formation and function of $CD8^+$ T_{RM} in murine and human skin tissue, making use of both existing and newly developed research techniques. The first part of this work (**chapter 2-3**) focuses on how genetic perturbation methods and a novel immunologically tolerant mouse model can be used to study various aspects in the 'lives of T cells'. The second part (**chapter 4-5**) reviews our current understanding on the formation of $CD8^+$ T_{RM} and investigates the clonal origin of these cells in murine skin. The final part of this thesis (**chapter 6-8**) examines the mechanisms by which $CD8^+$ T_{RM} mediate protection in murine skin, and presents a novel *ex vivo* imaging system that allows for the real-time study of $CD8^+$ T_{RM} and other immune cell behavior in fresh murine and human skin biopsies.

In this chapter, I would like to provide a broad perspective on $CD8^+$ T_{RM} biology and discuss a series of open questions that in my view deserve follow up.

$CD8^+$ T_{RM}: caught in between the adaptive and innate immune branch?

$CD8^+$ T_{RM} are considered part of the adaptive immune system as formation of this cell compartment requires clonal expansion, and as the cells carry a defined MHC-restricted pathogen- (or autoantigen-) specificity. However, given the parallels in the formation and function of $CD8^+$ T_{RM} with tissue-resident innate immune cells, it seems useful to explore to what extent $CD8^+$ T_{RM} can also be considered as part of the innate immune branch.

Tissue-resident memory T cells are not the only immune cell lineage that populates non-lymphoid tissues. For example, non-classical T cells (i.e. MAIT, NKT, γδ T cells), innate lymphoid cells (ILC, including natural killer (NK) cells), tissue-resident macrophages, and dendritic cells (DCs) can be found at different tissue sites, including the skin, lungs, and intestines[1-4]. Interestingly, several of these immune cell subsets show cell surface expression of molecules that are expressed by T_{RM}, such as CD49a, CD103 and CD69[5]. In addition, ILC1 and ILC3 make use of the same retinoic acid – gut-homing molecules – axis as $CD8^+$ T_{RM} to populate the intestines[6]. Similar to $CD8^+$ T_{RM}, many tissue-resident innate immune cell subsets require IL-7, IL-15, and TGFβ for their maturation and/or survival[2, 4, 7]. Notably, both $CD8^+$ T_{RM} and tissue-resident innate immune cells appear to adapt to tissue-specific metabolic demands, as illustrated by expression of free fatty acid-binding proteins (FABP) in these cell types in the skin[8, 9]. In line with the above data, resident-NKT and -NK cells express Hobit and Blimp-1, two transcription factors that are instrumental in instructing tissue-retention in $CD8^+$ T_{RM}[7]. Moreover, the development of ILC1 and ILC3 is dependent on

transcription factors (i.e. T-bet and RUNX3, and Ahr, respectively) that also play a role in CD8$^+$ T_{RM} formation[3]. Thus, many of the external factors and the molecular circuitry underlying the tissue retention and maintenance program are shared between CD8$^+$ T_{RM} and tissue-resident innate immune cell lineages. Since the latter populations are generally formed early during ontogeny as part of normal tissue development[10], I would argue that CD8$^+$ T cells make use of the similar 'default' or 'innate' (i.e. pathogen-independent) pathways to populate and inhabit the tissue. The recent study showing that migratory DCs pre-condition naïve CD8$^+$ T cells for epithelial T_{RM} cell fate via active TGFβ in the steady state[11], would support such a notion. As also outlined in **chapter 4**, in case of CD8$^+$ T_{RM}, such default pathways are by themselves insufficient to specify tissue fate, as pathogen-derived or -induced factors (e.g. cognate antigen, co-stimulation, cytokines, chemokines) are obviously necessary to initiate a CD8$^+$ T cell response. Moreover, presence of such factors at the inflamed tissue site can reinforce retention and maintenance programs and determine the clonal composition[12] of CD8$^+$ T_{RM} pools.

Next to similarities with regard to their formation, CD8$^+$ T_{RM} also resemble innate immune cells on a functional level. As shown in **chapter 6,** cognate-antigen dependent TCR triggering of a few virus-specific CD8$^+$ skin-T_{RM} is sufficient to induce a rapid (hours) and tissue-wide anti-viral and anti-microbial response via IFNγ. In addition, similar work on CD8$^+$ T_{RM} in the vaginal mucosa has shown that activation of these cells induces an innate (e.g. maturation of DCs) and adaptive (e.g. humoral immunity) immune response[13]. Thus, CD8$^+$ T_{RM} function as sentinels for foreign antigen and rapidly alarm their surroundings (i.e. tissue cells and immune cells) to initiate a broad-spectrum host defense response. Thus, from a conceptual point of view, the TCR of CD8$^+$ T_{RM} has a similar function as the monomorphic pathogen receptors and danger receptors, such as toll like receptors, on innate cells. In line with these studies, relative to their circulating counterparts, CD8$^+$ T_{RM} show the highest gene expression levels of effector (e.g. IFNγ) and cytotoxic (e.g. granzyme B) molecules[14]. Thus, much like innate immune cells, CD8$^+$ T_{RM} are poised for rapid action.

Taken together, CD8$^+$ T_{RM} clearly show overlapping requirements for their formation and similar functionality as tissue-resident innate immune cells, with the remark that in case of CD8$^+$ T_{RM} these behaviors are only initiated upon recognition of cognate antigen. Considering these characteristics, one could consider CD8$^+$ T_{RM} a population of pathogen-specific tissue-resident innate immune cells. More importantly, I believe that looking at T_{RM} biology from an innate immunity perspective could inspire new research questions. As an example, given that human skin generally contains a large fraction of αβ T cells but no dendritic epidermal T cells (DETC, γδ T cells) as is the case in murine skin[5, 15], one study questioned whether αβ T cells in human skin display functional characteristics of DETC. Intriguingly, similar to DETC[16], αβ T cells are capable of producing insulin-like growth factor 1 in wounded skin[17], suggesting that human αβ T cells are in part functional equivalents of murine DETC by contributing to skin repair. Following this line of thought, one could examine whether T_{RM}

share other characteristics with innate immune cells. For example, do $CD8^+$ T_{RM} display other functional properties of innate immune cells, such as an interaction with the nervous system like ILCs[3]? Also, do $CD8^+$ T_{RM} rely to a greater extent on pathogen and danger signal receptors, as compared to their circulating brethren? Finally, are there additional pathogen-independent mechanisms that imprint $CD8^+$ T cells for a tissue-resident T cell fate (e.g. via cytokines or metabolites that are important at the tissue site)?

How do $CD8^+$ T_{RM} relate to other T cell subsets?

Over the years, many studies have concluded that T_{RM} are a distinct memory T cell subset, as based on both their phenotype and transcriptional profile[14, 18, 19]. In **chapter 5**, we demonstrate that $CD8^+$ skin-T_{RM} are also different from systemic memory T cell subsets by descent. Specifically, we show that the capacity to form T_{RM} is – at least to some extent – imprinted on a clonal level prior to skin entry and remains fixed upon antigen re-encounter. These findings raise the question: what are the features of the clonal lineages that become T_{RM}? Notably, ongoing single cell transcriptomics and cellular barcoding experiments led by Lianne Kok have shown that a subset of systemic 'effector phase' T cells displays a T_{RM}-like transcriptional signature that is predictive of their capacity to form T_{RM} on a clonal level (i.e. high expression of *Itgae, Tgfb1, Cd101, Ccr10, Ahr*, and low expression of *Klrg1, Eomesodermin*) (personal communication). These data are consistent with the notion that $CD8^+$ skin-T_{RM} arise from a circulating T_{RM} memory precursor, as proposed in the model in **chapter 5**. In case a phenotypic profile of such precursors can be (further) distilled, the differentiation potential (i.e. degree of commitment) of this subset may be further investigated in adoptive transfer experiments in matched or non-matched infection settings (e.g. transfer of precursors activated by a skin infection into mice that received an infection to the lungs). In addition, although various cell intrinsic (e.g. stochastic gene expression[20, 21], developmental origin[22]) and extrinsic signals in the naïve cell stage and during priming (**chapter 4**) have been described, the mechanisms that lead to the divergence in memory potential of 'effector phase' $CD8^+$ T cells are incompletely understood. Single cell analysis of the transcriptomic and epigenetic landscape before and after local infection could provide insight in the intermediate cell stages that lead up to the formation of T_{RM} memory precursors. For instance, the reporter of genomic methylation (RGM) developed a few years ago, which allows for the *in vivo* monitoring of DNA methylation changes of endogenous genes on a single cell level[23], may be used to track the fate of individual $CD8^+$ T cells that underwent a particular epigenetic imprinting event. In addition, I see value in the use of gene expression reporter systems (**chapter 2**) that function as 'genetic recorders' and allow fate-mapping of cells that (have once) express(ed) a gene of interest (e.g. expression of KLRG1[24] or a transcription factor). As a $CD8^+$ T cell receives multiple internal and external signals on the road to tissue-residency, technologies to dissect the hierarchy of different input signals will also become valuable. The development of cell-signaling reporters (**chapter 2**) that provide a 'memory' of

the signals a cell has received, could provide valuable information. As gene expression levels are frequently proportional to the strength of the input signal, such reporters may also reflect the intensity of different internal/external signals.

Another research topic that will likely continue to gain significant interest in the coming years is the plasticity of $CD8^+$ T_{RM}. Upon secondary immune challenge, T_{RM} locally proliferate and contribute to the pre-existing T cell pool[25]. In addition, newly activated $CD8^+$ T_{RM} have been shown to be able to travel to the draining lymph node and can persist long-term at that site[26]. To date, it is unknown whether reactivated T_{RM} travel via the blood or only via lymph, however there are indications that these cells do have the capacity to enter the blood circulation (F. Behr, personal communication). If so, it would be interesting to determine whether the cells that have left the tissue of origin could take up residency at another non-lymphoid site. Next to T_{RM} themselves, T_{EM} and T_{CM} have both been described to contribute to secondary T_{RM} pools in various reinfection models[27] and in transplant models with human skin (T. Matos, personal communication). Given that T_{EM} and T_{CM} have different functional properties, it would be interesting to investigate whether the T_{RM} derived from either of these two T cell subsets would retain functional characteristics of their past (e.g. on an epigenetic and/or transcriptional level). I can envision that such a mechanism could contribute to the establishment of a functionally versatile secondary T_{RM} population. By analogy, 'exKLRG1' T_{RM} retain the cytotoxic and proliferative capacity of their 'effector past' and comprise the functionally most active T_{RM}[24].

What is the role of the local microenvironment on the behavior and formation of $CD8^+$ T_{RM}?

$CD8^+$ T_{RM} are integrated into the fabric of our tissues. As such, they interact with their surroundings including tissue cells, immune cells and extracellular skin components. As demonstrated previously by Silvia Ariotti[28] and by the work I present in **chapter 8**, $CD8^+$ T_{RM} are not immotile but actively patrol murine and human skin tissue. One intriguing paradox that remains in the field is how $CD8^+$ T_{RM} stay confined to areas of prior infection while they are constantly migrating. A straightforward explanation is that $CD8^+$ T_{RM} migrate relatively slow and make many turns ([15] and **chapter 8**) and therefore, their effective displacement is limited. However, it is unclear whether this model fully explains the observed $CD8^+$ T_{RM} confinement, and alternative explanations may still contribute. For example, physical or biochemical barriers (e.g. secreted by surrounding cells or by $CD8^+$ T_{RM} themselves) could keep T cells confined to certain areas. While we did not address this question specifically, we found evidence that extracellular skin components in the dermis (i.e. collagen type I and dermal vessels) can influence the motility of $CD8^+$ T_{RM} (**chapter 8**). Notably, due to limited cell numbers, we did not evaluate whether human dermal and epidermal $CD8^+$ T_{RM} differ in their migratory behavior. However, based on visual inspection, and on murine data on $CD4^+$ T_{RM} migration in the two skin compartments[5, 29], it seems possible that human $CD8^+$ T_{RM} in

the epidermis migrate relatively slower and have a more dendritic shape than in the dermis. Together, these data are consistent with the notion that the tissue-microenvironment may indeed influence the migratory behavior of $CD8^+$ skin-T_{RM}.

In contrast to epithelial tissues, subepithelial layers (e.g. dermis, lamina propria, interstitium) do not ubiquitously express factors that promote T cell retention and maintenance. As such, while epithelial $CD8^+$ T_{RM} are relatively dispersed at the site of initial pathogen entry, $CD103^{+/-}$ $CD8^+$ T_{RM} in subepithelial tissues often form clusters with antigen-presenting cells and $CD4^+$ T cells[4]. The establishment and stability of such mixed leukocyte clusters is critical for the induction of persistent T_{RM} populations. As an example, $CD8^+$ T_{RM} fail to form in the lung interstitium after a 'prime and pull' vaccination since the formation of the supportive repair-associated memory depots (RAMD) is dependent on cognate antigen signals[30, 31]. Notably, while this vaccination methodology is sufficient to induce $CD8^+$ T_{RM} in the skin epidermis and vaginal epithelium[32, 33], it does not induce a stable $CD8^+$ T_{RM} pool in the airway epithelium, as the epithelial $CD8^+$ lung-T_{RM} population requires replenishment from the RAMD in the interstitium[30, 31]. These data indicate that there are not only differences between epithelial and subepithelial tissues in the requirements for T_{RM} formation, but also between epithelial sites. One explanation for this difference is that the skin and vaginal epithelia are stratified tissues whereas the airway epithelium is a pseudostratified cell layer. Pseudostratified epithelia do not contain persisting immune cells such as DETC[34] and these cells have been shown to be displaced by $CD8^+$ T_{RM} after herpes simplex virus-1 infection[15]. Therefore, it has been proposed that $CD8^+$ T_{RM} may form best at sites where they can take over pre-existing niches of other tissue-resident immune cells[4]. Altogether, these data strongly argue that the anatomy of the tissue microenvironment should be taken into account when designing vaccines.

Are T_{RM}-like tumor infiltrating $CD8^+$ T lymphocytes suitable targets for anti-cancer therapies?

As outlined in **chapter 4**, tumor-infiltrating $CD8^+$ T lymphocytes (TIL) with a T_{RM}-phenotype (e.g. $CD103^+$, $CD69^+$) have been described in various human cancers and their presence has been associated with improved patient survival (i.e. $CD103^+$ $CD8^+$ TIL)[35]. In addition, these cells often display a dysfunctional phenotype, a property that is correlated with tumor-reactivity[36]. Furthermore, $CD103^+$ $CD8^+$ T cells substantially expand after treatment with anti-PD1 in melanoma patients[37]. Combined, these data are highly suggestive that T_{RM}-like $CD8^+$ TIL make good targets for anti-cancer therapies. However, important questions still remain. For example, does expression of single or a few T_{RM}-markers by $CD8^+$ TIL truly indicate that the cells should be considered tumor-resident T cells? Do T_{RM}-like $CD8^+$ TIL follow the same differentiation path as the 'classical' $CD8^+$ T_{RM} that stay behind in non-lymphoid tissues after local infection? How do resident memory $CD8^+$ T cells evolve during the various

stages of a progressing tumor? In-depth cell profiling studies (e.g. mass-cytometry, single cell transcriptomics) and lineage tracing experiments will aid in addressing these questions.

One functional property that appears to be shared between antigen-experienced $CD8^+$ T cells in murine skin and in tumor models is their ability to widely impact their environment after reactivation. Specifically, as shown in **chapter 6** and by others[13], cognate antigen-dependent triggering of a small number of $CD8^+$ T_{RM} alters the behavior of a large number of surrounding cells via the production of IFNγ and leads to the induction or amplification of innate and adaptive responses. Similarly, in recent work led by Mirjam Hoekstra and Laura Bornes, we have shown that cognate antigen-dependent recognition of a small part of the tumor by $CD8^+$ T cells leads to IFNγ-sensing by a large part of the tumor mass, reaching cells at >800 μm distances[38]. Moreover, this long-distance sensing of IFNγ modulates the behavior of tumor cells, including those that do not express the antigen[38]. This is particularly relevant in settings where presentation of tumor antigen may be heterogeneous. In light of this, it has been proposed that targeting $CD8^+$ TIL with a known specificity (e.g. viral epitopes) could be a way to modulate the tumor microenvironment and induce innate and adaptive immune responses (e.g. making a 'cold' tumor 'hot')[39].

How can we limit the density or functionality of existing T_{RM} populations?

Next to their protective function, T_{RM} have also been described to play a pathogenic role in a number of autoimmune, inflammatory and allergic diseases, such as diabetes, multiple sclerosis, asthma, Crohn's disease, psoriasis and vitiligo[40]. In these settings, the design of strategies that limit the number, or reduce the functionality, of existing T_{RM} populations should be a priority as patients are likely to enter the clinic with established lesions.

In addition to the approaches mentioned in **chapter 4**, another way to target $CD8^+$ T_{RM} is via Janus kinase (JAK1, JAK2, JAK3 and TYK2) inhibitors. JAKs are part of the signaling transduction pathway of multiple cytokines that phosphorylate STAT (signal transducer and activators of transcription) proteins to induce gene expression[41]. Currently, JAK inhibitors are being tested for various skin disorders including vitiligo and psoriasis. In vitiligo (which is IFNγ-mediated[42]), phase II clinical trials have shown that topical treatment with JAK1/2 inhibitor in combination with local UV-treatment induced repigmentation[43]. In psoriasis (which is IL-17- and IL-23-mediated[42]), oral treatment with the JAK1/3 inhibitor tofacitinib reduced psoriatic plaque formation and epidermal thickness in several phase III trials[43]. However, relapse was observed upon discontinuation of treatment. In addition, several patients showed reactivation of herpes simplex and herpes zoster infection at distant sites[43], suggesting that topical application of the drug may be a better strategy. As an alternative to targeting the effector functions of $CD8^+$ T_{RM}, selective JAK3 inhibitors could be used to inhibit the IL-7R- and IL-15R-signaling pathway[44], in order to interfere with T_{RM} maintenance. Taken together, although beneficial effects may be temporary, JAK inhibitors could provide a promising therapeutic approach to alleviate skin-T_{RM}-mediated pathology. If a local depletion

of a pathogenic T_{RM} pool could be combined with the generation of novel, non-pathogenic T_{RM} population, durability of response may conceivably be improved.

Another potentially interesting avenue to limit T_{RM} densities is to target the cytokine and/or chemokine signals that are required for the stability of the previously mentioned mixed leukocyte clusters. As an example, the chemokine CCL5 was shown to be important for the formation and stability of such clusters in the skin dermis and vaginal lamina propria[45, 46].

On a more general note, the *ex vivo* imaging system (**chapter 7, 8**) could provide a platform to study the behavior of resident immune cells within healthy or diseased human skin, including their response to immune stimuli or therapeutics (e.g. low-dose radiation therapy, antibody drug-conjugates, and cytokine or chemokine signaling inhibitors).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Together with my colleagues, I have established that the capacity to form skin-T_{RM} is imprinted in $CD8^+$ T cells on a clonal level prior to tissue entry. These data can be a starting point for other researchers to define the nature of the putative systemic T_{RM} memory precursor and investigate the signals that lead to formation of these cells. In addition, we demonstrated that skin-T_{RM} can mediate their protective effect over significant distances and function as a first line of defense against local (opportunistic) pathogens. Conceptually, this work puts T_{RM} at the bridge of the innate and adaptive immune system. In addition, in collaboration with my colleagues, I have uncovered that tissue-resident memory $CD8^+$ T cells patrol our skin.

Together, this work has contributed to a broader fundamental understanding of the formation and function of $CD8^+$ T_{RM} in murine and human skin tissue. In addition, the two novel technologies presented here, i.e. the 'Tol' mouse model and the *ex vivo* imaging system, could provide valuable tools for other researchers to dissect systemic and local immune responses.

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