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# Insights into phenotypic and functional CD8+ $T_{RM}$ heterogeneity

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## Summary

Cytotoxic $CD8^+$ T cells recognize and eliminate infected or cancerous cells. A subset of $CD8^+$ memory T cells called tissue-resident memory T cells ($T_{RM}$) resides in peripheral tissues, monitors the periphery for pathogen invasion, and offers a rapid and potent first line of defense at potential sites of re-infection. $T_{RM}$ cells are found in almost all tissues and are transcriptionally and epigenetically distinct from circulating memory populations, which shows their ability to acclimate to the tissue environment to allow for long-term survival. Recent work and the broader availability of single-cell profiling has highlighted $T_{RM}$ heterogeneity among different tissues, as well as identified specialized subsets within individual tissues, that are time and infection dependent. $T_{RM}$ cell phenotypic and transcriptional heterogeneity has implications for understanding $T_{RM}$ function and longevity. This review aims to summarize and discuss the latest findings on $CD8^+$ $T_{RM}$ heterogeneity using single-cell molecular profiling and explore the potential implications for immune protection and the design of immune therapies.

### Keywords

Tissue-resident memory T cell; CD8 T cell; immune memory; TRM heterogeneity

## Introduction

The immune system plays a crucial role in protecting the body from pathogens and cancerous cells. Upon antigen encounter, $CD8^+$ T cells proliferate rapidly and differentiate into a heterogeneous population of short-lived terminal effector cells (TE) and memory-precursor cells (MP) that mediate pathogen clearance. Following the resolution of infection, the population of effector T cells contracts. However, a small proportion of $CD8^+$ T cells persists and forms memory cells, which provide long-lived protection. $CD8^+$ memory cells have classically been studied in the blood and lymphoid organs. These long-lived circulating $CD8^+$ memory cells can be subdivided into central memory cells ($T_{CM}$) and effector memory cells ($T_{EM}$) based on their surface marker expression, trafficking patterns, and functional capabilities upon reinfection[1]. While $T_{CM}$ cells are predominately found in the blood and in

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secondary lymphoid tissues and are characterized by a high proliferative capacity, $T_{EM}$ cells patrol the blood and can transiently enter tissues during acute infections.

However, during acute infection, a subset of $CD8^+$ T cells migrates into tissues and gives rise to tissue-resident memory $CD8^+$ T cells ($T_{RM}$)[2–4] that provide local protection. $T_{RM}$ constitute a large portion of the $CD8^+$ T cell memory population and can remain lodged in tissues for the lifetime of the organism, without recirculating[5,6]. $T_{RM}$ cells scan tissues for pathogens and offer a rapid and potent first line of defense due to their enhanced effector functions and proximity to sites of reinfection[5].

## Defining tissue-residency

The strongest evidence that $T_{RM}$ reside in tissues without re-entering circulation and are a distinct memory T cell population arose from studies using parabiotic surgery in which the circulatory systems of two mice are joined. This procedure results in an equilibrium of circulatory T cells from the two animals. However, tissue-resident T cells did not reach homeostasis and remained tissue-specific in each mouse[2,7–10]. Studies in humans have exploited human leukocyte antigen (HLA)-mismatched allografts that have different HLA alleles between the organ donor and recipient to longitudinally follow donor-derived $T_{RM}$ cells in transplants. Using this approach, it has also been proven in humans that $T_{RM}$ cells can reside in organs for extended periods of time without draining to the lymph nodes or entering circulation[11–13]. Considering the experimental burden of performing parabiosis experiments in mice, many studies instead use an intravascular staining approach to mark T cells in the vasculature (thus inferred to be recirculating) with a fluorochrome-labelled antibody. T cells residing in tissues are not marked by the intravenous injection of the antibody, which has shown to faithfully reflect the residency of most antigen-specific $T_{RM}$ observed via parabiosis[14]. This strategy efficiently labels cells in the vasculature, but cannot discriminate between cells that reside in the tissue permanently and cells that only transiently enter the tissue. Nonetheless, for the purposes of this review, tissue-residency will be inferred via the absence of intravenous labelling.

## Common adaptations of memory T cells to tissue-residency

Although $T_{RM}$ cells share some transcriptional features with $T_{CM}$ and $T_{EM}$ cells, they are transcriptionally and epigenetically distinct from circulating memory populations[15–18]. A transcriptomic analysis of $T_{RM}$ across many tissues compared to their recirculating counterparts allowed the definition of “core” tissue-residency and circulatory gene expression signatures, which have helped define the tissue residency state[15–17,19]. Tissue residency is achieved by upregulation of adhesion and retention molecules and chemokine receptors (CD103, CD69, CCR9) and by reduced expression of lymphoid homing molecules (S1PR1, CCR7, CD62L) that help T cells to leave non-lymphoid tissues.

Multiple transcription factors that orchestrate the tissue-adaptation process have been identified. For example, CCR7, CD62L, and S1PR1 are controlled by the transcription factor KLF2, the expression of which is downregulated during $T_{RM}$ differentiation[3]. S1PR1 binds to sphingosine-1-phosphate (S1P), which is highly abundant in the blood and promotes tissue egress. Downregulation of S1PR1 is, hence, a necessity for tissue-residency. CD69

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is a c-type lectin protein that can form a complex with S1PR1 and inhibit binding of S1P to S1PR1, thus hindering egress from tissues of $T_{RM}$ cells[3]. A recent study showed that downregulation of S1PR5, another receptor for S1P, is essential for $T_{RM}$ formation in the skin[20,21], where S1PR5-deficiency leads to enhanced $T_{RM}$ formation. Interestingly, S1PR5 does not interact with CD69[21]. In contrast to S1PR1, which is directly controlled by KLF2, S1PR5 is regulated via T-bet and Zeb2[20], demonstrating how tissue-residency is the result of multiple coordinated adaptation processes by T cells. Downregulation of S1P-receptors is accompanied by the downregulation of the T-box transcription factors T-bet and Eomes, which subsequently renders $T_{RM}$ cells responsive to TGF-β[22,23]. TGF-β signaling induces the expression of CD103, thus promoting $T_{RM}$ cell retention, especially in epithelial tissues[24,25]. Furthermore, transcription factors that facilitate expression of the tissue-residency signature, while suppressing circulatory-associated genes, include Blimp1[15], Hobit[15], and Runx3[16]. In summary, while there is not a single $T_{RM}$-specific "marker," $T_{RM}$ cells are commonly characterized by expression of two surface molecules: CD103 and CD69. However, the expression of these two canonical $T_{RM}$ cell-surface proteins in tissues is neither uniform within a tissue, nor uniform across different tissue-resident T cells[17].

However, $T_{RM}$ cells do not constitute a homogenous memory T cell population. In fact, recent work has highlighted $T_{RM}$ heterogeneity within tissues and among tissues with differences in surface marker expression, transcriptional changes, functionality, and longevity[17,26]. $T_{RM}$ cells have been identified in almost every human and murine tissue and infiltration and long-term maintenance of these cells in non-lymphoid tissues requires T cells to acclimate to the specific environments that may differ in a broad range of ways, including the availability of nutrients, metabolite composition, cytokine milieu, cell composition, and matrix proteins. It is therefore not surprising that, although $T_{RM}$ cells share a common residency gene-expression signature, they also require tissue-specific acclimatization to persist in and patrol these unique and specialized environments. In addition to heterogeneity of $T_{RM}$ cells observed across different tissues, heterogeneity is additionally apparent within a single tissue, where $T_{RM}$ subsets resembling those observed in circulatory memory populations exist and change in their relative abundance over the course of an infection[27]. Furthermore, distinct infections can result in formation of different $T_{RM}$ populations; for example, $T_{RM}$ cells in the intestine that persist after *Yersinia pseudotuberculosis* or intravenous *Listeria monocytogenes* infection show different expression of cell surface proteins when compared to $T_{RM}$ cells that arise after acute *lymphocytic choriomeningitis* virus (LCMV) infection[28–30].

Single-cell RNA sequencing (scRNA-seq) is a powerful approach that can reveal heterogeneity within cell populations and has been used extensively in recent years to probe the dynamic gene expression patterns of a wide range of immune cell types in health and disease[1,31–33]. scRNA-seq combined with reporter- and fate-mapping approaches has led to important advances in the understanding of $T_{RM}$ intra- and inter-tissue heterogeneity with implications for $T_{RM}$ cytotoxicity, function, longevity, and plasticity. In this review, we will summarize and discuss the latest findings on $CD8^+$ $T_{RM}$ heterogeneity using single-cell molecular profiling and highlight the different levels of $T_{RM}$ heterogeneity as well as the potential implications for immune protection and the design of immune therapies.

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## Intertissue heterogeneity

Although most reports studying $T_{RM}$ cells focus on epithelial tissues like the intestine or the skin, $T_{RM}$ cells can be found in almost every organ. Besides the small intestine and the skin, $T_{RM}$ cells have, for example, been described in the kidney[17,34], liver[12,15], salivary gland (SG)[17,35,36], adipose tissue[17], pancreas[2], stomach[2], female reproductive tract[6], lung[37,38], and colon[2,26]. Although, $T_{RM}$ cells in these organs vary in their durability[6] – ranging from a half-life of 82 days in the uterus to no decay in the salivary gland – they lodge in these tissues for long periods of time, highlighting the necessity of recently migrating T cells to specifically acclimate to their new environment as they become long-lived $T_{RM}$[39]. Much work has focused on understanding how $T_{RM}$ cells become resident. Common adaptations include the downregulation of tissue egress molecules, such as S1PR1, and the upregulation of retention signals. However, considering that all organs fulfill distinct physiological roles, provide specialized tissue architectures, and have a unique composition of cells and structural tissue, it seems very likely that, in addition to common adaptations to tissue retention in general, $T_{RM}$ cells also need to make tissue-specific adjustments in gene expression, metabolic state, and homeostatic dependencies. In addition to a lineage $CD8^+$ T cell-defining signature and memory T cell signature, $CD8^+$ $T_{RM}$ cells need to acquire a “common” residency signature as well as tissue-specific adaptations that mediate long term survival and function in that environment (Figure 1).

Tissue-specific adaptations of other immune cell types that establish residency, such as tissue-resident macrophages, are well established and provide a basis for considering such changes for $CD8^+$ $T_{RM}$[40–42]. Specialized macrophages with distinct functions can be found in the spleen, skin (Langerhans cells), serosal tissues, lung, liver (Kupffer cells), gastro-intestinal tract, bones (osteoclasts), and the central nervous system (microglia)[40]. These tissue-specific adaptations are, by necessity, not reflected in the common $T_{RM}$ residency signatures observed when identifying the common adaptations of $T_{RM}$ originating from many tissues[15,16]. A careful comparison of gene expression across tissues in the same infection setting and the development of single-cell RNA sequencing (scRNA-seq) approaches has broadened our understanding of the differences in $T_{RM}$ populations, allowing differentiation between ubiquitous tissue-specific changes to the $T_{RM}$ cell population and changes in the abundance of shared, heterogeneous $T_{RM}$ cell populations[17]. How these specific adaptations are regulated is not completely understood, but may be initiated by distinct signals from cytokines, chemokines, cell-cell interactions, or metabolites, and could be reflected by unique patterns of transcription factor expression and lead to distinct expression patterns of surface proteins.

### Cytokines required for $T_{RM}$ differentiation

In accordance with different transcriptional acclimatization of $T_{RM}$ cells to tissues, $T_{RM}$ cells require distinct cytokines to both start and sustain their differentiation process into tissue-resident memory cells. For example, TGF-β, a pleiotropic cytokine, has been well established in its importance for $T_{RM}$ formation in the skin, intraepithelial lymphocyte (IEL), and salivary gland, but it is not required for liver or kidney $T_{RM}$ formation[17,22,23,25,36,43]. Furthermore, it has been shown, that IL-15, a common gamma

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chain cytokine that promotes homeostatic proliferation and survival of memory cells[44,45], is required for $T_{RM}$ survival in the skin[22,46], liver[47], salivary gland, and kidney[48] but is dispensable for $T_{RM}$ homeostasis in the pancreas, female reproductive tract, and the small intestine in mice[48,49]. However, even if some $T_{RM}$ populations do not require IL-15 for maintenance, these cells still proliferate in response to IL-15, possibly to transiently amplify memory populations[50]. Not only do the different requirements for $T_{RM}$ development highlight the necessity for tissue-specific adaptation, but they might also indicate the existence of distinct $T_{RM}$ subsets that are driven and maintained by distinct cytokines and cell interactions.

## $T_{RM}$ cells in different organs are dependent on distinct transcription factors

In line with the idea of tissue-specific adaptations of $T_{RM}$ cells, the transcription factors required for $T_{RM}$ differentiation differ among tissues. While some transcriptional adaptations (e.g., downregulation of KLF2) are shared among $T_{RM}$ populations, differential transcription factor dependence has been observed. For example, the transcription factor Hobit is required for skin, but not lung $T_{RM}$ generation[15]. Another example for tissue-specific transcription factors is the transcriptional repressor hypermethylated in cancer 1 (Hic1). Hic1 is induced during human iTreg differentiation[51] and was shown to regulate homeostasis of intestinal lymphocyte populations in mice, thereby preventing the development of intestinal inflammation[52]. In LCMV infection, Hic1 expression by T cells is largely restricted to the small intestine (Figure 2), and knockdown of Hic1 specifically reduces $T_{RM}$ cells in the small intestine, while other organs remain unaffected[17]. In line with that, overexpression of Hic1 leads to a specific accumulation of $T_{RM}$ cells in the small intestine[17]. Mechanistically, this can partially be explained by the finding that Hic1 regulates expression of P2RX7[17], an extracellular ATP receptor that enhances metabolic fitness[53] and contributes to small intestinal $T_{RM}$ survival[53,54].

In addition to tissue-specific transcription factors, the dependency of $T_{RM}$ on $T_{RM}$-defining transcription factors varies among tissues. Deletion of *Runx3* reduces $T_{RM}$ cell numbers in multiple non-lymphoid organs, including in kidney and salivary gland, but its deletion leads to the strongest reduction of $T_{RM}$ cells in the small intestinal epithelium and affects $CD103^{+}$ $CD69^{+}$ TRM cells more compared to $CD103^{-}$ TRM cells[16]. Similarly, *Blimp1* deletion impairs $T_{RM}$ formation in the IEL and SG more than the kidney[17]. Hence, tissue-specificity is not only achieved by the expression of tissue-defining transcription factors, but also by modulation of the expression of $T_{RM}$-inducing transcription factors. It is likely that an interplay of both mechanisms leads to the optimal adaptation to tissues that $T_{RM}$ require for persistence. Therefore, further understanding and identification of tissue-specific transcriptional networks will be the basis for engineering tissue-programmed T cells.

## $T_{RM}$ in different tissue sites are phenotypically distinct

Phenotypic comparisons of $T_{RM}$ in distinct tissues reveal tissue-specific patterns of expression of cell-surface receptors by $T_{RM}$ cells. While CD103 and CD69 expression, as well as loss of IL-18R expression, are commonly associated with and used for identification of $T_{RM}$ cells, substantial differences in expression of these molecules can be observed among different $T_{RM}$ populations. CD103 is an alpha integrin that, upon heterodimerization

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with a beta integrin, can bind to E-cadherin and facilitate retention in epithelial tissues. Thus, CD103 expression is mainly restricted to $T_{RM}$ cells associated with epithelial barrier tissues, including skin, small intestine, and salivary gland[17,22,26,36]. However, the integrin superfamily contains numerous subunits (with 18 alpha and 8 beta subunits)[55], and a recent study shows that expression of the beta integrins differs substantially among tissues (Figure 2). For example, there are multiple differences in alpha- and beta-integrins between the small and large intestine: αE integrin expression is higher in the small intestine compared to the colon, whereas expression of α4β1 is higher in $T_{RM}$ cells from the colon[26]. These findings indicate that tissue-retention modules are specifically adapted to distinct regions of the intestine. CD69, which antagonizes S1PR1 and thereby hinders tissue egress, is widely used to identify $T_{RM}$ cells. However, the expression levels of CD69 in $T_{RM}$ cells and the functional relevance of CD69 for formation or maintenance of $T_{RM}$ cells varies vastly among tissues. In murine acute LCMV infection, CD69 expression is not needed for $T_{RM}$ formation in the small intestine, but necessary for kidney $T_{RM}$ cells, and forced expression of CD69 increases $T_{RM}$ formation in the kidney[56]. Downregulation of IL-18R is associated with the establishment of $T_{RM}$ cells in the kidney[34]. However, similar to CD103 and CD69, its expression varies among $T_{RM}$ cells from different tissues: IL-18R expression is lost in IV-negative $T_{RM}$ cells from the IEL after LCMV infection in mice but only partially downregulated in $T_{RM}$ cells obtained from kidney and SG, and it is still expressed in visceral-adipose tissue and liver $T_{RM}$ cells[17]. Ly6C expression in reduced in $T_{RM}$ cells compared to $T_{CM}$ cells[49,57,58], but the expression of Ly6C also greatly depends on the studied tissue[17,57]. These findings demonstrate that the functional relevance of surface proteins commonly used for $T_{RM}$ identification largely depends on the tissue.

## $T_{RM}$ cell in different tissues exhibit specific metabolic adaptations

Another level of specialization of $T_{RM}$ cells to their given tissue environment is reflected in their usage of fatty acid binding proteins (FABP)[59] (Figure 2). $T_{RM}$ cells rely on fatty acid uptake for their survival[60]. However, the FABP family exists as a large family with many different isoforms that are expressed in a tissue-specific manner. Interestingly, $T_{RM}$ in different tissues express different isoforms of FABP, highlighting their specific adaptation to the tissue microenvironment[59]. Upon relocation, $T_{RM}$ cells adapt their FABP expression profile to the new location. Thus, isoform usage is determined by tissue-derived factors, and $T_{RM}$ cells actively and continuously sense their environment and adapt to it[59].

## $T_{RM}$ heterogeneity among organs in humans

Most studies of $T_{RM}$ heterogeneity among tissues have used murine models. Further, most studies rely on the adoptive transfer of T cell receptor (TCR)-transgenic antigen-specific T cells, and thus do not assess the contribution of a polyclonal endogenous T cell response and the relevance of the TCR clonotypes to $T_{RM}$ cells. A recent report, however, addresses these limitations and extends our knowledge of human tissue-resident T cells[61,62]. In this study, lymphocytes from the lung, jejunum, abdominal skin, their draining lymph nodes (LN) (pulmonary LN, mesenteric LN, and inguinal LN), and from blood and spleen were collected and used for a comprehensive profiling with CyToF, TCR-sequencing, and single-cell RNA sequencing. In accordance with findings from the murine $T_{RM}$ models, the authors found a unique composition of $CD4^+$ and $CD8^+$ $T_{RM}$ cells in the assessed barrier tissues:

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T cells lodged in the skin had high expression of *CCR4*, *CCR10* and *CXCR4*, and the Th2 lineage-defining transcription factor *GATA3*; T cells that reside in the intestine exhibited a high expression of integrins (*ITGAE*, *ITGA1*, *ICAM1*), and T cells isolated from the lung displayed a site-specific expression pattern of *CTLA4*, *IL10*, and *PDCD1*[61] (Figure 2). In addition to location-specific expression patterns, the authors identified common adaptations among all barrier tissues and expression patterns that are shared among only some tissues. For example, T cells in the jejunum and lung were characterized by high expression of *CXCR6* and Th17 signatures genes (*CCL20*, *RORA*, *RORC*, and *IL17A*)[61], and $T_{RM}$ cells in the lung and skin showed increased expression of genes encoding matrix- and adhesion-associated molecules as well as *PRDM1* encoding for BLIMP1, a transcription factor associated with tissue-residency and effector functions. These transcriptomic changes may indicate the specific needs of T cells to maintain immunity against various types of pathogens in threatened tissues throughout human life. Importantly, many of these site-specific adaptations are by human and mouse T cells; for example, expression of *ITGAE*, *CCR9* or *HIC1* and downregulation of *KLF2* in the intestine or high expression of *AHR* in the skin. These findings indicate that some of these transcriptional profiles are conserved across species (Figure 2). However, expression of some genes is not conserved; for example, T cells in the jejunum exhibit elevated expression of *IL17A* in humans, but not in mice. These differences might arise from differential exposure of humans to pathogens (in contrast to mice in a specific-pathogen free environment), but also might reflect variance due to polyclonal $CD3^+$ T cells for human versus TCR transgenic $CD8^+$ T cells for mice or additional differences. Considering an individual's age and history of infection also adds a new layer of complexity to understanding $T_{RM}$ phenotype, function, differentiation, and maintenance in humans. Insight into these topics is essential to improve targeted and tissue-adapted immune responses at barrier tissues in humans.

## Clonal relationships between circulatory and tissue-resident memory T cells

How T cell clones that contribute to circulatory memory populations are proportional to tissue-resident T cell populations and whether or not the TCR clones found in $T_{RM}$ population are unique to the $T_{RM}$ populations or even the specific tissue are all topics of great interest. Using TCR sequencing, a recent report described that certain TCR clones expand more in barrier sites compared to circulatory memory populations, indicating that the TCR pool in circulation is not representative of that seen in tissues[61] — an observation that has important implications in numerous areas, such as vaccine design and boosting strategies[63]. This finding is corroborated by a report using clonal tracing of T cell clones that showed that graft versus host disease (GvHD) in a murine model is maintained by expansion of a tissue-residing $TCF1^+$ subpopulation and not by recruitment of T cells from secondary lymphoid organs or the blood[64]. Furthermore, in humans, TCR clones for $CD4^+$ $T_{RM}$ cells segregated between the barrier tissues with only minimal overlap between the skin, lung, and jejunum, where $CD4^+$ T cell clones are less disseminated and more site-specific compared to $CD8^+$ T cell clones[61]. It is possible that this clonal segregation is a result of different infections or pathogens that T cells encounter at these three distinct barrier sites, but the possibility that certain TCR clones preferentially give rise to $T_{RM}$ cells in a specific organ cannot yet be ruled out. However, even within a naive T cell population that expresses the same TCR, a clonal population that possesses a heightened potential to form $T_{RM}$ cells was

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shown to exist[65]. In this study, barcode labelled naive OT-I T cells were transferred into recipient mice, and $T_{RM}$ cells were induced by vaccination or herpes simplex virus (HSV) infection. While T cell clones contributed proportionally to circulatory and skin effector T cells, a disparity in T cell clone distribution was seen at memory timepoints[65], suggesting that distinct T cell clones do preferentially give rise to $T_{RM}$ cells. Interestingly, there is evidence that these T cell clones might become preconditioned towards their $T_{RM}$ fate even before entering the tissue[65,66].

## Intratissue heterogeneity

The heterogeneity of cell surface receptor expression and overall gene expression indicated by scRNA-seq within a population of $T_{RM}$ cells from a given organ varies greatly. For example, $T_{RM}$ cells in the dermis are heterogeneous in their expression of CD69, with only approximately 70% of the dermal $T_{RM}$ cells expressing CD69, whereas almost all $T_{RM}$ cells in the epidermis express CD69[22]. In contrast, the majority of $T_{RM}$ cells in the dermis do not express CD103, but the expression pattern of CD103 on epidermal $T_{RM}$ is heterogeneous[22]. The heterogeneous expression of surface proteins of $T_{RM}$ within a tissue is not limited to the skin and has also been observed in other tissues. $T_{RM}$ in the salivary gland show a heterogeneous expression for both CD69 and CD103[17,36], and kidney $T_{RM}$ are characterized by an intermediate expression of CD69 but almost no expression of CD103[17,34] (Figure 3a). Similarly, liver-resident $T_{RM}$ exhibit low to intermediate expression of CD69 and no expression of CD103[17,36]. Similar findings are observed in human studies—whereas most of the intestinal T cells are positive for CD103 and CD69, expression of these two tissue-residency promoting molecules is heterogeneously expressed by T cells resident in the lung or skin and also varies between $CD8^+$ and $CD8^-$ T cells (Figure 3b). While most small intestinal intraepithelial $T_{RM}$ cells in mice express CD103 and CD69 after acute LCMV infection, only about 60% of small intestinal $T_{RM}$ cells from the lamina propria, and approximately 30% of colonic $T_{RM}$ cells, express CD103 and CD69[26]. In addition to CD69 and CD103, other $T_{RM}$ markers and transcription factors including *Il18r, Cd49a, Ly6c, Tcf1, T-bet*, and *Eomes* have been shown to exhibit variable expression within the tissue as well as among tissues[17,23,26,34,36]. The expression levels of these canonical $T_{RM}$ molecules are further subject to changes over time: e.g., expression of CD103 and CD69 in small intestinal $T_{RM}$ cells increases from the effector phase to memory timepoints, whereas expression of IL-18R decreases. Hence, the time after infection adds a new dimension to $T_{RM}$ heterogeneity. This idea is further supported by the finding that the importance of $T_{RM}$-driving factors can vary depending on the time after infection. For example, the inducible costimulator (ICOS) receptor is essential for $T_{RM}$ establishment, but not maintenance[67], and the transcription factor EOMES is not required for formation of small intestinal $T_{RM}$, but is needed for their long-term maintenance[26]. In summary, these findings support the hypothesis that different subtypes of $T_{RM}$ exist and that the composition of the $T_{RM}$ pool might vary over time.

### Parallel subsets between circulatory memory and tissue-resident memory T cells

The circulatory memory T cell compartment is phenotypically and functionally heterogeneous, with effector and memory T cell populations consisting of phenotypic

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and functional subsets that evolve over time. Terminally differentiated, short-lived effector cells ($CD127^{-}KLRG1^{+}$) and memory-precursor cells ($CD127^{+}KLRG1^{-}$) are predominantly found during the effector phase of infection[68,69]. These cells continue to differentiate over time and form circulatory memory T cells that can be broadly divided into $T_{CM}$ and $T_{EM}$, which derive from memory precursor cells and t-$T_{EM}$ cells that come from $KLRG1^{hi}$ effector cells. Recent data show that intratissue heterogeneity of $T_{RM}$ populations reflects $T_{RM}$ subsets that functionally resemble those observed in circulation[27,31,70].

Two distinct subsets of $T_{RM}$ cells that mirror those seen in circulatory memory populations have been identified[27]. While small intestinal T cells showed an enrichment for effector gene-signatures early after an infection, gene-expression at later timepoints was enriched for memory signatures, highlighting a continuous differentiation of $T_{RM}$ cells over time. Among the transcriptional regulators with differential expression over the course of infection were Blimp1 (*Prdm1*) and Id3[27]. Id3 is an inhibitor of E protein transcription factors. It regulates long-lived circulatory memory formation in $CD8^{+}$ T cells[71], and is most highly expressed in small intestinal $T_{RM}$ cells at late time points after infection. In contrast, Blimp1, which marks terminally-differentiated T cells, displayed an inverse expression pattern in studies, peaking at day 4 of infection and subsequently declining in small intestinal $T_{RM}$ cells[27] and skin $T_{RM}$ cells[15]. Using Blimp1 and Id3 reporter mice, two distinct subsets of small intestinal $T_{RM}$ cells were observed: $Blimp1^{hi}Id3^{lo}$ $T_{RM}$ cells expressed canonical effector genes (i.e., *Cx3cr1*, *Zeb2*, *Klrg1*, *Gzma* and *Gzmb*), and, in contrast, $Id3^{hi}Blimp1^{lo}$ $T_{RM}$ cells expressed genes reminiscent of memory-precursor cells (i.e., *Bach2*, *Tcf7*, and *Cd27*)[27]. Furthermore, $Id3^{hi}$ $T_{RM}$ cells have shown elevated degranulation capacity and cytokine production of IFNγ, TNF, and IL-2 and yield a greater frequency of both circulating and resident secondary effector T cell populations after rechallenge compared to the $Id3^{lo}$ subsets[27]. Thus, $Id3^{hi}$ small intestinal $T_{RM}$ cells exhibit multifunctionality and memory potential, which is consistent with the greater proliferation capacity of $Id3^{hi}$ circulatory memory T cells.

Analysis of $T_{RM}$ differentiation in tissues has largely relied on bulk RNA sequencing and a small number of phenotypic markers, limiting the ability to discern functional differences and intermediate states of differentiation that might arise within the $T_{RM}$ population[15,16]. scRNA-seq overcomes this limitation and has identified clear functional and phenotypical subsets among $T_{RM}$ cells within the same tissues and highlighted similarities to the memory differentiation of memory $CD8^{+}$ T cells in blood and secondary lymphoid organs. A longitudinal scRNA-Seq study[31] of the T cell response to the Armstrong strain of LCMV (LCMV Armstrong) analyzed small intestinal $T_{RM}$ in comparison to circulatory memory cells and found a transcriptional memory signature that is used by both circulatory and tissue-resident subtypes of memory $CD8^{+}$ T cells throughout their differentiation[31]. In addition to this shared signature, intestinal $T_{RM}$ cells exhibited a distinct signature, which may contribute to their adaptation to the intestinal environment (Figure 1*, tissue-specific adaptations*). Importantly, by focusing the analysis on $T_{RM}$ cells, the study also showed that the $T_{RM}$ population is heterogenous throughout the course of an infection and exists in multiple clusters, many of which were present at several time points. These findings suggest that multiple subtypes of $T_{RM}$ cells exist. For example, at day 60 of infection, two distinct types of clusters existed within the small intestinal $T_{RM}$ population: one with higher

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expression of memory-associated transcription factors, such as *Id3*, *Jun*, *Fos*, and *Klf2*, and higher expression of *Il7r* (encoding for CD127), and another with expressed transcription factors, such as *Zeb2*, that might indicate a more terminally differentiated cell state and have been shown to promote terminal differentiation of circulatory effector and memory T cells[72] and lower expression of *Il7r*[31]. The difference between these two $T_{RM}$ subsets was also highlighted by higher cytokine production after restimulation with a cognate antigen in the $CD127^{high}$ $T_{RM}$ cells compared to $CD127^{low}$ $T_{RM}$ cells. Therefore, these subsets seem to phenotypically and functionally mirror the $Id3^{hi}$ and $Blimp1^{hi}$ subsets that were identified using reporter mice and confirm the presence of functionally relevant $T_{RM}$ subsets that change in their relative abundance over time.

A recent study using flow-cytometry and scRNA-seq to analyze donor-derived $T_{RM}$ cells from intestinal transplant recipients reveals substantial heterogeneity within the $T_{RM}$ compartment in a single tissue can be observed in humans as well[13]. Remarkably, by exploiting HLA-mismatches between the organ donor and recipient, the authors prove that donor-derived $T_{RM}$ can survive in the organ graft for at least 5 years following intestinal transplant[13]. Much like the mouse $T_{RM}$ compartment, two distinct populations were observed among the donor-derived intestinal $T_{RM}$ cells: a first population expressed higher levels of *ITGAE*, *IL7R*, *KLRB1* and *CCR6*, indicating a more memory-like cell state, and a second population expressed higher levels of *KLRG1*, cytotoxic granules and the transcription factor *ZEB2*, thus resembling more terminally differentiated effector T cells[13]. Extending the characterization of intestinal $T_{RM}$ to the disease state, a different study analyzing intestinal T cells from patients with ulcerative colitis and healthy controls revealed enrichment of $T_{RM}$-like cells with inflammatory properties (higher expression of ZEB2, TBX21, and PRF1) compared to controls[70]. These data suggest, that $T_{RM}$ cells normally exist in an equilibrium of multiple distinct differentiation states and that an imbalance of these $T_{RM}$ differentiation states might be associated with, or even causative for, autoimmune-mediated diseases such as ulcerative colitis.

A recent complementary study highlights heterogeneity within the human skin $T_{RM}$ population[61]. Using scRNA-seq of skin-resident T cells from two individuals, the authors were able to identify clusters of skin $T_{RM}$ cells that were transcriptionally poised towards Th1-like (TBX21), Th17-like (RORA), and cytotoxic (GZMA, GZMK, NKG7, PRF1, IFNG) responses[61]. The concept of skin $T_{RM}$ heterogeneity is further corroborated by previous studies that identified functionally and phenotypically discrete populations of resident and recirculating memory T cells in the skin[73,74]. $CD103^{-}$ $T_{RM}$ cells mainly localized in the dermis and a higher proliferative capacity compared to $CD103^{+}$ $T_{RM}$ cells, which were enriched in the epidermis and had higher effector functions[73].

In summary, these studies support the idea that multiple subsets within the $T_{RM}$ population with distinct functional capacities and transcriptomic programs exist in both mice and humans. This heterogeneity observed within the $T_{RM}$ population is also likely reflected in the $T_{RM}$-mediated protection from secondary infection. More cytotoxic $T_{RM}$ cells might preferentially act on infected cells, and their reduced proliferation capacity might limit immunopathology caused by activated $T_{RM}$ cells. In contrast, $T_{RM}$ cells with higher stemness or "memory potential" might replenish and sustain the $T_{RM}$ pool over longer time periods.

In line with this idea, recent studies have identified that CD103⁻ $T_{RM}$ cells have the capacity to replenish and augment the CD103⁺ $T_{RM}$ pool upon a secondary infection[75–77]. Given the strong evidence for distinct subsets within intestinal $T_{RM}$ cells, it will be interesting to see which factors drive and maintain differentiation of these subsets. It is tempting to speculate that these cells might be localized in different areas of the tissue and that the tissue microenvironment, the level of inflammation, the abundance of antigens, the presence of cytokines, chemokines, and metabolites, and the interactions of T cell partners might govern the $T_{RM}$ cell adaptation to the tissue to allow for optimal tissue surveillance.

## Infection-mediated variation in tissue-resident memory T cells

### Infection type dictates the $T_{RM}$ phenotype

In addition to the environmental influences derived from different tissue that may regulate $T_{RM}$ formation and maintenance within a tissue[5,49], the type of infection also shapes the outcome of $T_{RM}$ differentiation. For example, LCMV Armstrong causes an acute and systemic infection that leads to formation of $T_{RM}$ populations in many tissues. In this infection setting, the $T_{RM}$ population within the small intestine almost entirely expresses CD69 and CD103. In contrast, following *Yersinia pseudotuberculosis* infection, two distinct $T_{RM}$ populations form in the small intestine[28]: a CD103⁺ $T_{RM}$ population and a CD103⁻ $T_{RM}$ population. The CD103⁺ subset constitutes the majority of $T_{RM}$ cells in the intraepithelial layer, whereas both populations are equally abundant in the lamina propria. Unlike CD103⁻ $T_{RM}$ cells that develop in the absence of TGF-β, cluster around areas of bacterial infection, and are enriched in the lamina propria, CD103⁺ cells require TGF-β for their development and are scattered throughout the intestine[28]. Importantly, CD103⁻ $T_{RM}$ cells in this setting are not precursor cells of CD103⁺ $T_{RM}$ but represent a distinct population that is stable over time. It has been shown that a single infection can give rise to two independent CD8⁺ $T_{RM}$ populations and that the phenotypic composition of the $T_{RM}$ population induced by different pathogens may reflect the unique signals associated with the infection type.

### Route of infection alters the $T_{RM}$ phenotype

The phenotypic and transcriptional features of $T_{RM}$ cells are not only a result of the infection model used, but also depend on how the pathogen is administered. For example, oral infection with a mouse-adapted *Listeria monocytogenes* strain that contains a mutation in the internalin A protein to facilitate invasion of murine epithelial cells efficiently induced $T_{RM}$ cells in the small intestine[30]. However, mice infected with the same bacterial strain via an intranasal method, had fewer intestinal $T_{RM}$ cells, and these cells did not fully convert to a memory phenotype[78]. Similarly, infection of mice intranasally with an influenza virus expressing ovalbumin did not induce memory cells in the intestine[30]. Thus, the route of infection, and thereby the location of T cell priming, dictates where and to what extent $T_{RM}$ cells form in peripheral tissues.

Another study comparing how T cell priming in the spleen versus T cell priming in the mesenteric lymph node contributes to T cell differentiation into CD103⁺ intestinal $T_{RM}$ cells[29,79] provides further evidence that the location of T cell priming regulates $T_{RM}$ formation. Comparing intravenous infection, where T cells were primed in the spleen,



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to foodborne infection with *Listeria monocytogenes*, where T cells were primed in the mesenteric lymph node, revealed that only mesenteric lymph-node-primed T cells give rise to CD103+ intestinal $T_{RM}$ cells[29]. This difference in $T_{RM}$ licensing is attributed to the presence of retinoic acid in the mesenteric lymph node, which regulates expression of intestinal homing receptors, such as CCR9 and α4β7-integrin, as well as genes that are part of the $T_{RM}$ gene-expression signature (*Hic1*, *Xcl1*, *Itgae*, and *P2rx7*)[29].

These findings are of direct therapeutic relevance. While intramuscular, intravenous, or intranasal administration of an mRNA vaccine targeting the influenza A virus nucleoprotein were all capable of establishing memory cells in the lung and draining lymph nodes, the combination of intramuscular priming with an additional intranasal boost archived the highest frequencies of lung $T_{RM}$ cells[38]. This “prime-and-spike” approach has also been successfully used in mouse models of COVID-19 infection[63], but, surprisingly, it was not able to induce an effective immune response in a human phase I trial[80]. It is not entirely clear why these differences occur, but it might be partially explained by different dosages of the vaccine or the way the vaccine is administered[80]. However, these results highlight the need for a better understanding of how $T_{RM}$ can be induced at specific tissue sites for therapeutic purposes. Thus, both tissue microenvironment and type of infection, and, therefore, inflammatory microenvironments, significantly impact $T_{RM}$ development in response to acute infection and have direct implications for vaccination strategies to archive infection experience and induce optimal tissue protection.

## Common adaptations of immune cells to tissue residency in specific tissues

In addition to CD8+ $T_{RM}$ cells, many other immune cells, including CD4+ T cells, innate lymphoid cells (ILCs), macrophages, and natural killer (NK) cells, form tissue-resident populations similar to those described for CD8+ T cells[15,16,40,41,81–84]. Interestingly, some of the transcriptional programs inducing tissue residency and inhibiting tissue egress seem to be shared among the different tissue-resident immune cell types. For example, Blimp1 and Hobit have well-established roles in both CD8+ $T_{RM}$ and NK and NKT tissue-resident memory cells[15], and suppression of Klf2 and S1PR1 and upregulation of Hobit is shared between CD4+ and CD8+ $T_{RM}$ cells[84]. These findings suggest that mechanisms for immune residency are shared between different immune cells and could reflect the adaptation to the unique environment of a specific tissue.

Runx3, which drives $T_{RM}$ formation in CD8+ $T_{RM}$ cells through a TGF-β-dependent transcriptional mechanism, is not required for tissue residency of CD4+ $T_{RM}$ cells in the dermis, which instead relies on Runx1[16,85]. This discrepancy might, in part, be explained by different localization and expression profiles of CD8+ versus CD4+ $T_{RM}$ cells in the skin. CD4+ $T_{RM}$ express less CD103 compared to CD8+ $T_{RM}$ cells, preferentially reside in the dermal layer of the skin, and display a higher dynamic movement compared to CD8+ $T_{RM}$ cells[86]. Notably, overexpression of Runx3 in CD4+ $T_{RM}$ cells changes the localization of CD4+ $T_{RM}$ in the skin with a preference to the epithelium, similar to CD8+ $T_{RM}$ cells, and enhances effector functionallity[85]. These findings show that ectopic expression of Runx3 in

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CD4 T cells induces a residency program similar to that seen in CD8 $T_{RM}$ cells, which is usually not acquired by CD4 $T_{RM}$ cells due to the lack of Runx3 expression.

In addition to a core residency program shared among tissues, organ-specific adaptations seen in $T_{RM}$ cells are also mirrored by other immune cell types (Figure 1). For example, the transcription factor Hic1, which was first identified as a tumor suppressor in human cancers[87,88], is relatively specifically expressed in intestinal $T_{RM}$ cells[17]. Interestingly, the intestine-specific expression pattern of this transcription factor is also seen in other immune populations, including innate lymphoid cells (ILCs), $CD4^+$, $T_{reg}$, and macrophage resident cells[17,52,89,90]: Loss of Hic1 reduced accumulation of T cells and ILC3 in the lamina propria and intraepithelial lymphocyte compartments[52,89]. These findings suggest, that Hic1 could serve a broad role in establishing or maintaining tissue-residency in the small intestine and that its function is shared among many cell types. More generally, these findings support the idea that tissue-specific adaptations can be shared across multiple types of resident immune cells (Figure 1).

In a similar example of common adaptations of immune cells to a tissue, the tissue-specific isoforms of fatty acid binding proteins (FABP) of $CD8^+$ $T_{RM}$ cells mimic the expression pattern seen in other resident immune cells for skin, small intestine, and liver[59]. Thus, even though resident immune cells exhibit distinct differentiation trajectories and are derived from distinct precursor cells, they can share common adaptation methods to the tissue they reside in. Intriguingly, understanding tissue-specific adaptations in one resident population might be extrapolated to other immune cells. Considering that an effective immune response requires coordination of many different immune cells, each with a specific function, the ability to boost tissue-acclimatization of multiple immune cells simultaneously within a given tissue could be a promising therapeutic concept. In contrast, inhibiting tissue influx of immune cells by inhibiting common adaptation processes or, conversely, directing regulatory T cells to a tissue, could be an effective therapy for autoimmune and immune-mediated diseases, highlighting the need to further understand how immune cells acclimatize to tissues.

## $T_{RM}$ heterogeneity is reflected in T cell function

The pathognomonic function of memory cells is to protect from reinfection, where the memory cells re-encounter their cognate antigen. Among the memory cell population, $T_{RM}$ cells provide the first line of defense at barrier tissues. Upon activation, $T_{RM}$ cells not only produce cytokines, such as IFN$\gamma$ or TNF, but also, and maybe even more importantly, they can alert the neighborhood and recruit circulating lymphoid and non-lymphoid cells to the tissue to aid in the response[49,78,91]. Importantly, $T_{RM}$ cells can rapidly proliferate, traffic to regional lymph nodes, and even contribute to secondary circulatory memory populations[26,36,92–94]. Thus, $T_{RM}$ cells exhibit features of effector cells as well as features that are reminiscent of $T_{CM}$ cells.

By comparing $T_{RM}$ cells from the small intestine to the colon after acute LCMV infection in mice, a recent study identified higher expression of granzyme A and B in small intestinal $T_{RM}$ cells compared to $T_{RM}$ cells residing in the colon[26]. However, the highest expression

of IFNγ was observed in colon $T_{RM}$ cells and for both the small intestine and the colon, IFNγ was higher in intraepithelial $T_{RM}$ cells compared to $T_{RM}$ cells located in the lamina propria[26]. Importantly, this functional heterogeneity among intestinal $T_{RM}$ cells did not appear to be primarily driven by differences in CD69 and CD103 expression[26] but reflected the specific functional adaptation of cells to their residing tissue.

The functional capacity of $T_{RM}$ cells is diverse among tissues and even among subsets within a distinct tissue. This diversity adds another layer of complexity to understanding how $T_{RM}$ adapt to barrier tissues and optimally respond to different types of antigens to sustain a long-lived memory population.

## $T_{RM}$ in secondary infections

Memory T cells are functionally specialized to provide enhanced protection against subsequent infection. $T_{RM}$ cells locally surveil non-lymphoid tissues and provide the first line of defense upon reinfection at barrier sites. $T_{RM}$ can undergo *in situ* proliferation[48,92–95] independent of help from $CD4^+$ T cells[93] and can autonomously amplify local immune surveillance and memory in various barrier sites in response to a range of stimuli[93].

Secondary systemic adaptive immune responses are commonly attributed to circulatory memory cells. Recent reports suggest, however, that $T_{RM}$ cells themselves can undergo retrograde migration and substantially contribute to secondary circulatory memory cells, thus forming an "outside-in" immune response[36,92,94]. For example, in one experiment, congenically distinct OT-I $T_{RM}$ engrafted skin was transplanted to infection-matched mice, and $T_{RM}$ cells were then reactivated locally with their cognate peptide. After 2–3 weeks, ex-$T_{RM}$ cells were detectable in the draining lymph node and gave rise to circulating $T_{CM}$ and $T_{EM}$ cells. Interestingly, this egress from the non-lymphoid tissue could be reduced by treatment with FTY720, a S1P-receptor modulator, suggesting that the tissue egress is at least partially mediated by S1P[92]. In contrast, another report studying the recall response of skin $T_{RM}$ cells did not find a contribution of $T_{RM}$ cells to the circulatory memory response[95]. In this study, HSV-specific T cells were injected into mice, and skin-resident $T_{RM}$ cells were induced with DNFB, which acts as a non-specific stimulus to recruit T cells into the skin. Thereafter, these mice were rechallenged with local HSV infection[95]. In this experimental setting, local proliferation of $T_{RM}$ cells that provided localized protection from HSV-infection could be observed. However, skin $T_{RM}$ cells were constrained to their location and did not migrate towards the lymph node after HSV infection[95]. It is unclear, if these differences were a result of the different infections used or how $T_{RM}$ cells were induced, but these findings highlight the diversity of site-specific immunity.

Most studies focusing on $T_{RM}$ "stemness," or ability to contribute to secondary responses and memory formation, rely on adoptive transfer experiments of $T_{RM}$ cells into new recipient mice. Using the transfer $T_{RM}$ has revealed that small intestinal intraepithelial $T_{RM}$ cells can lose the expression of their hallmark molecules CD69 and CD103 and transdifferentiate to secondary circulatory memory populations[26,36,49,92,94]. Phenotypically, these cells mostly give rise to $T_{EM}$-like memory cells (as identified by $CD127^+CD62L^-$), but few ex-$T_{RM}$ cells also upregulate L-selectin (CD62L), a marker that is classically expressed by $T_{CM}$



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cells[49,92]. After transfer of equal numbers of $T_{RM}$, $T_{EM}$, and $T_{CM}$ cells into freshly infected recipient mice, cell numbers of secondary circulatory memory cells derived from ex-$T_{RM}$ cells were higher than those derived from ex-$T_{EM}$, but lower than those derived from ex-$T_{CM}$ cells. Thus, $T_{RM}$ cells can display “developmental plasticity” and are not terminally-differentiated[92]. This finding is also supported by a machine-learning approach trained on a whole-genome bisulfite sequencing dataset using naive T cells as a reference for the highest stemness and exhausted T cells as a reference for the lowest plasticity[92]. The authors found that $T_{RM}$ cells had an intermediate plasticity score between $T_{EM}$ and $T_{CM}$ cells. However, despite their ability to contribute to secondary memory populations, these ex-small-intestinal $T_{RM}$ cells continued to exhibit phenotypic traces of their tissue origin and had a higher expression of Ly6C and CCR9 compared to ex-$T_{CM}$ and ex-$T_{EM}$ cells. In line with this, and despite their phenotypic plasticity, small intestinal ex-$T_{RM}$ cells retain a bias to their tissue of origin[92].

Additional studies comparing the capacity of $T_{RM}$ cells from different tissues to contribute to secondary memory responses found substantial differences between skin ex-$T_{RM}$ cells and liver ex-$T_{RM}$ cells: skin ex-$T_{RM}$ cells were unable to expand and repopulate the splenic or hepatic T cell pool to the same extent as ex-liver $T_{RM}$ cells[36]. This difference is partially explained by the finding that $T_{RM}$ cells from the skin depend on TGF-β, whereas liver $T_{RM}$ can form independently of TGF-β. Furthermore, using a tamoxifen-inducible knockout of TGFBRII in $T_{RM}$ cells after acute LCMV infection, has shown that $T_{RM}$ cells in the small intestine and salivary gland require ongoing TGF-β signaling, whereas $T_{RM}$ cells in the kidney, liver, and visceral adipose tissue can persist without TGF-β[17]. This finding suggests that TGF-β imprinting may be one underlying mechanism repressing $T_{RM}$ memory potential. This is further supported by the finding that TGF-β-dependent $CD103^+$ salivary gland ex-$T_{RM}$ cells are disadvantaged in repopulating the splenic memory population compared to their $CD103^-$ counterparts, which do not require TGF-β[36], and also by a report showing that TGF-β-dependent $T_{RM}$ formation limits the response to tumor vaccines[96]. Reflecting the intra-tissue heterogeneity of $T_{RM}$ cells, it has been shown that the $Id3^{high}$ $T_{RM}$ populations, which exhibit increased multifunctionality, yield a greater frequency of circulatory resident populations after rechallenge compared to their $Blimp1^{high}$ counterpart[27]. This study showed that the observed heterogeneity within the $T_{RM}$ populations is functionally relevant in primary and secondary immune responses and that different $T_{RM}$ subsets may be differently poised to contribute to secondary infection.

In conclusion, $T_{RM}$ cells need distinct signals, cytokines, and transcriptional programs to adapt to their specific niche in their tissue of residence. This leads to specialized $T_{RM}$ cells that exhibit different phenotypic traits, distinct functions, and also different potential to contribute to secondary infection response. Hence, by both studying the differences and commonalities between $T_{RM}$ from a broad range of tissues, we can infer what makes a $T_{RM}$ cell responsive to secondary infections. It will be interesting to see how tissue-specific adaptations by primary $T_{RM}$ cells influence their ability to respond to secondary infection and contribute to secondary memory and whether tissue-specific adaptations require a trade-off with future memory potential.

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However, one main limitation of analyzing the contribution of $T_{RM}$ cells to secondary infections by transferring sorted populations into new hosts is that $T_{RM}$ cells are subjected to harsh methods of isolation that might alter their biology due to the necessity of isolating them from the tissue. Further insights were gained by studies focusing on secondary $T_{RM}$ responses in situ. Using intravital mucosal imaging, $T_{RM}$ cells were found to proliferate within their residing tissue upon antigen restimulation, and it was found that the pool of secondary $T_{RM}$ cells was mostly derived from primary $T_{RM}$ cells independently of $T_{CM}$ cells and proliferation in lymphoid tissues[93]. Furthermore, using reporter and fate-mapping mouse models has brought many new insights into how $T_{RM}$ cells within barrier sites respond to reinfection. These reporter mice models rely on genes that are uniquely expressed in $T_{RM}$ cells and are hence used to mark and track $T_{RM}$ cells during secondary infection without the need to isolate the memory cells, disrupt them from their residing environment, and transfer them into a new host.

For example, the transcription factor Hobit can be expressed in $T_{RM}$ cells in the liver, the small intestinal IEL, and LPL $T_{RM}$ cells, but not in circulatory or mesenteric lymph node memory T cells[15]. Using a Hobit-reporter mouse (tdTomato integrated into the *Hobit* locus) and a Hobit fate-mapping mouse (Hobit-Cre R26-LSL-eYFP), it has been confirmed that reinfection causes local expansion of $T_{RM}$ cells. These cells can also drain into lymph nodes and contribute to circulatory memory where they resemble $T_{EM}$ cells expressing KLRG1 and CX3CR1, and only a small proportion expresses the $T_{CM}$ marker CD62L[94]. These secondary ex-$T_{RM}$ cells downregulate the expression of Hobit, which is in line with the finding that IEL ex-$T_{RM}$ cells can lose expression of CD103 and CD69 after transfer and reinfection. These results underscore that at least certain parts of the $T_{RM}$ adaptation program are not permanently imprinted and that $T_{RM}$ cells can adapt to new environments and transdifferentiate into other T cell memory subsets.

Hobit expression is neither restricted to $T_{RM}$ cells from a specific organ nor specifically expressed in a functionally distinct $T_{RM}$ subset. Two new complementary studies addressed this by developing $CD103^+$ fate-mapping mice that specifically and permanently mark $CD103^+$ $T_{RM}$ cells, thereby allowing dissection of the differences between $CD103^+$ and $CD103^-$ $T_{RM}$ populations[75,76]. Using a CD103-CreER$^{T2}$ mouse crossed to a flox-STOP-flox fluorescent reporter mouse, both groups could selectively mark cells that express CD103 at a specific time by treatment with tamoxifen at memory timepoints, thus excluding cells that might only temporally express CD103 during early activation or the effector phase. Despite using different infection models (VSV, *Listeria monocytogenes*, LCMV, and *Yersinia pseudotuberculosis*) and different TCR transgenic mice, both studies found, that $CD103^+$ $T_{RM}$ cells residing in the intestinal epithelium were poorly reactivated upon reinfection. Surprisingly, reporter positive $CD103^+$ cells did not retreat to the draining mesenteric lymph nodes. Instead, the $CD103^-$ $T_{RM}$ population responded to secondary infection, showing an increase in TCR signaling (as measured by Nur77) and in proliferation compared to their $CD103^+$ $T_{RM}$ counterparts[75–77]. Since $CD8^+$ $CD103^+$ $T_{RM}$ cells do not show an increase in TCR signaling activity and seem to respond in a TCR-independent manner after antigen reencounter, it will be interesting to determine what signals activate these cells. A recent report found that $CD4^+$ $T_{RM}$ cells in the lung that formed after *Bordetella pertussis* infection in mice, were able to respond to non-cognate immune challenges[97,98]. These findings

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show that the TCR-independent activation of $T_{RM}$ can occur in both $CD4^+$ and $CD8^+$ $T_{RM}$ cells and suggest that $T_{RM}$ might acquire innate-like features to respond to secondary infection at barrier sites. The lack of proliferative capacity of $CD103^+$ $T_{RM}$ cells might be a physiological adaptation to limit immunopathology.

Supporting the idea that $CD103^-$ $T_{RM}$ cells possess a higher proliferative capacity, these studies found that the percentage of reporter-positive cells within the $CD103^+$ $T_{RM}$ population decreased after reinfection, suggesting that the pool of $CD103^+$ cells is re-seeded by $CD103^+$ $T_{RM}$ cells. To exclude a competition for antigen and space limitations, CD103-Cre-$ER^{T2}$ mice were crossed to $DTR^{floxed}$ mice, thus, after treatment with tamoxifen, all $CD103^-$ cells could be depleted with diphtheria toxin to efficiently eliminate competition between $CD103^+$ and $CD103^-$ $T_{RM}$ cells, as well as circulatory memory cells during reinfection. Interestingly, even in these artificial conditions, $CD103^+$ $T_{RM}$ cells only marginally contributed to circulatory memory populations, showing that the limited expansion capacity is an intrinsic feature of $CD103^+$ $T_{RM}$ cells[76]. In summary, these studies suggest, that $CD103^-$ $T_{RM}$ cells in the small intestine might represent a precursor $T_{RM}$ cell population with the ability to replenish and sustain the $CD103^+$ $T_{RM}$ pool in the tissue.

In previous reports, $T_{RM}$ cells were shown to locally proliferate upon antigen re-encounter[92,93,95], and it is still unclear what causes these divergent results. This might be explained in part by different infection models, different induction of $T_{RM}$, or different organs studied. However, these differences make clear that the heterogeneity within a $T_{RM}$ population in a single tissue results in different functional capacities and that $CD103^+$ and $CD103^-$ $T_{RM}$ subsets need to be assessed separately. In terms of cytokine production, $CD103^+$ cells predominately produced Granzyme A, whereas $CD103^-$ $T_{RM}$ cells secreted higher levels of IFN$\gamma$, TNF, and IL-2, which is in line with the higher production of these cytokines previously seen in $Id3^{hi}$ cells, which also exhibited a greater plasticity upon transfer and reinfection[27,75]. However, in contrast, these $Id3^{hi}$ cells exhibited more expression of $CD103^+$, indicating that one marker alone might not be sufficient to delineate among the functionally distinct subsets of $T_{RM}$ cells in the small intestine.

## Outlook

Localized tissue-immunity is an integral component of our body's immune defenses. Understanding, how immune cells enter and persist within a tissue has direct therapeutic potential. Recent studies highlight that $T_{RM}$ cells need to acquire tissue-specific changes to allow for optimal acclimatization to these tissues. Currently, most vaccination strategies focus on generating pathogen-specific memory cells or antibodies. However, recent studies suggest that by directing these memory cells to the exposed tissue, protection against infection can be enhanced. A better understanding of how memory cells adapt to the tissue microenvironment will hopefully allow for tissue-tailored vaccination strategies. Additionally, releasing $T_{RM}$ cells from tissues could boost secondary systemic immune responses. Besides prevention of diseases, T cell therapies provide a novel treatment strategy for cancer and autoimmune diseases. Directing T cells to solid tumors remains a challenge, and mechanisms learned from tissue-resident cells could be extrapolated to

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improve tumor-infiltration and survival of T cells. Alternatively, allowing tissue egress of exhausted tumor-infiltrating T cells could render them more susceptible to checkpoint therapy, since they would be removed from the immunosuppressive tumor environment. Similarly, selectively targeting resident immune cells in autoimmune-diseases or after organ transplantation provides an exciting new treatment approach where alloreactive T cells could be depleted or functionally impaired[16,17].

Single-cell RNA sequencing has helped to dissect $T_{RM}$ heterogeneity, but we are only beginning to understand the complexity of $T_{RM}$ cell populations and the underlying mechanisms resulting in these specialized T cell subsets. By studying common mechanisms across multiple $T_{RM}$ populations and dissecting individual adaptations of $T_{RM}$ from a broad range of tissues, we can infer what signal, cytokines, cell-cell interaction, or environmental sensing mechanisms are required to initiate $T_{RM}$ differentiation. Recent technological advances that allow the study of cellular transcriptomes in intact tissues, combined with computational methods creating cell-cell communication networks, will deepen our understanding of the signals driving T cell residency. This knowledge on how $T_{RM}$ differentiation is embedded in a cellular and environmental network will bring us one step closer to tissue-directed immune-cell therapies.

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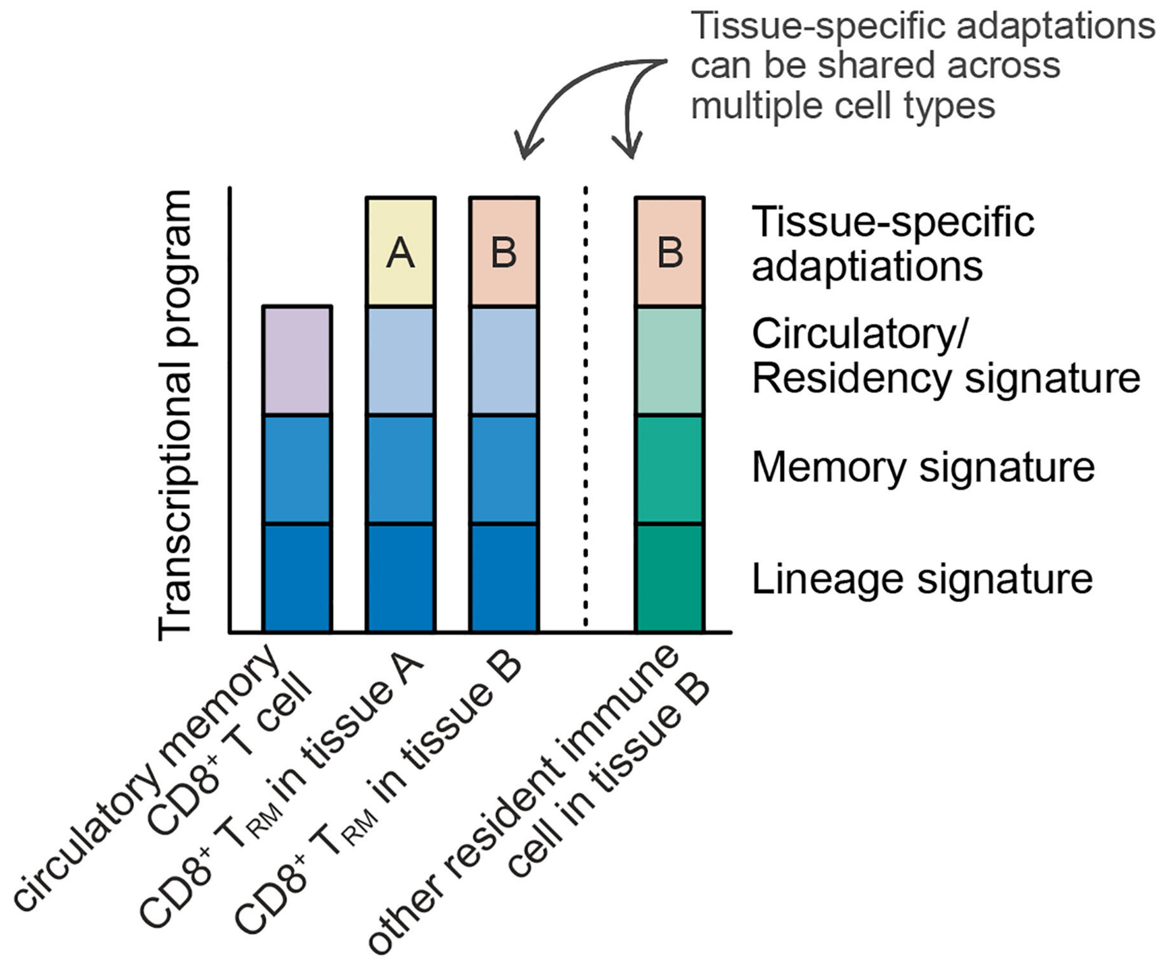


**Figure 1.**

In addition to a shared lineage $CD8^+$ T cell-defining signature and a memory T cell signature, $T_{RM}$ cells need to acquire a transcriptional residency signature. Long term survival and function within the tissue are mediated by tissue-specific adaptations of $T_{RM}$ cells that allow them to optimally acclimatize to their tissue of residence. These tissue-specific adaptations can be shared across multiple cell types.

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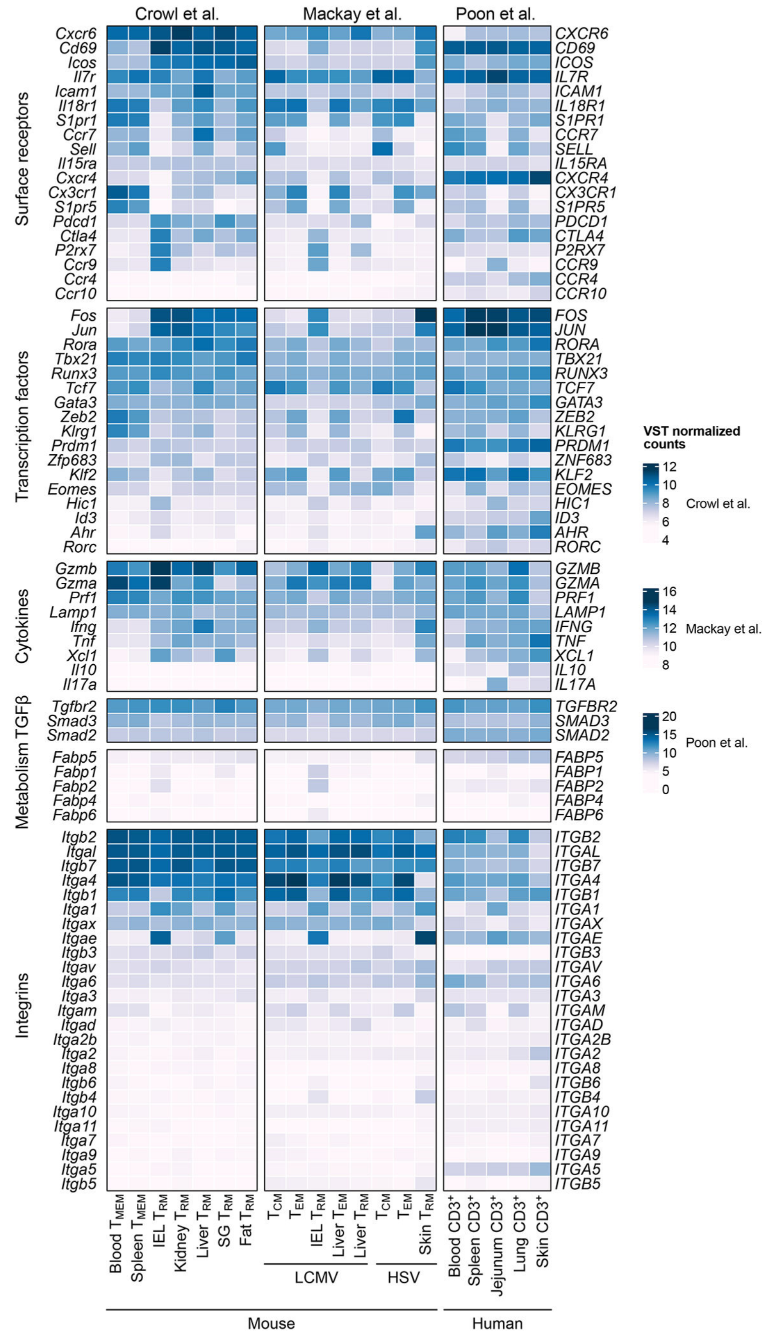


**Figure 2.**

Expression of $T_{RM}$ molecules, lineage defining transcription factors, cytokines and integrins. Although $T_{RM}$ share common adaptation signatures, expression of $T_{RM}$ associated genes is heterogeneous across tissues and infections. Data from Crowl et al., 2022 ($CD8^+$ P14 T cells isolated from spleen and blood ($T_{MEM}$) and $IV^-$ $CD8^+$ P14 T cells from IEL, kidney, liver, SG, and fat ($T_{RM}$) at day 30 post infection with LCMV Armstrong, $T_{MEM}$: all P14 cells); Mackay et al., 2016 (gp33 and np396 tetramer LCMV-specific CD8 T cell populations day 40 post LCMV Armstrong infection, and gBT-I cells 40 days post HSV infection); and Poon

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et al., 2023 (scRNA-seq of $CD3^+$ T cells of two donors, aggregated to pseudo-bulk samples per donor). Mean expression values are plotted.

For generation of the heatmaps, data was downloaded from GEO (GSE182274, GSE70813, and GSE206507). For bulk RNA Seq data (GSE182274 and GSE70813), fastq files were processed using the nextflow pipeline (nf-core/rnaseq: 3.10.1[99]). Variance stabilizing transformation (VST) was performed using DESeq2[100]. Mean VST values for selected genes were plotted using the R package ComplexHeatmap. For GSE206507 raw counts were downloaded from GEO and pseudobulk samples were created using the aggregateAcrossCells function from scuttle[101] in R with the groups tissue and donor. Pseudobulk samples were then processed in DESeq2 and visualized with ComplexHeatmap as described above.

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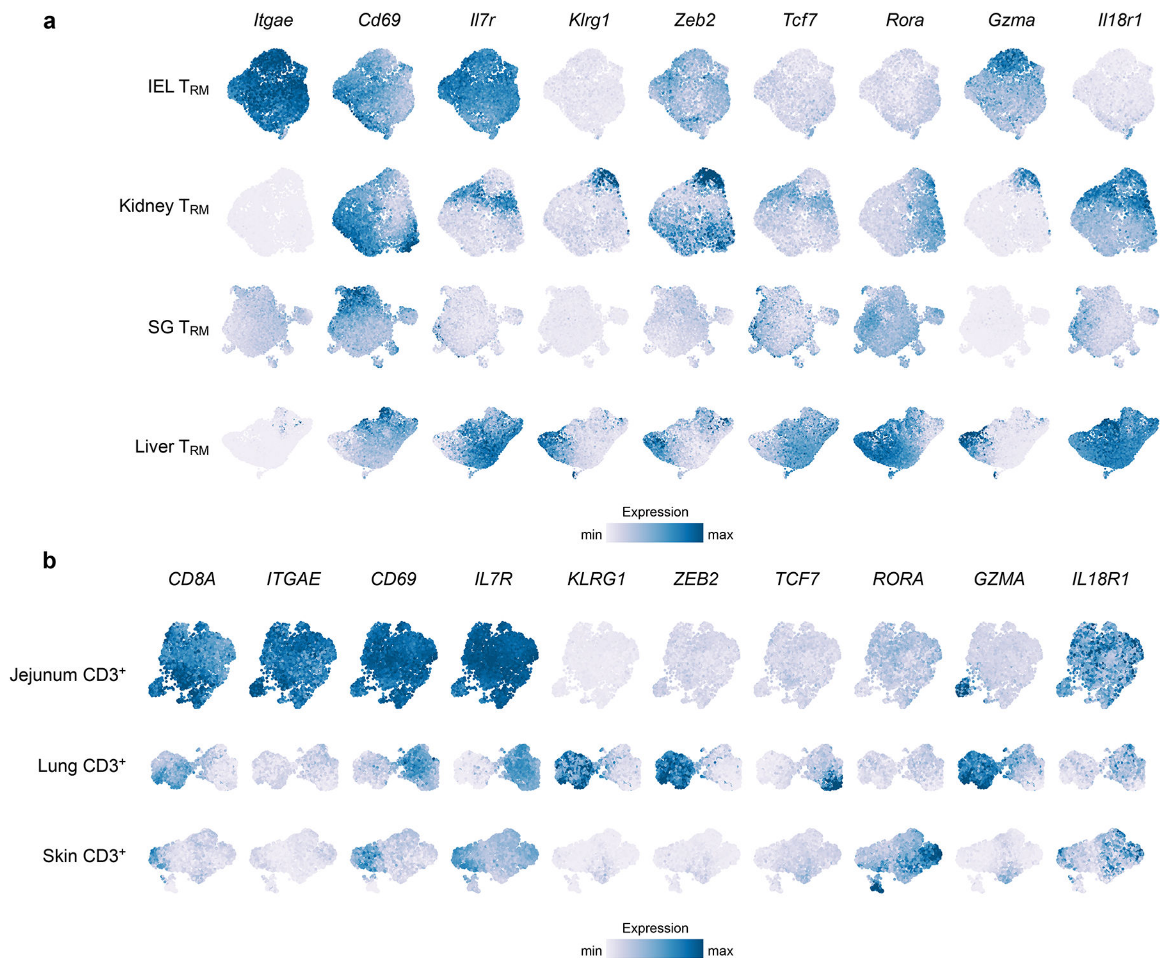


**Figure 3.**

scRNA-seq reveals intertissue and intratissue heterogeneity. $T_{RM}$ cells within a single tissue display varying expression of genes associated with effector and memory potential, showing that distinct subsets of $T_{RM}$ cells exist. Scales for each gene are consistent across tissues to allow for comparison within and among tissues. Data from (a) Crowl et al. 2022 (scRNA-seq of murine $IV^-$ $CD8^+$ P14 T cells from IEL, kidney, SG, and liver at day 32 post infection with LCMV Armstrong) and (b) Poon et al., 2023 (scRNA-seq of human $CD3^+$ T cells of two donors). Donor D551: Jejunum and Lung; Donor D492: Skin.

For visualizing the intra-tissue heterogeneity, each tissue dataset (downloaded from GEO GSE182275 and GSE206507) was normalized separately using sctransform with method = "glmGamPoi" in Seurat[102]. Dimensional reduction was performed using the RunPCA function in Seurat following by RunUMAP using the first 20 PCAs. In addition, data imputation was performed using MAGIC[103] using the default settings and the exact solver. Manually selected genes to highlight inter- and intratissue heterogeneity were plotted. Color scale was limited to the 98th expression percentile.