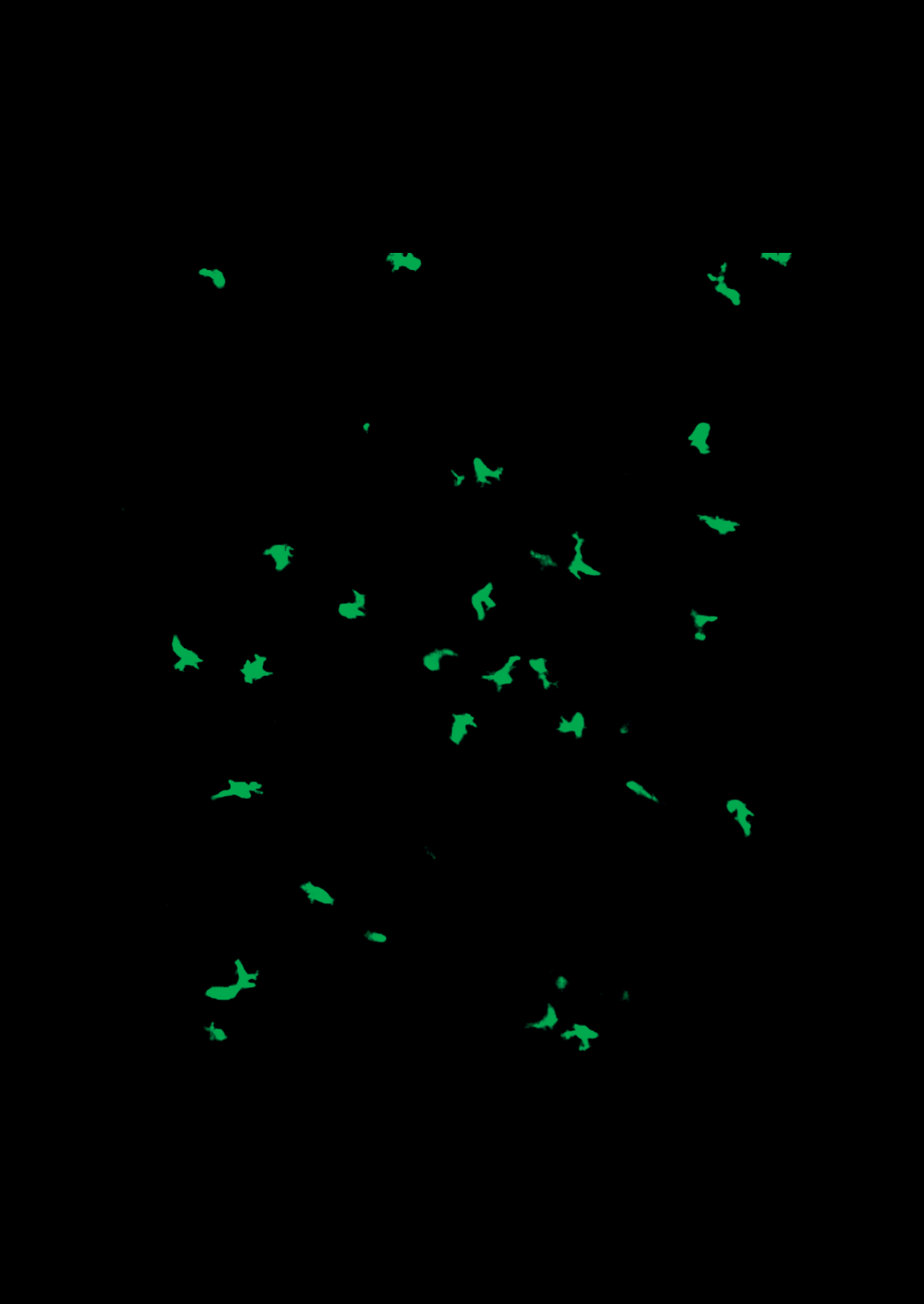

Chapter 4

Formation of tissue-resident CD8$^+$ T cell memory

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Cold Spring Harbor – Perspectives in Biology, on invitation, manuscript under review

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ABSTRACT

Resident memory CD8$^+$ T cells (T_{RM}) permanently reside in non-lymphoid tissues where they act as a first line of defense against recurrent pathogens. How and when antigen-inexperienced CD8$^+$ T cells differentiate into T_{RM} has been a topic of major interest, as knowledge on how to steer this process may be exploited in the development of vaccines and anti-cancer therapies. Here, we first review the current understanding of the early signals that CD8$^+$ T cells receive before they have entered the tissue and that govern their capacity to develop into tissue-resident memory T cells. Subsequently, we discuss the tissue-derived factors that promote T_{RM} maturation *in situ.* Combined, these data sketch a model in which a subset of responding T cells develops a heightened capacity to respond to local cues present in the tissue-microenvironment, that thereby imprints their ability to contribute to the tissue-resident memory CD8$^+$ T cell pool that provide local control against pathogens.

INTRODUCTION

Characteristics of tissue-resident memory CD8$^+$ T cells

Upon local infection of a tissue site, dendritic cells (DCs) that have taken up pathogen-derived antigens migrate to the draining secondary lymphoid organs (SLO) where they interact with antigen-specific naïve CD8$^+$ T cells. During this encounter, DCs present peptide-major histocompatibility complexes (pMHC) to the T cell receptors (TCR) on naïve T cells, while also providing co-stimulatory ligands and cytokine signals. As a consequence of these signals, CD8$^+$ T cells specific for an MHC class I-presented pathogen-derived antigen undergo rapid clonal expansion and differentiate into effector phase T cells that leave the secondary organs to enter the blood. Part of the effector phase CD8$^+$ T cell population will subsequently enter the affected body site to contribute to pathogen clearance, both by the direct lysis of infected cells, and by the antiviral and antibacterial activities of the interferon gamma (IFNγ), and tumor necrosis factor alpha (TNFα) that effector phase T cells secrete upon antigen encounter. Following pathogen clearance, approximately 90-95% of the effector phase T cells dies due to apoptosis [1], leaving behind a small population of long-lived memory CD8$^+$ T cells. These memory CD8$^+$ T cells persist for many years in the body to provide rapid protection in case of reinfection.

Traditionally, two major subsets of memory CD8$^+$ T cells, referred to as central memory T cells (T_{CM}) and effector memory T cells (T_{EM}), have been distinguished on the basis of their trafficking abilities. Similar to naïve T cells, central memory T cells (T_{CM}) show cell surface expression of C-C chemokine receptor type 7 (CCR7) and L-selectin (CD62L) that allow for entry into lymphoid tissues. T_{CM} can be found circulating in blood, efferent lymph and SLO. In contrast to T_{CM}, effector memory T cells (T_{EM}) lack expression of CCR7 and CD62L, and this memory T cell population primarily recirculates in blood and peripheral tissues. Work over the past two decades has however identified a third memory CD8$^+$ T cell subset that permanently resides at sites of previous pathogen infection. Such tissue-resident memory CD8$^+$ T cells (T_{RM}) have now been described in a series of non-lymphoid tissues in mice and humans, including the skin, lung, intestines, vaginal mucosa and brain [2, 3]. In addition, T cell populations with T_{RM}-like characteristics have been identified in mouse models of solid cancers and in human malignancies [4, 5]. T_{RM} are generally characterized by cell-surface expression of CD69, a molecule that is transiently expressed on recently activated T cells at other body sites, but that shows sustained expression on T_{RM}. CD69 binds to and antagonizes cell surface expression of the G-coupled protein sphingosine-1-phosphate receptor 1 (S1PR1) [6]. As the sensing of S1P, which is present in high concentrations in blood and lymph but low in tissues, by S1PR1 is a major factor driving T cell egress, regulation of S1PR1 expression by CD69 forms a mechanism to achieve tissue residency. Next to the constitutive expression of CD69, T_{RM} express a core 'tissue-residency' transcriptional profile that is shared between tissue-resident CD8$^+$ T cells at different body sites, and that distinguishes these cells from

circulating CD8$^+$ T cell subsets [7, 8]. Notably, the core transcriptional signature of human CD69$^+$ T_{RM} shows a substantial overlap with that of murine CD8$^+$ T_{RM} generated upon herpes simplex virus 1 or acute lymphocytic choriomeningitis virus infection [8], supporting the validity of mouse models to understand human T_{RM} biology. Next to the CD69-S1PR1 axis, two other pathways have been shown to influence the capacity of T_{RM} to remain tissue resident. Specifically, CD69$^+$ T_{RM} in epithelial tissues (e.g. skin epidermis and brain epithelium) often express the alpha E subunit (CD103) of the αEβ7 integrin. CD103 is an adhesion molecule that interacts with E-cadherin expressed on epithelial cells, thereby contributing to T_{RM} retention [7, 9]. In addition to this receptor-ligand pair, a sizable fraction of CD69$^+$CD103$^+$ T_{RM} also expresses the very late antigen-1 integrin (CD49a), which binds collagen type IV present in basement membranes [8, 10, 11]. Next to CD69, CD103 and CD49a, other molecules have been described to distinguish T_{RM} from circulating memory T cell subsets, including CXCR3, CXCR6 and CD101 [8, 12, 13]. It is important to note that presence of neither of these markers on CD8$^+$ memory T cells, including CD69 or CD103, is sufficient to unambiguously classify CD8$^+$ T cells as tissue resident [14, 15, 16]. Conversely, absence of any of these molecules does not necessarily indicate a lack of residency [15, 17]. In the majority of studies, CD8$^+$ T_{RM} are defined as CD69$^+$CD103$^+$ memory T cells at tissue sites and we will also utilize this definition. However, it is important to keep in mind that additional T_{RM} subsets do exist, and that these may show subtle differences with respect to both differentiation pathways and functional characteristics.

Protective function of resident memory T cells

Once established at local sites, T_{RM} play a key role in protecting the tissue from reinfection. Intravital and *ex vivo* imaging studies in murine skin [18, 19], liver [13, 20], vaginal mucosa [21] and also in human skin [22], have shown that in the absence of infection, CD8$^+$ T_{RM} actively patrol their surroundings. This tissue patrol allows T_{RM} to efficiently identify antigen-positive cells at the moment reinfection occurs [18]. In mice, T_{RM} have been shown to provide superior protection against local reinfection with acute lymphocytic choriomeningitis virus [23], oral *Listeria monocytogenes* [24], vaccinia virus [25] and herpes simplex virus-1 [11, 18, 26], as compared to circulating memory T cells. Consistent with expectations, such protection has been shown to correlate with local T_{RM}-density in mouse models [27], providing a compelling argument to try to boost local T_{RM} numbers by vaccination. Furthermore, in patients with herpes simplex virus-2 infection, the number of CD8$^+$ T_{RM} positively correlated with viral clearance in the vaginal mucosa [28, 29, 30], suggesting that also in human tissues T_{RM} densities determine their protective effect. The residency of T_{RM} in human tissue, and also their protective capacity, has been illustrated in patients with cutaneous T cell lymphoma that received low-dose alemtuzumab (anti-CD52) treatment. Anti-CD52 treatment selectively depletes circulating T cells while sparing T cells in skin, and the preservation of the cutaneous T cell pool was shown to be associated with local protection [31]. Next to protection from viral- and bacterial reinfections, emerging

evidence also indicates a beneficial role for CD8$^+$ T_{RM} in human solid cancers (as reviewed by Park et al. [4] and Amsen et al. [5]). In mice, pre-formed tumor antigen-specific T_{RM} and T_{RM} that developed during tumorigenesis were shown to control tumor growth, also in the absence of circulating memory T cells [32]. In humans, CD69$^+$CD103$^+$CD8$^+$ tumor infiltrating lymphocytes (TIL) have been observed in several solid cancers[4, 5] and CD103$^+$CD8$^+$ TIL densities, as inferred by phenotypic or transcriptional analysis, have been associated with improved patient survival in different cancer types including melanoma[33], lung[34, 35], bladder[36], ovarian[37], endometrial[38], cervical[39], breast[40], and colorectal cancers[41]. Interestingly, single cell profiling studies in various cancers have demonstrated that CD103$^+$CD8$^+$ TIL, or in one case CD39$^+$CD103$^+$CD8$^+$ TIL, often display a dysfunctional transcriptional state, characterized by high expression of co-inhibitory receptors such as PD-1, TIM-3, and Lag-3 [34, 40, 42], a process thought to be driven by tumor-antigen recognition [43]. Although these data suggest a central role for T_{RM} in cancer biology and cancer immunotherapy, it is important to bear in mind that expression of T_{RM}-associated markers does not necessarily imply the presence of true resident memory T cells. Specifically, as CD69 and CD103 expression are both induced by TCR triggering [44, 45], the presence of these molecules may also indicate recent antigen encounter [7]. In addition, CD69 expression is reported to be induced in oxygen deprived milieus, as one may expect to be present in larger tumors[46]. Thus, an appropriate level of care is required when aiming to translate findings on T_{RM} that arise after local viral infection to tumor-resident T cells, as the mechanisms that control their formation and function may differ.

T_{RM} exert their protective effect by both direct and indirect mechanisms. Direct killing of cognate antigen-expressing target cells by T_{RM} can occur by the secretion of cytotoxic molecules such as granzyme B and perforin [45, 47]. Importantly, activated T_{RM} also provide tissue protection through indirect means, by the secretion of cytokines and the resultant activation and recruitment of other (immune) cell types. Specifically, transcriptional studies have shown that upon cognate antigen triggering, skin-T_{RM} rapidly (within hours) instruct the surrounding tissue to express molecules involved in broad-spectrum host defense. This 'tissue-conditioning' is to a large extent dependent on IFNγ and also provides protection against antigenically unrelated pathogens [48], in essence forming a reverse link between adaptive and innate immunity. Furthermore, IFNγ production by activated T_{RM} in the vaginal mucosa leads to the rapid recruitment (within 12 hours) of circulatory memory T cells and B cells [49, 50]. In addition, when activated by antigen, T_{RM} produce TNFα and IL-2, thereby inducing maturation of local DCs and activation of natural killer cells (NK), respectively [50]. Moreover, T_{RM} contribute to secondary T_{RM} pools by local proliferation after reinfections [27]. Next to their role at the tissue site, a fraction of activated T_{RM} in the vaginal mucosa and skin has been shown to leave the primary tissue site and populate the draining LN (dLN) upon reinfection, potentially to contribute to protection in the dLN and/or to repopulate the downstream non-lymphoid tissue after reinfection [14]. Together, these data sketch a role for

T_{RM} as tissue sentinels that rapidly alarm their surroundings when antigen is encountered, to both establish local protection and to contribute to secondary T_{RM} populations. In view of the role that T_{RM} play in preventing or limiting local disease, an understanding of the signals that determine T_{RM} formation is of interest from both a fundamental and therapeutic perspective.

FORMATION OF TISSUE-RESIDENT MEMORY CD8$^+$ T CELLS: FATE-IMPRINTING SIGNALS PRIOR TO TISSUE ENTRY

While a number of studies have demonstrated the importance of tissue-derived factors in T_{RM} formation, more recent work has started to highlight the contribution of signals that CD8$^+$ T cells receive already prior to tissue entry and that help control T_{RM} cell fate. Below, we will discuss such signals and the contribution of these signals during the different phases in the development of an antigen-specific CD8$^+$ T cell response (see also **Figure 1**).

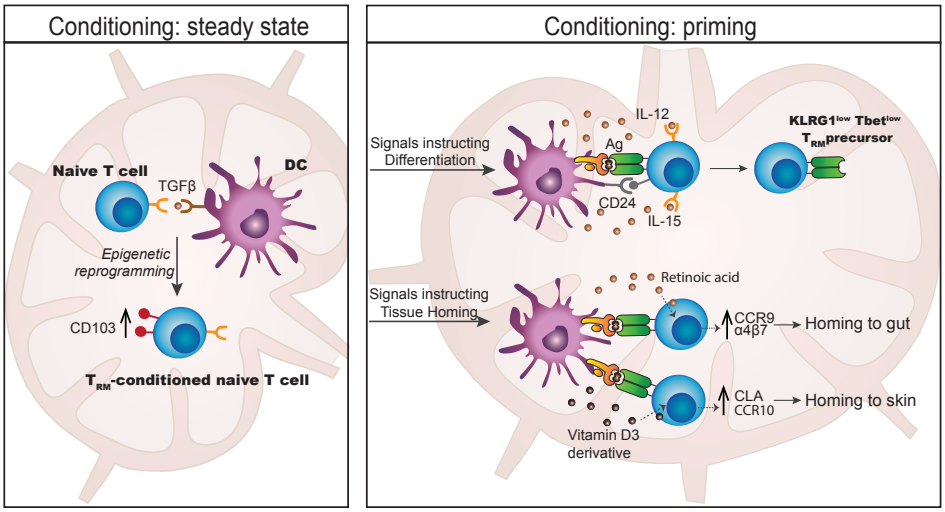

Figure 1 | T_{RM} conditioning in lymphoid tissues by dendritic cells. Dendritic cells (DCs) are critical players in T_{RM}-fate conditioning of naïve T cells. **Left panel**: in the absence of infection, αV$^+$ migratory DCs present active transforming growth factor beta (TGFβ) that promotes epigenetic modifications in naïve T cells and poises them for epithelial (i.e. CD103$^+$) T_{RM} cell fate. **Right panel**: in case of infection, certain signals that DC provide to naïve T cells during activation can increase their capacity to form T_{RM} (i.e. yielding effector phase T cells with low expression of killer-cell lectin like receptor G1 (KLRG1) and transcription factor T-bet), such as antigen cross-presentation, co-stimulation via CD24, and cytokines such as IL-12 and IL-15. In addition, DCs may process and present metabolites to induce expression of lymphocyte homing molecules (e.g. CCR9 and α4β7) on activated T cells, thereby instructing them to migrate to specific tissue sites.

Pre-conditioning at the naïve cell stage

As demonstrated by single cell lineage tracing studies using cellular barcoding or congenic markers, individual naïve CD8$^+$ T cells are capable of producing phenotypically and functionally distinct effector phase T cells [51, 52, 53, 54, 55]. Likewise, individual naïve CD8$^+$ T cells yield both T_{EM} and T_{CM} as progeny. These data demonstrate that commitment to a short-term effector or long-term memory cell fate is not fully determined at the naïve cell stage. In line with these data, sequencing of the *Tcrbv* gene encoding the TCRβ CDR3 region of CD8$^+$ T cells demonstrated that every abundant T_{RM} clone present in the skin after local immunization was also detected in the LN [56]. Thus, as is the case for T_{EM} and T_{CM}, T_{RM} and T_{CM} share a common precursor in the naïve T cell pool. Furthermore, analysis of the TCRαβ repertoire of antigen-specific T cells isolated from human lung tissue demonstrated a substantial overlap between the CD69$^+$CD103$^+$ T_{RM} pool and both the CD69$^+$CD103$^-$ and CD69$^-$CD103$^-$ memory T cell pool. As the latter population is generally considered to be a circulating subset [57, 58, 59], these data suggest that also human T_{RM} share a common precursor with circulating memory T cells.

While these data establish that individual naïve T cells are not fully committed to a particular memory T cell fate, a recent study by Mani et. al has provided the first evidence that naïve T cells can be conditioned to preferentially yield epithelial T_{RM} cells as output [60]. Prior work demonstrated a requirement for active transforming growth factor (TGFβ) to induce the CD103 expression that contributes to tissue residency of CD8$^+$ T cells [7, 9]. Activation of TGFβ requires release from the latency-associated protein (LAP), and this release can be mediated by the binding of αV-integrins to the TGFβ-LAP complex [61]. Making use of mice bearing DCs that lack αV-integrin expression, Mani et al. showed that efficient formation of epithelial T_{RM} depends on migratory αV-integrin$^+$ DCs that present active TGFβ to resting naive CD8$^+$ T cells during non-cognate interactions in the dLN. This interaction was shown to introduce epigenetic modifications at transcription factor (TF) binding sites (e.g. of RUNX3 and KLF family members, see also **Box 1**) in genes that are implicated in T_{RM} formation, such as *Itgae* (encoding CD103), *Ccr8* and *S1pr5* [60]. Together, these data indicate that cell contact-dependent delivery of active TGFβ to individual naïve CD8$^+$ T cells introduces heterogeneity within the naïve T cell pool and poises cells for epithelial T_{RM} cell fate.

Signals during T cell priming

During the initiation of the CD8$^+$ T cell response, T cells get activated and shift to a 'primed' state upon interaction with antigen-presenting DCs in the SLO. The type, duration and severity of the infection determine the amount of antigen, and also the nature and magnitude of co-stimulatory signals and cytokine signals the DC provides to the CD8$^+$ T cell [62]. Together, these signals shape the magnitude and kinetics of the antigen-specific CD8$^+$ T cell response, and also the composition of the effector- and memory T cell pools [62, 63]. While it is currently

unclear how the signals provided during priming exactly impact T_{RM} cell fate decisions, several reports are starting to provide insight on this matter.

As mentioned above, single cell lineage tracing studies have demonstrated that one naïve $CD8^+$ T cell gives rise to both effector and memory T cell subsets. However, at the same time is has become apparent that individual naïve $CD8^+$ T cells bearing the same TCR differ in type of output and the number of progeny they produce [51, 52, 53, 54, 55]. In addition to this TCR-independent variability in cellular output, several studies have shown a relationship between the strength of antigenic stimulation and the ability to yield different memory T cell subsets. Specifically, low affinity TCR interactions have been shown to preferentially result in the formation of T_{CM}, whereas high affinity TCR interactions generally favor the formation of T_{EM} with an enhanced expression of molecules associated with terminal differentiation (i.e. killer-cell lectin like receptor G1 (KLRG1) [64] and CX3C chemokine receptor 1 (CX3CR1)) [65, 66 67, 68]. Recent studies also suggest a role for TCR-signal strength in the formation of T_{RM}, although the results in part appear contradictory. First, T_{RM} formed in the brain and kidneys after local persistent mouse polyoma virus (MPyV) infection showed a ±20-fold higher affinity than circulating memory T cells, indicating that increased TCR-signal strength can positively correlate with the capacity to yield T_{RM} [69]. In line with these data, $CD8^+$ T cells bearing a high-affinity TCR showed improved tissue entry and local persistence in the brain as compared to low-affinity T cells in mouse models of chronic toxoplasma gondii infection [70]. In contrast, systemic infection with MPyV strains expressing variants of a subdominant $CD8^+$ T cell epitope was used to show that weak TCR stimulation yielded increased numbers of $CD103^-$ T_{RM}, and functionally superior $CD103^-$ T_{RM}, in the brain as compared to strong TCR stimulation [71]. In line with the latter observations, a recent study in which T_{RM} formation was assessed after infection with influenza A strains bearing either a high-affinity cognate epitope or low-affinity variants showed that recognition of low-affinity antigen by $CD8^+$ T cells favored formation of lung-T_{RM} relative to systemic $CD8^+$ T memory, as compared to high-affinity interactions [68]. Interestingly, this preferential T_{RM} formation by weakly stimulated $CD8^+$ T cells was also observed at the distant vaginal mucosa tissue that lacked cognate antigen, suggesting that the antigenic signals provided during priming are relevant for T_{RM} formation irrespective of possible additional effects at the tissue site. Together, these data support the notion that early antigenic stimulation can impact T_{RM} differentiation. Notably, the TCR$\alpha\beta$ repertoire of T_{RM} in human lung tissue specific to the same epitope was shown to be clonally diverse [57]. While it cannot be excluded that these different TCRs have a similar affinity for antigen, these data are consistent with the possibility that a range of TCR-signal strengths can lead to T_{RM} cell formation in humans.

With respect to other signals that may influence T_{RM} formation, a study by Iborra et al. has highlighted that the type of antigen-presenting DC that T cells encounter during priming determines the efficiency of T_{RM} formation in murine skin and lung [72]. In this work, mice lacking DCs capable of antigen cross-presentation (i.e. DNGR1- or Batf3-deficient) showed

impaired T_{RM} cell formation after vaccinia virus or influenza A infection, while systemic memory T cell subsets were unaffected. Effector phase CD8$^+$ T cells raised in cross-priming deficient mice egress the dLN earlier than responding T cells in wild type animals, and populate the skin with enhanced expression of terminal differentiation markers (i.e. KLRG1, and the transcription factor T-bet, see **Box 1**). Cross-priming CD103$^+$ and CD8α^+ DCs were shown to prolong antigen-presentation, provide co-stimulation via CD24, and secrete IL-12 and IL-15. These signals transiently induce high levels of T-bet in CD8$^+$ T cells in the dLN and expression of the downstream target CXCR3, causing the cells to populate the tissue as KLRG1$^-$ and T-bet^{low} effector phase T cells [72, 73], which were shown to possess an increased ability to generate T_{RM} [7].

In order for T_{RM} to form, entry of effector phase T cells into peripheral tissues is an obvious requirement, and regulation of the capacity to extravasate can therefore comprise a means to control T_{RM} formation. Instructions for lymphocyte homing can be provided by DCs during priming and are influenced by the location of the tissue-associated SLO. For example, priming within the mesenteric LN induces $\alpha4\beta7$ integrin and CCR9 expression on T cells, thereby allowing their homing to the gut, whereas priming in the inguinal LN induces expression of the cutaneous lymphocyte-associated antigen (CLA), which contributes to homing to the skin [24, 74, 75]. Notably, gut homing by effector phase T cells after systemic lymphocytic choriomeningitis virus infection was shown to be instructed in the spleen [74], demonstrating that homing instructions are not absolute, and also providing a mechanistic explanation for the formation of local T_{RM} after systemic infections. Interestingly, $\alpha4\beta7$ and CCR9 expression is induced by the vitamin A derivative retinoic acid (RA) that is produced by antigen-presenting DCs in gut-associated SLO [76]. In addition, DCs in skin-associated SLO process ultraviolet-induced vitamin D and present the active form (i.e. 1,25$(OH)_2$D3) to activated T cells, thereby inducing expression of the skin-homing receptor CCR10 [77, 78], providing another example of metabolite-induced tissue-tropism.

Interestingly, a study by Sowell et al. has described a role for the mammalian target of rapamycin kinase (mTOR) signaling pathway in T_{RM} homing [79]. In this work, inhibition of the mTOR-signaling pathway (i.e. through knockdown or treatment with rapamycin) impaired migration of effector phase T cells to the gut and vaginal mucosa, while the number of circulating effector phase T cells was enhanced [79]. As the effector phase T cells in the gut showed decreased levels of $\beta7$ and CCR9 expression upon mTOR pathway inhibition, and the metabolite RA can induce components of the mTOR pathway [80], the authors propose that mTOR-induced expression of homing molecules plays a key role in T_{RM} formation.

Heterogeneity with the effector T cell pool: evidence for a T_{RM} precursor?

As established many years ago, the pool of effector phase T cells that arises following T cell priming shows heterogeneity with respect to its capacity to develop into memory T cells. Specifically, within the effector phase T cell pool two subsets, terminal-effector T cells

(TE) and memory precursors (MP), can be distinguished. TE show cell-surface expression of KLRG1 but lack expression of CD127 (i.e. the IL-7 receptor alpha chain, IL-7Rα). TE generally undergo apoptosis after clearance of the infection and hence exhibit a low potential to persist long-term [64]. Conversely, MP express very low levels of KLRG1 but do express IL-7Rα and preferentially give rise to long-lived memory T cells (i.e. T_{EM} and T_{CM}) [81].

Adoptive transfer studies have demonstrated that, similar to T_{EM} and T_{CM}, T_{RM} originate from MP. Specifically, KLRG1$^+$ effector phase T cells failed to produce T_{RM} in the skin and intestines, while KLRG1$^-$ precursors were shown to yield T_{RM} in both skin, liver and small intestines [7, 24, 82]. In addition, a recent study by Herndler-Brandstetter et al. in which a genetic reporter for *klrg1* expression was used, demonstrated that the KLRG1$^+$ precursors that lose expression of this molecule during the contraction phase (referred to as 'exKLRG1 cells') are able to generate circulating memory T cell subsets and also significantly contribute to the T_{RM} pool in the liver and small intestines after *Listeria monocytogenes* infection [82]. Thus, circulating memory T cells and T_{RM} both arise from precursors that either never acquired KLRG1 expression or that lost such expression prior to memory formation.

Work by Masopust et al. has demonstrated that T_{RM} arise from early effectors that have seeded the tissue before the peak of response. Specifically, transient expression of gut-homing molecules was shown to peak around day 4.5 after infection, and day 7 effector phase T cells from the spleen were not able to form gut-T_{RM} anymore, while still being able to develop into circulating memory T cells [74]. Consistent with the possibility that there may be heterogeneity in KLRG1$^-$ effector phase T cells with respect to the capacity to form tissue-resident memory, a recent report showed that day 7 effector phase T cells present in non-lymphoid tissues are transcriptionally and epigenetically distinct from MP in the spleen [83]. In addition, MP are transcriptionally more diverse than TE [84], providing some evidence for the notion that multiple MP lineages may exist. While jointly these data suggest the possible existence of a T_{RM} precursor, direct evidence, e.g. in the form of single cell lineage tracing experiments, is thus far lacking.

FORMATION OF TISSUE-RESIDENT MEMORY CD8$^+$ T CELLS: LOCAL FACTORS THAT PROMOTE T_{RM} DIFFERENTIATION INSIDE THE TISSUE

Following the generation of effector phase T cells, a number of tissue-derived signals influence their capacity to enter the tissue and subsequently differentiate into T_{RM}. These factors can be grouped into a number of classes such as antigen, chemokines, cytokines and metabolites. Here, we will discuss the key factors that promote the entry of effector phase T cells into the tissue, their differentiation into T_{RM} and their local long-term maintenance (i.e. the balance between proliferation and cell death) within tissues (see also **Figure 2**).

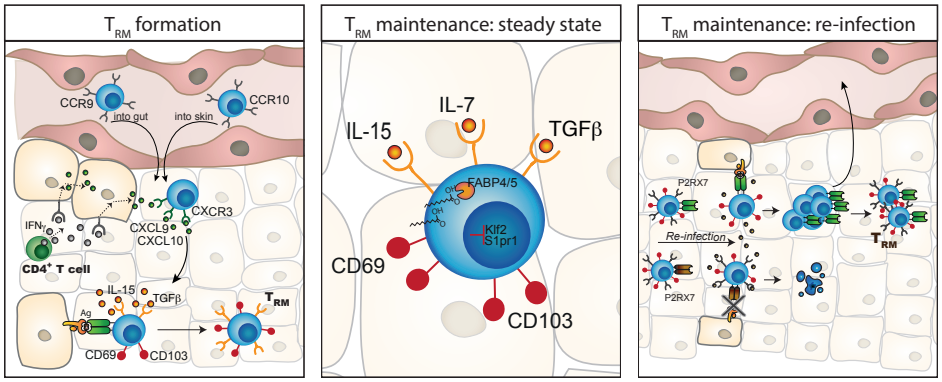

Figure 2 | Establishment and maintenance of T_{RM} in peripheral tissues. Left panel: effector phase T cells expressing tissue-specific homing molecules (e.g. CCR9 and CCR10) will enter the inflamed tissue. $CD8^+$ T cells expressing the chemokine receptor CXCR3 selectively penetrate the tissue through sensing of the tissue-derived ligands CXCL9 and/or CXCL10. Activated $CD4^+$ T cells can increase production of these ligands through local IFNγ secretion. The subsequent exposure to antigen, IL-15 and TGFβ during inflammation facilitate retention and survival of the infiltrated T cells, ultimately leading to the formation of $CD69^+CD103^+$ T_{RM}. **Middle panel**: In the absence of infection, $CD69^+CD103^+$ T_{RM} possess the ability to persist within peripheral tissues, relying on local factors that facilitate their retention and maintenance. Firstly, T_{RM} require IL-15 and IL-7 for low homeostatic proliferation and survival, but also active TGFβ, which facilitates adhesion to E-cadherin positive tissues through the induction of CD103 expression. Secondly, to allow sustained survival, T_{RM} have been reported to require the uptake and metabolism of exogenous free fatty acids, for which the expression of FABP4 and FABP5 is essential. Together, these and other signals instruct a tissue-residency transcriptional profile that prevents the cells from exiting the tissue (i.e. with low KLF2 and S1PR1 expression) and facilitates long-term persistence. **Right panel**: Local reinfection, which coincides with tissue damage, leads to the release of extracellular nucleotides. These factors induce cell death via activation of the damage/danger-associated molecular pattern receptor P2RX7 expressed on T_{RM}. However, upon TCR-triggering, T_{RM} downregulate P2RX7, thereby promoting selective survival of pathogen-specific T cells. T_{RM} that are activated by cognate antigen can also proliferate locally and, after pathogen clearance, give rise to a secondary pool of memory T cells. In addition, upon reinfection, antigen-specific T_{RM} may exit the peripheral tissue and migrate to the draining lymph nodes.

Factors that promote tissue entry

One of the key chemokine receptor-ligand pairs that facilitates localization of effector phase T cells into tissues is CXCR3 and its ligands CXCL9 and CXCL10. Adoptive transfer experiments using CXCR3-deficient effector phase T cells demonstrated impaired T_{RM} formation in the skin by such cells, while formation of circulatory memory T cells was increased[7]. In line with these data, CXCR3 expression is higher on $KLRG1^-$ effector phase T cells that have been shown to preferentially yield T_{RM}, as compared to $KLRG1^+$ effector phase T cells [7]. In addition, the $KLRG1^-$ effector phase T compartment shows an increased capacity to migrate in response to CXCL10 in vitro, which is produced together with CXCL9 at inflamed tissue sites, such as in herpes simplex virus-1 infected skin [7]. Notably, topical application of CXCL9 and CXCL10 is sufficient to 'pull' effector phase T cells to the vaginal mucosa in the

absence of infection [85]. In the vaginal mucosa and lung tissue, IFNγ production by activated $CD4^+$ T cells present at the inflamed site promotes tissue-entry of T_{RM}, presumably via local induction of CXCL9 and CXCL10 [86, 87]. Interestingly, these findings align well with data from a human study in which menstrual blood of healthy females or human immunodeficiency virus (HIV)-infected females that bear low $CD4^+$ T cell counts was sampled, showing that $CD4^+$ T cell-derived IFNγ levels were correlated with $CD103^+$ T_{RM} numbers in the vaginal mucosa [88].

Factors that promote tissue retention

Upon entry into the tissue, effector phase T cells can encounter several factors that promote tissue retention, one of which is the local presence of antigen. Local antigen abundance has been shown to promote T_{RM} numbers in all tissues examined [44, 89, 90]. However, only the formation of $CD103^+$ T_{RM} in the brain [45] and both $CD103^+$ and $CD69^+$ T_{RM} in the lung [91] was shown to fully depend on *in situ* TCR-triggering. In addition, antigenic signals were shown to reduce the mean velocities of effector phase T cells in the skin [92], thereby potentially prolonging their exposure to additional local factors that may promote T_{RM} cell fate. As a third mechanism to explain the link between *in situ* antigen encounter and T_{RM} formation, recognition of antigen at the tissue site may induce local proliferation [93].

In addition to antigen, tissue-derived cytokines play a crucial role in promoting T_{RM} retention. One of the major drivers of CD103 expression, and to a lesser extent CD69, is TGFβ [9, 94]. The different TGFβ isoforms are ubiquitously expressed in several tissues including the skin [95] and intestines [24], and formation of $CD103^+$ T_{RM} at these sites was shown to be dependent on this cytokine [7, 94]. TGFβ signaling regulates T-bet and Eomes levels (see **Box 1**), thereby rendering cells responsive to local retention and survival signals [7, 16]. In contrast, the formation of $CD103^-$ T_{RM} in the intestines after bacterial infection is independent of TGFβ. Unlike $CD103^+$ T_{RM}, $CD103^-$ T_{RM} in the lamina propria shown an uneven tissue distribution, with an apparent colocalization with $CD4^+$ T cells and $CX3CR1^+$ DCs and macrophages [17]. Of note, production of pro-inflammatory cytokines (i.e. IFNβ and IL-12) by macrophages within these niches was shown to counteract TGFβ-induced CD103 expression on effector phase T cells, thereby explaining the phenotype of these cells [17 96]. At present, the niche-derived signals that are required for the local retention of this T_{RM} subset have not been established. *In vitro* work has shown that several other pro-inflammatory cytokines may promote retention of $CD8^+$ T cells (i.e. type I IFN, TNFα and IL-33), by decreasing the expression of KLF2 and its target S1PR1 and increasing expression of CD69 on effector phase T cells [97, 98], but the *in vivo* situations in which these pathways play a role have to our knowledge not been elucidated. Finally, next to antigen and cytokines, metabolites may also play a role in tissue retention. Specifically, hypoxia was shown to induce CD69 expression on activated murine and human T cells *in vivo*. Since the CD69 locus is a direct target of hypoxia-inducible factor 1-α [46], it is plausible that T cell retention is promoted in hypoxic environments such as the skin.

Box 1 | Transcription factors involved in T_{RM} formation

In mice, the transcription factors (TF) that have been shown to either positively or negatively influence T_{RM} formation include Eomesodermin (Eomes), T-bet, KLF2, RUNX3, Notch, Nr4a1, aryl hydrocarbon receptor (Ahr), Bhlhe4, Blimp-1 and the Blimp-1 homologue Hobit.

Whereas the formation of circulatory memory T cells depends on **Eomes**, T_{RM} differentiation in situ requires a complete shutdown of Eomes expression [16]. Likewise, high **T-bet** expression on responding T cells in the tissue is a negative regulator of T_{RM} formation [86], however skin-T_{RM} do require low transcript levels of T-bet for the induction of CD122 to sense IL-15 [16]. **KLF2** drives S1PR1 expression in circulating memory T cells to provide access to the blood or lymph [119]. Reduced expression of KLF2 and its target S1PR1 is a prerequisite for T_{RM} formation, as shown by the impaired skin-T_{RM} formation upon forced expression of these molecules in effector phase T cells [97]. **RUNX3** acts as a central promotor of resident memory T cell fate in the small intestines and kidney, by supporting the expression of tissue-residency genes (i.e. *CD69, Nr4a1*) and suppression of genes involved in tissue egress and recirculation (i.e. *S1pr1, Klf2*) [83]. **Notch** directly regulates CD103 transcript levels in lung-T_{RM} and has been implied in T_{RM} maintenance through regulation of metabolic processes [120]. **Nr4a1** is expressed in T_{RM}, but not in circulatory memory T cells, and has beem proposed to be involved in residency of T_{RM} in the small intestine and liver. However, as downstream targets of Nr4a1 have not been identified to date, the precise working mechanism remains to be elucidated [121, 122]. The Aryl hydrocarbon receptor **Ahr** is highly expressed in human skin-T_{RM} and was shown to be necessary for maintenance of T_{RM} in murine skin [19, 122]. Ahr ligands are environmentally derived small molecules (e.g. diet, microorganisms) [123]. Ahr expression is required for homeostasis of $\gamma\delta$ T cells in the skin [124] and CD8$\alpha\alpha^+$ intraepithelial intestinal lymphocytes [125], suggesting that similar pathways drive environmental adaptation of T_{RM} and tissue resident innate immune cells. **Bhlhe4** is critical for the survival and function of lung-T_{RM} and CD8$^+$ tumor infiltrating lymphocytes. Bhlhe4 induces expression of multiple mitochondrial genes thereby supporting oxidative phosphorylation and mitochondrial fitness. These processes promote acetylation of genes involved in tissue-residency (e.g. *Itgae* (encoding CD103), *Runx3*) and effector function (e.g. *Ifng*, *GzmB*) [126]. **Blimp-1**, rather than **Hobit**, is required for T_{RM} formation in lung tissue [127], whereas both TFs are required for the formation of T_{RM} in the skin, liver, kidney and small intestine [128]. Both Hobit and Blimp-1 directly bind the *Klf2*, *S1pr1* and *Ccr7* loci, thereby regulating tissue residency programs [129]. Interestingly, other tissue-resident immune cells such as natural killer T lymphocytes (NKT) also show elevated Hobit expression [130], suggesting that this TF is a central regulator of tissue-residency. However, Hobit transcript levels are low in CD69$^+$ T_{RM} isolated from human lung [8] and Hobit expression has also been observed in effector T cells and effector memory T cells isolated from human peripheral blood [131], suggesting that Hobit may play a different role in human T_{RM}.

Factors that promote T_{RM} maintenance

In the absence of infection, T_{RM} in mice and humans show a limited degree of proliferation as measured by BrdU incorporation and Ki67 staining [8, 11, 99]. Furthermore, in most tissues, the maintenance of T_{RM} pools does not rely on influx from circulating memory T cells, as shown by antibody-mediated depletion of the circulating memory T cell pool and adoptive transfer experiments [49, 74]. As an exception, T_{RM} generated upon influenza A infection in murine lung tissue fail to persist long-term due to apoptosis and require replenishment from the circulation [100, 101].

As T_{RM} numbers remain stable in murine and human tissues up to several years [11 102] in spite of this limited proliferation and limited steady state tissue entry, T_{RM} can be considered long-lived cells in most tissues. Although T_{RM} show phenotypic similarities with recently

activated T cells and chronically stimulated T cells (i.e. CD69 and PD-1 expression) [7, 8, 103], their persistence appears to be independent from antigenic stimulation in several tissues, including the lung [26, 101]. While continued antigen encounter appears not required, homeostatic cytokines do play a crucial role in maintaining T_{RM} populations. IL-15 has previously been shown to promote the proliferation and survival of T_{EM} and T_{CM} [104], and was subsequently shown to be required for the maintenance of virus-specific T_{RM} in the skin [7], salivary glands, and kidney [105]. In murine epidermis, T_{RM} localize close to hair follicles that constitutively produce IL-7 and IL-15. Accordingly, both cytokines are required for the maintenance of T_{RM} in these epithelial niches [106]. IL-15 is not required in all tissues, as T_{RM} in the vaginal mucosa, pancreas and small intestines can develop normally in mice that lack this cytokine [105]. Next to these cytokines, it has been proposed that activation of the integrin signaling pathway of CD103 and CD49a, which can be induced by TGFβ [9, 94], provides survival signals to epithelial T_{RM} [10, 45]. The chemokine receptor CXCR6 has also been implied in T_{RM} survival, as intradermally injected effector phase T cells deficient in CXCR6 showed impaired skin-T_{RM} formation, whereas memory T cell formation in spleen was unaffected [12].

In contrast to circulating memory T cells, the survival of $CD8^+$ T_{RM} in skin relies on the consumption of exogenous free fatty acids (FFA). This metabolic pathway is dependent on expression of intracellular fatty-acid-binding protein 4 (FABP 4) and FABP 5, and appears conserved between mice and humans [107]. Given that FABP 4 and -5, but also other FABP family members, are expressed in a tissue-specific manner by multiple cell types (e.g. macrophages, enterocytes) [108], T_{RM} maintenance likely requires a specific metabolic state at other tissue sites as well. Finally, extracellular nucleotides (i.e. ATP^+, NAD^+) released upon tissue damage and infection, have been shown to regulate T_{RM} maintenance in the liver and intestines. Specifically, activation of the damage/danger-associated molecular pattern receptor P2RX7 by extracellular nucleotides was shown to promote cell death in T_{RM}. As TCR triggering in T_{RM} leads to decreased expression of P2RX7, this process may be viewed as a mechanism to bias the local T cell repertoire, by allowing the selective survival of antigen-specific T_{RM} relative to bystander T_{RM} [109].

THERAPEUTIC STRATEGIES TO MANIPULATE T_{RM} BIOLOGY

As illustrated above, the formation of T_{RM} pools is indispensible for protection against recurrent local pathogens. For this reason, induction of these populations at non-lymphoid tissues should be a primary objective in T-cell directed therapeutic or prophylactic vaccines. One strategy that has already proven successful in generating enhanced T_{RM} is the 'prime and pull' methodology [85]. In this approach, conventional vaccination with an attenuated virus takes place to elicit a systemic T cell response ('prime'), followed by topical application of chemoattractants to recruit effector phase T cells to the tissue ('pull'). Application of this

strategy has been shown to generate protective T_{RM} against herpes simplex virus-2 in the vaginal mucosa of both mice and guinea pigs [85, 110]. Given the more recent data indicating that, next to tissue-derived factors, also early signals that CD8$^+$ T cells receive prior to tissue entry contribute to T_{RM} formation, additional avenues may be considered. For example, a temporary pre-conditioning regimen in which active TGFβ is delivered to naïve CD8$^+$ T cells in SLO before vaccination may potentially increase formation of epithelial T_{RM} [60]. In addition, the targeting of cross-presenting DCs during priming, for instance by coupling antigens to monoclonal antibodies against CD103 or DNGR1 [72, 111], could enhance formation of epithelial and mucosal T_{RM}. Notably, the suggested application of rapamycin to enhance CD8$^+$ T cell responses during vaccination [112] may be contra-indicated in strategies where the induction of local T cell immunity is the primary objective.

While T_{RM} are necessary for protection against local reinfections, their presence is undesirable in auto-immune diseases, such as vitiligo and psoriasis, where these cells play a pathogenic role [113]. As a pre-formed pathogenic T_{RM} compartment is already present in these diseases, approaches that prevent the formation of novel T_{RM} may be of modest value as monotherapies. However, strategies aimed at reducing the survival or retention of established T_{RM} pools could potentially be attractive. Low-dose radiation therapy has been shown to eradicate malignant T cells in the skin and improve survival of patients with early stage mycosis fungoides (i.e. lymphoma of T cells with a T_{RM} phenotype) [114], but more defined strategies to target the T_{RM} compartment would clearly be preferable. Conceivably, T_{RM} pools may be targeted by administration of bi-specific single domain antibody drug conjugates (e.g. targeting CD69 and CD103) [115], which are expected to penetrate into dense tissues such as the skin [22]. Alternatively, disruption of the signal transduction pathways that control T_{RM} retention or maintenance, such as the IL-15 receptor signaling pathway, could be attractive, especially of local inhibition can be achieved.

CONCLUDING REMARKS

Data that has emerged over the past years makes it evident that T_{RM} formation is regulated at several stages of the T cell response. At the naïve T cell stage, and also during T cell priming, the encounter of specialized DCs that provide specific signals plays a central role in the imprinting of the capacity to yield tissue-resident progeny. At the tissue site, the presence of local factors determines whether effector phase T cells with heightened sensitivity to such factors are retained locally and form a stable T_{RM} compartment. Factors that regulate these processes include antigen, co-stimulatory molecules, cytokines, chemokines and metabolites. Importantly, the presence of these factors may differ between individuals [116, 117, 118], between pathogens and tissue types [2], and even locally within tissue sites [17]. Further insight into the key signals that create and maintain T_{RM} populations in diverse tissues and under

different (patho-)physiological conditions, will help us to steer T_{RM} immunity. Early efforts already indicate that it is feasible to therapeutically target T_{RM}, and a further effort to design strategies that may be used to either boost or deplete tissue-specific resident $CD8^+$ T memory is clearly warranted.

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