TISSUE-RESIDENT MEMORY T CELLS

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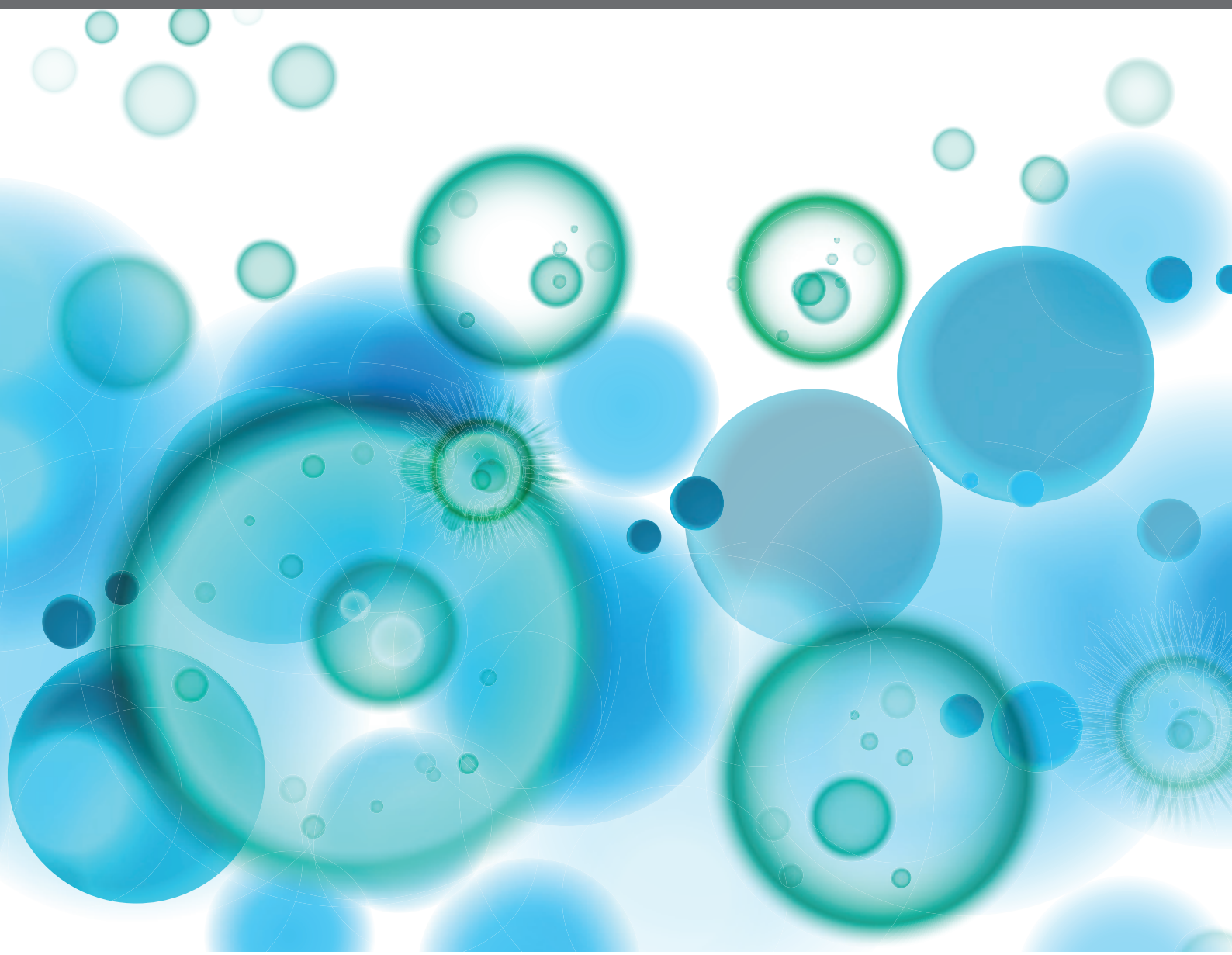

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TISSUE-RESIDENT MEMORY T CELLS

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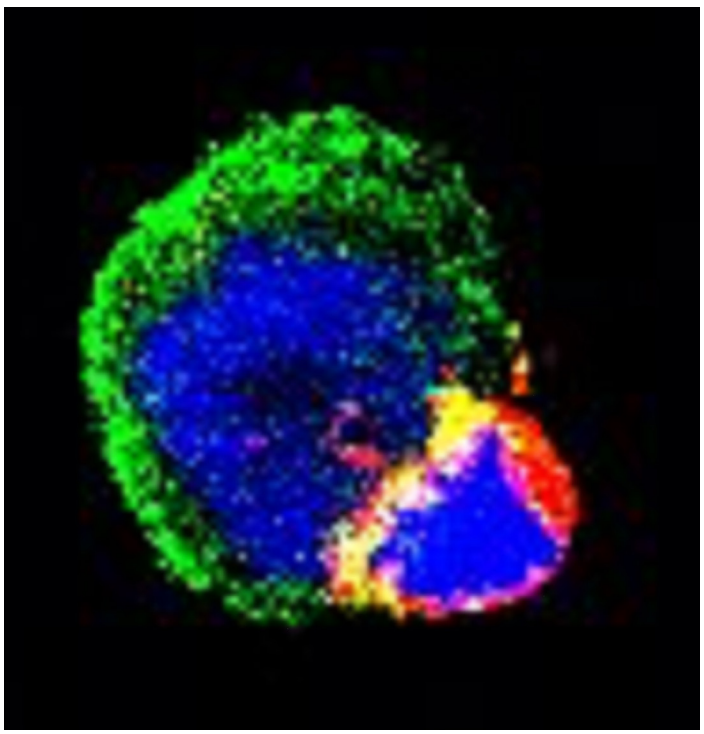

An immune synapse formed between a CD103+CD8+ TRM cell freshly isolated from human NSCLC TIL and autologous lung tumor target cell.

Image: Stéphanie Corgnac and Fathia Mami-Chouaib

Tissue-resident memory T (TRM) cells play a major role in control of viral infections. Their involvement in cancer diseases has been more recently demonstrated. This non-circulating T-lymphocyte subset lacks molecules enabling egress from the tissue and migration to lymph nodes, expresses specific markers of residency and displays specific transcription factors. The present special issue elucidates our current knowledge on CD8+ TRM cells and explores less frequently described resident subsets, such as CD4+ TRM and innate-like cells, as well as their specific metabolism and niches for their formation in infectious and cancer diseases.

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EDITORIAL
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Editorial: Tissue Resident Memory T Cells

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Editorial on the Research Topic

Tissue Resident Memory T Cells

Resident memory T cells (T_{RM}) were identified about 10 years ago following the discovery of tissue-resident T cells that do not recirculate. The role of this population of T cells in control of viral infections was rapidly demonstrated. This population is considered to represent a new T-lymphocyte lineage, in that it lacks molecules enabling egress from the tissue and migration to lymph nodes (Klf2, S1Pr1, CCR7, CD62L, etc.) and expresses specific markers of residency (CD103, CD49a, CD69). However, not all T_{RM} cells express these surface markers and their residency feature remains the main characteristic. T_{RM} cells have a distinct differentiation profile dependent on certain cytokines (TGF-β, IL-15, Type I IFN, IL-12) and specific transcription factors (Runx3, Hobit, Blimp-1, Notch, etc.) [Behr et al., (1)]. More than 130 articles were published in 2018 on this population, covering all areas of pathology (infection, allergy, autoimmunity, transplantation, cancer, etc.). The moment thus seemed appropriate for publishing a special issue on this T-cell subset so as to elucidate our current state of knowledge, as well as exploring less frequently addressed issues, such as the specific metabolism of T_{RM} cells (Pan and Kupper), subpopulations of $CD4^+$ T_{RM} (Oja et al., Wilk and Mills) and resident lymphocyte populations different from conventional T cells, such as innate lymphocytes or innate-like cells (Chou and Li). The major niches for T_{RM} maintenance and persistence, which is an important issue for this population, are also discussed (Takamura). It is interesting to note that, while this T-cell subset was initially studied in the context of infectious diseases, its role in oncology has recently been demonstrated (2–5). Nevertheless, in the present special issue, the number of articles and reviews dedicated to T_{RM} cells in infection (Wilk and Mills, Morabitoet et al., Muruganandah et al.) is fewer than those dealing with their role in cancer diseases (Oja et al., Blanc et al., Corgnac et al., Dhodapkar, Dumauthioz et al., Smazynski and Webb). This is not surprising; indeed, cancer immunotherapy targets the tumor microenvironment in which T_{RM} cells are located, presumably due to their expression of CD103 integrin, allowing an interaction with tumor epithelial cells expressing E-cadherin (6–11).

The search for cellular targets mediating the therapeutic effects of anti-PD-1 and anti-PD-L1 antibodies is the subject of intense worldwide investigation. This is a medical challenge, and goes hand in hand with the identification of biomarkers predictive of a response to these immunotherapies so as to more effectively select patients likely to respond. The role of T_{RM} has been rapidly addressed; indeed, they represent cells that express high levels of inhibitory receptors (PD-1, Tim-3, etc.) (2, 12), and it has been shown that these lymphocytes proliferate after treatment with anti-PD-1/-PD-L1 (13). Despite expression of high levels of checkpoint receptors, these cells have a cytotoxic capacity, especially after blocking of the PD-1-PD-L1 axis, indicating that they can be reactivated (2, 14). Expression by T_{RM} cells of high levels of granzyme B and TNF-α, as well as the presence of preformed RNA coding for IFNγ, may explain the particular reactivity of

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these lymphocytes (Behr et al.). A strongly documented hypothesis concerning the mechanism of action of anti-PD-1/-PD-L1 relies on the presence of pre-existing anti-tumor T cells (15, 16). Interestingly, when T_{RM} ($CD103^+CD8^+$ T cells) were separated from the other T cells isolated from the tumor microenvironment, these lymphocytes were enriched in tumor-specific cells (2, 12). In different preclinical tumor models, the presence of these T lymphocytes enables maintaining an equilibrium between the host and tumor, and protects against cancer progression (17). In line with these previous results, mice deficient in T_{RM} cells display accelerated tumor growth (17). In humans, tumor infiltration with this T-cell subset is associated with a favorable prognosis in both univariate and multivariate (2, 12, 14, 18) analyses. T_{RM} cells can be characterized by different techniques (transcriptomic, single cell RNAseq, cytof, etc.) requiring high quality when performing cell isolation. In the present issue, Rissiek et al. report that blocking ARTC2.2 by preventing P2X7 ribosylation improves cell vitality during their *ex vivo* isolation.

Various reviews in this issue are also devoted to a better understanding of mechanisms involved in T_{RM} differentiation *in vivo* and new strategies for inducing them, especially after vaccination (Morabito et al., Muruganandah et al.). T_{RM} cells can be generated from naive T lymphocytes, and a T_{RM} precursor phenotype ($KLRG1^{low}$) has been reported (19). Nevertheless, central memory T (T_{CM}) cells and effector T (T_{EFF}) cells can also differentiate into T_{RM} cells in peripheral tissue, suggesting a certain plasticity of the pool of memory T lymphocytes (Enamorado et al.). This mode of generation may explain why a common T-cell receptor (TCR) repertoire has been pointed out between T_{CM} cells and T_{RM} cells (20). Differentiation of T_{RM} cells can be inhibited using an anti-TGF-β or an inhibitor of the mTor pathway during T-cell priming (12, 21). Specific parameters might influence generation of T_{RM}, such as the high affinity of TCR for the HLA-Class I-peptide complex or a strong inflammatory stimulus (22, 23). In some tissues, but not in others, such as the lung, it has been shown that an inflammatory stimulus without the presence of the antigen may be sufficient to induce differentiation of T_{RM} (5). Finally, in mice, Batf3-dependent type I dendritic cells (DC), corresponding to DNGR-1-expressing DC, appear to be required for priming of T_{RM} (24). In contrast, in humans, $CD1c^+$ DC and, to a lesser extent, $CD141^+$ DC, play a crucial role in differentiation of T_{RM} cells (25). The need for these local DCs for priming T lymphocytes may explain why the mucosal route of immunization is most effective in priming T_{RM} (26, 27). Vectors targeting certain DC subtypes (4, 28) and some mucosal adjuvants (IL-1β, αGalCer, zymosan. etc.) also boost generation of T_{RM} cells (29–31). The present issue provides the most up-to-date information on T_{RM} cells, but the field is very rapidly evolving. A recent article from Neurath MG's group shows that CD4 T_{RM} cells also play a pathogenic role in models of intestinal inflammation, thus opening up a new field of investigation and indicating a direct role for these lymphocytes in human pathologies (32).

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Tissue-Resident Lymphocytes Across Innate and Adaptive Lineages

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Lymphocytes are an integral component of the immune system. Classically, all lymphocytes were thought to perpetually recirculate between secondary lymphoid organs and only traffic to non-lymphoid tissues upon activation. In recent years, a diverse family of non-circulating lymphocytes have been identified. These include innate lymphocytes, innate-like T cells and a subset of conventional T cells. Spanning the innate-adaptive spectrum, these tissue-resident lymphocytes carry out specialized functions and cross-talk with other immune cell types to maintain tissue integrity and homeostasis both at the steady state and during pathological conditions. In this review, we provide an overview of the heterogeneous tissue-resident lymphocyte populations, discuss their development, and highlight their functions both in the context of microbial infection and cancer.

Keywords: tissue resident, innate lymphocyte, innate-like T cells, conventional T cells, cancer, infection

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INTRODUCTION

A fundamental role of the immune system is to maintain host integrity. For metazoan species, an effective immune response must address invading threats in a rapid and specific manner such that the afflicted tissues remain uncompromised and continue to carry out their vital functions for the host. The innate immune system provides the first line of defense through the recognition of stereotypic motifs associated with a broad spectrum of pathogens (1–3). In contrast, the adaptive immune system, equipped with antigen receptors of near-limitless diversity, exerts its effector functions in an antigen specific manner (3). This expanded population of antigen-specific adaptive lymphocytes in turn forms the basis of immunological memory, bestowing the hosts with long-lasting immunity against previously encountered pathogens (3).

For mammalian species, the adaptive immune response is initiated in secondary lymphoid structures by antigen presenting cells (APCs). Upon activation by danger-associated signals, APCs migrate from the site of insult to draining lymph nodes, carrying with them components of the menacing agents. There they present these captured antigens to naïve T lymphocytes, which in turn triggers the successive rounds of cell division by T lymphocytes and initiates their differentiation into effector and memory subsets. Whereas, effector T cells home back to the primary sites of insult, mediate clearance of pathogen and undergo population contraction, memory T cells persist after the resolution of infection and are poised to mount recall responses. Under this classical view, the secondary lymphoid tissues are the integral component of the adaptive immune system, for the constant migration of adaptive lymphocytes within such a network maximizes their chance of antigen encounter (4). Teleologically, this circulatory behavior of naïve adaptive lymphocytes is a necessary consequence of their anticipatory antigen receptor repertoire (5). The antigen receptor genes of adaptive lymphocytes are assembled through random somatic recombination without prior knowledge of their cognate antigen. This anticipatory nature of the adaptive antigen receptor

repertoire underlies its tremendous diversity, but greatly limits the frequency of lymphocytes with a given specificity. As such, a given naïve T cell clone cannot be present in all tissues at once. By necessity, they patrol strategically placed lymph nodes, which collect information on the statuses of their associated tissues, to efficiently survey the antigen landscape of the whole organism.

Our understanding of lymphocyte responses has broadened significantly in the past decade by the successive discovery of many non-circulating lymphocyte populations. These lymphocytes predominantly reside in non-lymphoid tissues in stark contrast to naïve adaptive lymphocytes, which constantly recirculate between secondary lymphoid organs. In fact, it is now well-appreciated that many, if not all, non-lymphoid organs harbor a sizable population of tissue-resident lymphocytes. These include tissue-resident memory T (T_{RM}) cells; unconventional T cells such as invariant natural killer T (iNKT) cells, intraepithelial lymphocytes (IEL), and γδ T cells; and a diverse family of innate lymphocytes. This property of tissue residency spans across the innate-adaptive spectrum and may be essential for the tissue-specific functions of its respectively resident lymphocyte populations. In this review, we introduce the defining features of tissue-resident lymphocytes, provide an overview of their characteristic features, summarize recent findings on their ontogeny, and discuss their functions in the context of cancer.

DEFINING TISSUE-RESIDENT LYMPHOCYTES

The defining feature of tissue-resident lymphocytes is their distinct migration pattern. In contrast to naïve adaptive lymphocytes which frequently travel between secondary lymphoid organs, tissue-resident lymphocytes constitutively reside in non-lymphoid tissues and generally do not re-circulate through blood (6, 7). This blood-tissue disequilibrium can be conveniently approximated by intravascular staining (8–10). Intravenous administration of fluorescently-conjugated antibody labels vasculature-associated cell populations in a short period of time. Unlabeled cells are thus presumed to reside in the tissue parenchyma and are unlikely to re-circulate. The tissue resident property is most formally demonstrated by parabiosis experiments in which the circulatory systems of two animals are surgically joined, allowing for free exchange of their cell populations (11). Over time, half of the re-circulating lymphocyte compartment in one animal will be derived from its parabiont (6, 11). In contrast, the non-circulating compartment remains dominated by endogenous lymphocyte populations with little to no input from the parabiont (6, 11). This restricted migratory pattern of tissue-resident lymphocytes is often associated with their lack of lymphoid tissue homing chemokine receptors and elevated expressions of several adhesion molecules (7, 12). The sphingosine-1-phosphate receptor (S1PR1) and the chemokine receptor CCR7, whose ligands, S1P, and CCL19/21 are abundantly found in the blood and secondary lymphoid organs, respectively, facilitate re-circulation of lymphocytes and are downregulated as part of the tissue residency program (13–15). On the contrary, CD69, which antagonizes S1PR1 signaling, is reciprocally upregulated (16, 17). In addition, increased expression of integrin molecules, such as CD49a (encoded by *Itga1*) and CD103 (encoded by *Itgae*), whose ligands are collagen and E-cadherin, respectively, promotes interaction with tissue constituents, further reinforcing retention of lymphocytes (18, 19). Whereas, the downregulation of CCR7 and S1PR1 seems to be universal for tissue-resident lymphocytes, the usage of integrin molecules is more diverse. CD103 is specifically found on lymphocytes associated with epithelial tissues, such as the small intestine epithelium and ductal epithelium in glandular organs (20–23). CD49a and CD69 also have their own tissue-restricted expression patterns (18, 24–26). These observations highlight the substantial heterogeneity within the tissue-resident lymphocyte compartment. Thus, defining tissue-resident populations solely based on phenotypic markers may not reliably identify all cells. Instead, parabiosis experiments remain the gold standard to properly define tissue residency.

OVERVIEW OF TISSUE-RESIDENT LYMPHOCYTE POPULATIONS

So far, tissue-resident populations have been identified for all known types of lymphocyte across the innate-adaptive spectrum (6), strongly suggesting that the acquisition of the tissue residency program represents a state of differentiation rather than commitment to a distinct lineage. Resident lymphocyte populations are hypothesized to sense in their home organs tissue disturbances stemming from infection, stress and other deviations from the norm. In turn, they initiate the necessary immune responses to restore homeostasis. Below we briefly describe the characteristic features of various tissue-resident lymphocyte populations and their functions in maintaining tissue integrity.

Innate Lymphocytes

Innate lymphocytes are characterized by their lack of functionally re-arranged antigen receptors. This population includes the prototypic member, natural killer (NK) cells, and the emerging family of innate lymphoid cells (ILCs) (27, 28). Under steady-state conditions, NK cells are recirculating while ILCs are not (6). Emerging evidence suggest that ILCs can be further parsed based on their cytotoxic potential into two subsets: helper ILCs, which are IL-7R-expressing cytokine producers, and killer ILCs, which express cytotoxic molecules but have little to no IL-7R expression (28). Helper ILCs are enriched at mucosal sites and include ILC1, ILC2, and ILC3, each of which produces signature cytokines not unlike their helper T cell subset counterparts (27). The killer ILCs, on the other hand, are mostly found in the liver and epithelium of glandular tissues, such as the salivary, prostate, and mammary glands, and can mediate direct cytolysis of target host cells through granzyme secretion or Fas ligand engagement (23, 29–31).

The exact function of tissue-resident type 1 innate lymphocytes remains contentious. Because of their striking resemblance to NK cells at the phenotypic level, studies aiming

to test NK cell functions by depleting NK marker-expressing populations through antibodies or diphtheria toxin system may have inadvertently eliminated type 1 ILCs as well. Hence it is difficult to pinpoint which population mediates the observed phenotypes. This caveat has only been recognized recently but nevertheless precipitated the development of new genetic tools to selectively target either populations. For instance, a recent study utilized animals deficient for the transcription factor *Zfp683*, or *Hobit*, to specifically reduce the number of liver ILCs, leaving the NK compartment intact (32). In these animals, control of early viral replication in the liver was impaired, supporting the idea that resident type 1 ILCs function as first line defenders.

Type 2 ILCs are the most homogenous among the innate lymphocytes and produce signature cytokines of the type 2 response, such as IL-5, IL-13, and amphiregulin, in a transcription factor *Gata3*-, *Bcl11b*, and *Rora*-dependent manner (33–35). ILC2s control normal immune responses through cross-talk between stroma and other immune cell types. For instance, during helminth infection, intestinal tuft cell-derived IL-25 activates ILC2s to secrete IL-13, which feedbacks on the epithelium to promote tuft cell differentiation (36). The alarmin IL-33 produced upon tissue injury also stimulates IL-5 production by ILC2s, which in turn recruits eosinophils and enhance their innate effector functions (37). This pathway can be antagonized by a secretory product of the helminth *H. polygyrus*, HpARI, which prevents the release of IL-33 by tethering it to necrotic cells (38), further demonstrating the evolutionary benefit of ILC2-dependent responses.

Group 3 ILCs are highly complex and can be roughly unified by their dependency on the transcription *Rorc* for development and function (39). Upon activation by IL-23, a subset of ILC3s produce IL-22, which in turn triggers the anti-microbial peptide production by intestinal epithelium (40–42). Mice with an impairment in the IL-23-ILC3-IL-22 axis succumb to infection by *Citrobacter rodentium*, a gut effacing bacterium (42–44). Furthermore, IL-22 in concert with IL-18 is essential for control of murine norovirus infection (45). Together, these data demonstrate a critical role for ILC3s in maintaining gut homeostasis.

Innate-Like T Cells

Innate-like or unconventional T cells express functionally re-arranged T cell receptors (TCRs) of limited diversity. In contrast to conventional T cells whose TCRs strictly recognize peptides in the context of classical polymorphic major histocompatibility molecules (MHCs), the mode of antigen recognition by innate-like T cells is diverse, with TCRs recognizing antigen in the context of canonical MHCs, non-classical non-polymorphic MHC-like molecules, or even independently of MHCs altogether (46). The most well-characterized members of this family of lymphocytes include IELs, iNKT cells, and γδ T cells.

Many epithelial tissues contain resident IEL populations (47). The most studied are the small intestinal IELs, which consist of both TCRαβ- and TCRγδ-expressing subsets (48). The TCRαβ$^+$ IELs can be further divided into two major populations based on the surface expression of CD8αβ heterodimer. CD8αβ$^-$ IELs, typically expressing the CD8αα homodimer, develop early in life, but its population dwindles as the animal ages and is progressively replaced by CD8αβ$^+$ IELs (48). Thus, the CD8αα$^+$ subsets are often termed "natural" or "unconventaionl" IELs, to distinguish them from the more conventional CD8αβ$^+$ subsets, or the "induced" IELs. In addition to the TCR, CD8αα$^+$ IELs also express panoply of activating and inhibitory receptors typically found on innate lymphocytes. These include the Ly49 and other NK receptor family members (49–51). Recently, another subset of IELs, characterized by the expression of both CD4 and CD8αβ co-receptors was identified (52, 53). A series of experiments demonstrate that these CD4$^+$CD8αβ$^+$ IELs are in fact converted from conventional CD4$^+$ T cells by intestinal tissue-specific signals, such as TGFβ (54). So far, the exact functions of IELs remain elusive, although in specific settings, IELs contribute to anti-pathogen responses in the gut (52, 53, 55, 56).

iNKT cells express an invariant TCRα chain paired with a TCRβ chain of limited diversity (46, 57). Distinct from other TCRαβ$^+$ T cells, iNKT cells recognize lipid antigens presented in the context of the MHC class I-like molecule, CD1d (58–60). The synthetic glycolipid, alpha-galactosylceramide, has been one of the prototypic stimulators of iNKT cells (61). Since then, a plethora of structurally homologous lipids capable of activating iNKT cells have been identified (62). These range from foreign substances, such as certain bacterial cell wall components (63–65) to endogenous sources, such as intermediates in lipid metabolism (66, 67), although the latter is often only transiently present, rare, and less potent. Nevertheless, sensing of endogenous lipid ligands may be the major mechanism by which iNKT cells detect a breach of tissue integrity. Two studies demonstrate an essential role for iNKT cells in controlling infection by pathogens that lack potent agonist ligands (68, 69), supporting the idea that iNKT cells may primarily survey host cells for altered metabolism as a result of pathogen invasion. Similar to ILCs, iNKT cell subsets analogous to the T_H1, T_H2, and T_H17 conventional CD4 T cells have been described (70). Not unlike these T helper cells, each iNKT cell subset produces its signature cytokines driven by distinct master transcription factors (70).

T cells expressing the TCRγδ are present at barrier sites with a particular enrichment at the skin and intestinal epithelium (71, 72). In mice, rearrangement of the TCRγ locus follows a strict temporal order, resulting in the sequential appearances of distinct γδ T cells bearing monoclonal or oligoclonal TCRs that seed various epithelial tissues during fetal development (71–73). For instance, dendritic epithelial T cells (DETC), characterized by their monoclonal TCR composed of Vγ3 and Vδ1, develop between embryonic days 14 and 16 (73, 74). In contrast, intestinal Vγ7$^+$ γδ T cells arise between 2 and 3 weeks after birth (75). It is conceivable that developmental stage-dependent tissue-derived signals permit temporally ordered colonization by distinct γδ T clones. In support of this, two studies demonstrate that *Skint1* and *Btnl* molecules, which are expressed by epithelium during specific stages of development, induce the maturation and potentiate the responses of Vγ5$^+$ DETCs and Vγ7$^+$ intestinal γδ T cells, respectively (75, 76). The cognate antigens for γδ TCRs are still elusive. Whether MHC molecules are involved in γδ TCR recognition is also unresolved. Similar to innate lymphocytes, γδ T cells rapidly produce cytokines, including interferon gamma (IFNγ) and IL-17, when activated (77). A recent study revealed an unconventional role of skin resident γδ

T cells in antagonizing carcinogen-induced melanoma (78). In an IL-4-dependent manner, these γδ T cells promote extrafollicular production of autoreactive IgE, which in turn activate basophils.

Tissue-Resident Memory T (T_{RM}) Cells

The term tissue-resident memory T cells specifically describe populations of conventional T cells that acquire tissue-resident properties. Both CD4 and CD8 T cells can adopt tissue-resident phenotypes (12). Because the $CD8^+$ subset has been better characterized, T_{RM} hereafter refers to $CD8^+$ T_{RM} cells unless noted otherwise. T_{RM} cells have been commonly regarded as first line of defense in peripheral tissues especially against previously encountered threats (79–81). They are hypothesized to provide timely control of tissue threats before the participation of circulatory memory populations. For instance, a report showed that pre-existing herpes simplex virus (HSV) 2 antigen-specific T_{RM} cells at the vaginal mucosa protect hosts from lethal HSV-2 challenge by restricting viral replication at the site of infection as well as preventing the spread of virus to the peripheral nervous system (81). T_{RM} cells engage in diverse effector functions to mediate host protection. As $CD8^+$ T cells can directly lyse infected target cells through the release of granzymes and perforin, several studies reported granzyme B expression in T_{RM} cells as well (19, 23, 82, 83). Notably, T_{RM} cells in the brain can lyse antigen-loaded targets *in situ* (84), suggesting their cytotoxic potential and direct killing as their means of immunosurveillance. By contrast, lung T_{RM} cells protect hosts from influenza virus infection through a process involving IFNγ rather than cytotoxicity (85). More strikingly, recent studies highlighted the innate-like effector property of T_{RM} cells (83, 86, 87). Local activation of T_{RM} cells resulted in their chemokine production, which potently recruited non-antigen specific T cells and initiated an innate immune cascade. Such a bystander response resulted in near-sterilizing immunity against antigentically unrelated pathogens. Thus, in this context, T_{RM} cells can serve as alarm-sounders rather than front line defenders.

ORIGIN OF INNATE AND INNATE-LIKE TISSUE-RESIDENT LYMPHOCYTES

Adaptive lymphocytes are naturally circulatory and only acquire tissue residency program upon activation. In contrast, innate and innate-like lymphocytes migrate directly to their home tissues after exiting sites of development, bypassing this recirculatory step. We postulate that this difference in trafficking between adaptive and innate/innate-like lymphocytes is imprinted during their development. The developmental pathway of thymocytes to mature T cells is punctuated by several checkpoints, one of which occurs at the double-positive (DP) stage (**Figure 1**). Here, DP thymocytes test their functionally assembled TCRs for reactivity against self-derived antigens in the context of MHC molecules (88). Strong self-reactivity instructs DP thymocytes to adopt innate-like T cells fates whereas weakly reactive clones are diverted into conventional T cell lineages (88). For instance, thymocytes expressing a transgenic TCR predominantly develop into unconventional IELs when its cognate ligand is expressed in the thymus, but into conventional T cells when otherwise. This process of agonist selection instructs a phenotypic change on DP thymocytes characterized by the downregulation of both CD4 and CD8 co-receptors and the concomitant upregulation of PD-1 (89–92). This population, when adoptively transferred into lymphopenic recipients, exclusively become $CD8\alpha\alpha^+$ unconventional IELs, and is thus named IEL progenitor (IELp; **Figure 1**) (89). Consistently, thymocytes expressing TCRs isolated from natural IELs also adopt the IELp phenotypes (90, 91). In a similar fashion, the endogenous agonist selection ligand, isoglobotrihexosylceramide (iGb3), which strongly stimulates the invariant NKT TCR, drives the lineage commitment of DP thymocytes into iNKT cells (**Figure 1**) (93). The homotypic interaction between SLAM family receptors is also essential for iNKT development, presumably by complementing TCR-driven selection signals (94, 95). Thus, strong self-reactivity underlies the innate-like T cell fate choice.

Because innate lymphocytes do not express antigen receptors, their self-reactivity is difficult to gage. However, there exist several striking parallels between innate lymphocyte and T cell development. All innate lymphocytes appear to arise from an early innate lymphoid progenitor (EILp; **Figure 1**). One defining feature of EILp is downregulation of IL-7 receptor (IL-7R), which also occurs in DP thymocytes, presenting a peculiar similarity between the two progenitors among the otherwise IL-7R-dependent intermediates during lymphopoeisis (96, 97). Just as agonist selection signals drive PD1 expression, a PD1-expressing innate lymphoid cell progenitor (ILCp) downstream of EILp has been identified (**Figure 1**) (35). Like NKT cells, ILCp expresses the transcription factor PLZF and can differentiate into all subsets of helper ILCs (98). The transient upregulation of PD1 on ILCp suggests that all ILCp-derived ILCs engage in a brief but strong stimulation during their development, which parallels the autoreactive TCR-mediated signals that drive IEL commitment. Notably, NK potential is lost in ILCp, although a dedicated NK progenitor remains unidentified (**Figure 1**) (98). The default circulatory behavior of NK cells aligns them more with the conventional T cells than ILCs. Conceivably, NK cells, like conventional CD8 T cells, may not have experienced a $PD1^{high}$ state during development. In fact, the lack of PD1 expression may help distinguish such NK-dedicated progenitors from their ILC-committed counterparts. The developmental path of cytotoxic ILCs is less understood. In contrast to IL-7R-expressing helper ILCs, which require the transcription factor *Gata3* and *Nfil3* for development, cytotoxic ILCs in the salivary gland are marginally affected upon loss of either transcription factors (29, 31, 99–101). Furthermore, while the vast majority of IL-7R-expressing ILCs develop from the PLZF-expressing ILCP, a substantial fraction of cytotoxic ILCs in the salivary gland do not (102). Additionally, whereas conventional NK cells are critically dependent on *Eomes* and *Nfil3*, cytotoxic ILCs again are not (103–105). These genetic data suggest the existence of yet another innate lymphocyte lineage, which is distinct from both the ILCh and conventional NK cells, and is tentatively named ILCk (**Figure 1**).

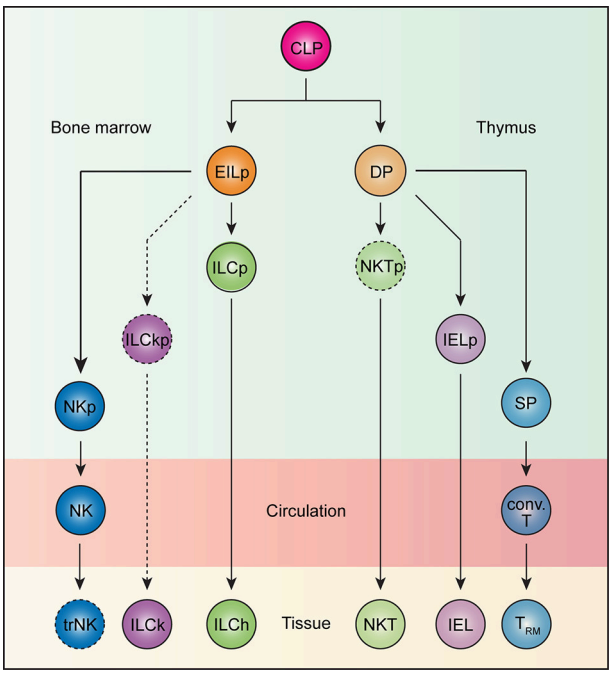

FIGURE 1 | Ontogeny of tissue-resident lymphocytes. All lymphocytes develop from the common lymphoid progenitor (CLP). In the bone marrow, an early innate lymphoid progenitor (EILp) can give rise to natural killer (NK) cells and innate lymphoid cells (ILCs). Whereas, the identity of an NK-restricted progenitor (NKp) remains unknown, a committed innate lymphoid cell progenitor (ILCp), which can give rise to all helper ILCs (ILChs), but not NK cells has been described. Less understood, ILCs with cytotoxic potential, or killer ILCs (ILCk) may arise from a hypothetical killer ILC progenitor (ILCkp) that have lost ILCh and NK potential. While ILCs are inherently tissue-resident, NK cells recirculate. Whether NK cells can acquire tissue-resident features remains unknown. Thus, the term tissue-resident NK (trNK) cells is better kept until such a possibility can be unequivocally ruled out. Beside innate lymphocytes, CLP also gives rise to T lineage-committed progenitors that complete their differentiation in the thymus. The vast majority of TCRαβ-expressing T cells undergo a double positive (DP) stage, during which MHC-based selection takes place. DP thymocytes bearing strongly self-reactive TCRs develop into unconventional intraepithelial lymphocyte (IEL) and natural killer T (NKT) cell lineages through agonist selection, while those with weakly self-reactive TCRs are diverted into single positive (SP) thymocytes, which subsequently give rise to conventional T (conv. T) cells. Whereas, IELs and NKT cells are naturally tissue-resident, conventional T cells recirculate but can become tissue-resident (T_{RM}) upon activation.

ILCks in fact resemble IEL in their constitutive expression of cytotoxic molecules and inherent tissue-resident nature (23). Provocatively, ILCk progenitor may develop from EILp and assume IELp-like phenotypes such as high PD1 but little PLZF expression.

ACQUISITION OF TISSUE RESIDENT PROGRAM BY CIRCULATING LYMPHOCYTES

Best exemplified by T_{RM} cells, re-circulating lymphocytes can acquire tissue resident properties upon activation. The exact time point at which the tissue-resident program is launched during the activation history of a T cell is still unknown. Several lines of evidence suggest that tissue tropism of an activated T cells can be imprinted by dendritic cells (DCs) during priming. For instance, T cells activated by DCs isolated from peripheral lymph nodes upregulate E- and P-selectin while those primed by DCs from mesenteric lymph nodes express gut-homing molecules, such as α4β7 integrin and CCR9 (106, 107). Furthermore, the expression of skin- and gut-homing receptors can be enhanced by metabolites specific to these two tissues, such as retinoic acid (108, 109). These data collectively suggest that activated T cells acquire tissue tropism and specific homing capacity during priming. Contrary to this model, recent studies demonstrated that T cell migration is rather promiscuous during the effector phase of the immune response. In fact, T cells primed at any site can access almost every tissue in the organism. For instance, priming of T cells during systemic LCMV infection leads to the migration of antigen-specific T cells to many peripheral tissues (110). More strikingly, intranasal immunization with Sendai virus also results in the migration of antigen-specific T cells to other peripheral tissues (110). Further examination revealed that T cells primed in any secondary lymphoid organs can in fact upregulate homing receptors for non-lymphoid tissues (111). Thus, the entry of a T cell into non-lymphoid tissues can be instated regardless of priming locations. Once inside the tissue, local signals then orchestrate the tissue resident program. Indeed, adoptive transfer of *in vitro* activated CD8 T cells into the dermis is sufficient to induce their differentiation into long-lived $CD103^+CD69^+$ T_{RM} cells, phenotypically indistinguishable from those generated *in vivo* (18). These data suggest that entry into the tissue is a stochastic but pivotal event that marks the initiation of tissue resident program. Recently, fate-mapping experiments using KLRG1-Cre revealed further heterogeneity within the T_{RM} population with contribution from both KLRG1-fate mapped and non-fate mapped precursors (112). This is in contrast to previous studies where $KLRG1^+$ CD8 T cells fail to give rise to $CD103^+$ T_{RM} when adoptively transferred (18). The discrepancy may be caused by the use of different infection models. Interestingly, although both KLRG1-fate mapped and non-fate mapped precursors lost KLRG1 expression when entering the tissue, the progeny of the two exhibits nuanced but discernable differences in effector functions (112), suggesting that other events before tissue entry can impact the functional capacity of T_{RM}.

Often deemed as the counterpart to conventional CD8 T cells, whether NK cells can acquire tissue resident features like T_{RM} differentiation is less understood. In one study, adoptive transfer of hepatic $DX5^+$ conventional NK cells into lymphopenic mice did not result in their upregulation of tissue resident markers, such as CD49a in the liver (105). In contrast, when transferred into tumor-bearing lymphopenic recipients, $DX5^+$ cells infiltrate the tumor and assume tissue resident phenotypes in a TGFβ-dependent manner (113). These results suggest that re-circulating conventional NK cells possess the tissue resident potential, but its manifestation requires tissue-specific signals. Further studies, such as fate-mapping experiments, are needed to formally test this hypothesis.

MAINTENANCE OF TISSUE RESIDENT LYMPHOCYTES

Long-term parabiosis experiments revealed that under steady-state conditions, tissue resident lymphocytes are long-lived and replenish their population predominantly by local expansion (6). Consistently, other studies in mice and rhesus macaques showed that the tissue memory CD8 T cell populations are stable for 300–700 days, with little to no input from the circulatory memory pool (114–116). These observations suggest that while the concerted actions of adhesion molecules and chemokine receptors enforce tissue retention, additional cell-extrinsic signals promote the maintenance of tissue resident lymphocytes.

IL-7 and IL-15, both of which signal through the common gamma chain (γ_c), have pleiotropic roles during lymphocyte development and maintenance. While mice deficient for γ_c (encoded by *Il2rg*) lack B, T, NK, and ILCs, innate lymphocyte progenitors, such as EILp and ILCp were minimally affected (97), suggesting that the depletion of NK and ILCs in *Il2rg*$^{-/-}$ mice most likely stem from defective maintenance of the mature populations. In the absence of IL-7, bone marrow ILC2p, intestinal ILC2 and ILC3, but not ILC1 are drastically reduced (97, 117–119). In contrast, IL-15 deficiency predominantly impairs ILC1 in the liver, salivary glands, and the small intestine lamina propria (29, 119, 120), although intestinal NKp46$^+$ ILC3 are dually dependent on IL-7 and IL-15 (119, 120). While the NK-restricted progenitor remains elusive, a Lin$^-$CD127$^+$CD122$^+$ population has been identified to contain NK cell precursors and develop normally in the absence of *Il2rg* (121). The profound ablation of mature CD127$^-$ NK cells in these animals are attributed to the lack of IL-15 signaling as IL-15, but not other γ_c cytokines, deficiency can solely recapitulate this defect (121–123). In the thymus, a minute population of CD127$^+$NK1.1$^+$ innate lymphocytes, currently called thymic NK cells, require IL-7 for development (124).

The critical roles of homeostatic cytokines IL-7 and IL-15 for the maintenance of re-circulating naïve and memory T cells, respectively have been long appreciated. The dependency on IL-15 for T_{RM} varies by their locations. T_{RM} in the non-lymphoid tissues, such as the skin, are critically dependent on IL-15 (18) whereas those in the secondary lymphoid organs are not (125). Like T_{RM}, CD8αα$^+$ intestinal IELs are also maintained by IL-15 and enterocyte-expressed IL-15 in an otherwise IL-15-deficient animal is sufficient to restore unconventional IELs (126), suggesting that IL-15 critically sustains mature IELs rather than their precursors. In support of this, PD1$^+$ IEL progenitors develop independent of IL-15 in the thymus (127). While T_{RM} are induced in an antigen-dependent manner, they can be maintained in the absence of cognate antigen in the skin, reproductive tract, and salivary glands (18, 19, 21). In other tissues, persisting antigens contribute to T_{RM} differentiation (19, 26, 82, 84, 128). Thus, the requirement for antigen during T_{RM} maintenance may be tissue-specific. Lastly, given the similar requirement for IL-7 and IL-15 during their homeostasis, resident lymphocytes may occupy overlapping tissue niche. Pinpointing the source of these cytokines in the tissue may help elucidate the redundant and non-redundant roles of each resident lymphocyte population in maintaining tissue integrity.

TISSUE-RESIDENT CYTOTOXIC LYMPHOCYTE RESPONSES IN CANCER

The vertebrate immune system has evolved to exquisitely distinguish self from non-self, thereby achieving effective anti-pathogen responses while curbing autoreactivity. Cancer presents a unique challenge to this fine-tuned system as transformed cells are pathogenic agents derived from the host itself. Yet prevailing evidence has demonstrated that the immune system exerts constant pressure on tumors (129). These observations underlie the preponderant concept of cancer immunosurveillance (130–132). Mechanistically, increased somatic mutation as a result of genomic instability in transformed cells may generate neo-epitopes that can be recognized by conventional adaptive lymphocytes (133, 134). Although these T cells often exhibit "exhausted" phenotypes, their effector functions may be restored by checkpoint blockade therapies (134–136) (**Figure 2**). Targeting this mode of immunosurveillance certainly has been fruitful. However, not all cancer types sustain high mutation burden (137, 138). In such cases, CD8 T cell responses elicited by unmutated self-antigen often fail to restrict tumor growth (139, 140). These findings thus highlight the need to explore other immunosurveillance mechanisms for effective cancer immunotherapies.

Just as pre-existing T_{RM} populations are essential for restraining previously encountered pathogens, prophylactically induced T_{RM} cells by cancer vaccines provide superior control of tumor growth over re-circulating memory T cells (141, 142). In fact, the presence of circulating tumor antigen-specific CD8 T cells alone is not sufficient to control tumor growth (141, 143), highlighting the potential therapeutic benefit of targeting tissue-resident lymphocytes. Strategies to enhance the differentiation and maintenance of these vaccine-induced T_{RM} cells may decrease the relapse rate as well as restrict metastasis. However, prophylactic vaccination with tumor-associated antigen may not always be feasible in clinical settings, as it requires knowing the antigen ahead of time when patients who seek medical attention often have developed tumors already. Notwithstanding, tumorigenesis does naturally elicit tissue-resident lymphocyte responses (23, 144–148). Importantly, a substantial fraction of participating lymphocyte populations appear to have cytotoxic potential (23, 145, 148). These include conventional T cells of the CD8 lineage as well as more recently identified unconventional T cells and group 1 innate lymphocytes (**Figure 2**). Below, we summarize the latest findings on their characterization and potential cancer immunosurveillance functions.

Conventional and Unconventional αβT Cells

In many murine tumor models, αβ T cells can make up a substantial fraction of infiltrating lymphocytes. Among them,

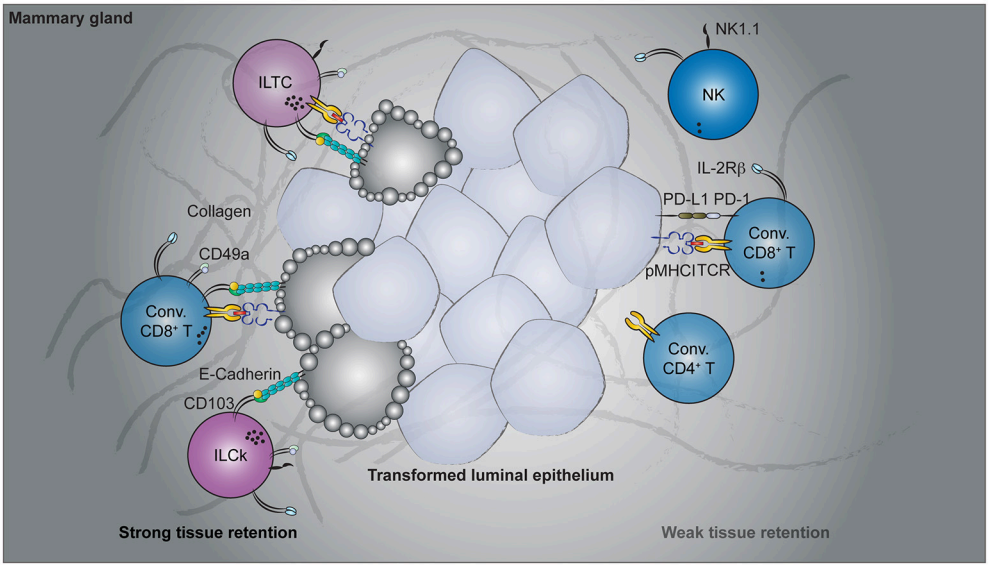

FIGURE 2 | Cancer immunosurveillance by tissue-resident lymphocytes. Spontaneous oncogene-driven breast tumors are infiltrated by group 1 innate lymphocytes, conventional, and unconventional T cells. Parabiosis experiments revealed the tissue-resident nature of CD49a- and CD103-co-expressing lymphocytes, including the innate-like T cells (ILTCs), killer innate lymphoid cells (ILCks), and some conventional (Conv.) $CD8^+$ T cells. In contrast, natural killer (NK) cells, PD1-expressing conventional $CD8^+$ T cells recirculate through blood. Functionally, $CD49a^+CD103^+$ tissue-resident lymphocytes abundantly express lytic granules and can potently lyse transformed target cells. Despite their cytotoxicity, therapies targeting these tissue-resident populations are lacking while rapid advancement has been made to target conventional NK and T cells.

populations expressing tissue-resident markers are abundantly found (23, 144, 145, 148–152). These include T cells of both the conventional and unconventional lineages.

Our understanding of tissue-resident T cell responses in the context of cancer has only begun to advance in recent years. Much of the foundation is in fact built upon extrapolating observations from T_{RM} cells in infectious settings. While these studies provide an invaluable conceptual framework to start with, cancer and acute infection differ fundamentally. Tumorigenesis is a continuous process without a defined time course. In contrast to acute infections where the pathogen load peaks and wanes within a week's time, tumor-associated antigen is continuously present and, in most oncogene-driven cancer models, persist until the endpoint of disease. Thus, there is no well-defined memory phase in the context of cancer and the term "tissue-resident memory T cells" seems to be a misnomer. In a sense, tumorigenesis is more analogous to chronic than acute infections. Indeed, the induction and accumulation of dysfunctional cytotoxic T lymphocytes (CTLs) by persistent antigen stimulation is a shared feature in both settings (153). To what extent the $PD1^{hi}$ CTLs are tissue-resident remains to be determined. Beside the $PD1^{hi}$ population, which appears to dominate in multiple cancer types, tumor-infiltrating $CD8^+$ T cells that express tissue-resident markers have also been reported in several mouse cancer models (**Figure 2**). In a B16-F10 mouse melanoma transplantable tumor model, a fraction of antigen-specific tumor-infiltrating CD8 T cells acquired CD69 and CD103 expression 3 weeks after tumor engraftment (149). Furthermore, administration of blocking antibodies against CD103 resulted in a slight but significant acceleration in tumor growth (149), implying a CD103-dependent cancer immunosurveillance mechanism by these putative tissue-resident tumor-infiltrating lymphocytes (TILs). Using a similar transplantable melanoma model, another study demonstrated a CD8 T cell-intrinsic requirement for the transcription factor *Runx3* in the development of tumor-resident CTL responses (144). CD8 T cells with reduced levels of *Runx3* expression failed to constrain tumor growth (144), further implicating a tumor surveillance role for tissue-resident CTLs. In a spontaneous oncogene-driven breast tumor model, a proportion of intratumoral $CD8^+$ T cells co-express CD49a and CD103 (23). Unlike in the transplantable tumor models, some $CD49a^+CD103^+$ T cells co-express natural killer (NK) receptors, such as NK1.1 and have innate-like features (**Figure 2**). These $NK1.1^+CD49a^+CD103^+CD8^+$ T cells are distinct from iNKT cells as they developed in the absence of CD1d, and thus represent a novel tissue-resident T cell population with no currently known counterpart in the T_{RM} field (23). For this, $NK1.1^+CD49a^+CD103^+CD8^+$ T cells are termed innate-like T cells (ILTCs) to distinguish them from their $NK1.1^-$ counterparts. Parabiosis experiments confirmed the tissue-resident property of both ILTCs and $NK1.1^-$ tumor-infiltrating T cells, with the former being significantly less circulatory (23). Further studies demonstrated that these ILTCs produce little to no IFNγ, but abundantly express the cytotoxic molecule granzyme B (23). Indeed, ILTCs exhibit potent cytotoxicity toward transformed target cells *in vitro*, suggesting their potential role in anti-tumor responses (23). Thus, using infection-induced T_{RM} cells as a template, these seminal works demonstrated the presence of tissue-resident cytotoxic T cells in

mouse tumor models and implicated their immunosurveillance functions.

In human patients, CD103-expressing tumor infiltrating $CD8^+$ T cells are abundantly present in multiple types of epithelium-derived cancers (145–147). In many cases, the accumulation of intratumoral $CD103^+CD8^+$ T cells is associated with favorable prognosis (145–147, 154, 155). Although the exact mechanisms by which these TILs contribute to restraining cancer progression remains elusive, emerging evidence unveil their similarity to T_{RM} cells and suggest cytotoxicity as their mechanism of immunosurveillance. Whether $CD103^+$ TILs are indeed tissue-resident cannot be easily established in humans. Nonetheless, whole genome transcriptome analysis reveals that these TILs share a gene expression program typically associated with pathogen-induced T_{RM} cells and tumor-elicited $CD49a^+CD103^+$ TILs in mouse models (23, 142, 148, 156). For instance, $CD103^+$ TILs from non-small cell lung carcinoma co-express CD49a and CD69, but little to no S1PR1 and the lymphoid tissue homing receptor CCR7 (145, 148). In addition to potentially increased tissue retention, $CD103^+$ TILs appear to be in a distinct activation state compared to their $CD103^-$ counterparts. Not only do more $CD103^+$ TILs exit quiescence, as measured by Ki67 expression (148), they also express higher levels of granzymes (148) and possess increased degranulation potential relative to $CD103^-$ TILs in response to stimulation (145). When incubated with autologous tumor cells, $CD103^+$ TILs potently induced cytolysis of target cells (145). Whether this $CD103^+$ population also contains innate-like T cells, such as the ILTCs found in mice, remains an outstanding question although NK receptor-expressing $CD8^+$ T cells in human cancer patients have been documented (157–159). Nevertheless, these data demonstrate that the tissue-resident cytotoxic T cell response is a conserved cancer immunosurveillance mechanism between mouse and human and represents a promising target for tumor immunotherapy.

Group 1 Innate Lymphocytes

The protective role of group 1 innate lymphocytes against tumors has been repeatedly demonstrated in chemically-induced sarcoma and transplantable tumor models (160–163). However, most of these seminal works were done before the distinction between NK cells and ILCs was recognized. Most studies in this genre made use of depleting antibodies against NK1.1 or genetic systems in which diphtheria toxin is specifically expressed in $NKp46^+$ cells. These approaches effectively eliminated NK cells, but also depleted ILC1s and ILCks as they too express NK1.1 and NKp46. Thus, one cannot conclude which of the affected population contributes to the reported phenotype (164). Having recognized this ambiguity, some studies further subset the $NK1.1^+NKp46^+$ innate lymphocyte populations with a set of markers conventionally used to distinguish between NK cells and ILC1s/ILCks (113, 165). Adoptive transfer of each subset into tumor-bearing lymphopenic hosts then allowed them to identify the population responsible for the protective phenotypes. In these studies, most anti-tumor activity appears to reside within the conventional NK cell compartment (75, 113). Non-NK tissue-resident innate lymphocytes, on the other hand, were shown to dampen anti-tumor immune responses (113). This is in contrast to their roles in oncogene-driven spontaneous tumor models (23, 166). For example, in a breast tumor model, early control of tumor progression is critically dependent on innate lymphocytes, as IL-15 deficient animals, which lack group 1 innate lymphocytes showed accelerated tumor growth (23). However, conventional NK cells were dispensable for this innate lymphocyte-dependent anti-tumor responses because *Nfil3*-deficient mice, which have profoundly diminished NK cell compartment, did not exhibit accelerated tumor growth (23). These data collectively imply that non-NK group 1 innate lymphocytes, most likely ILCks, assume a dominant role in early anti-tumor responses (**Figure 2**). Despite these tumor model-specific discrepancies, the immunosurveillance potential of tumor-infiltrating group 1 innate lymphocytes has garnered much therapeutic interest in recent years.

Many types of human solid tumors are also infiltrated by group 1 innate lymphocytes. Although collectively called NK cells, they in fact consist of two populations distinguished by the makers CD56 and CD16 (167–170). The $CD56^{bright}CD16^-$ subset outnumbers their $CD56^{dim}CD16^+$ counterpart in tissues, both at steady state and during inflammation. In contrast, the $CD56^{dim}CD16^+$ population is far more abundant in the blood. Not surprisingly, the $CD56^{bright}CD16^-$ innate lymphocytes express several tissue-resident markers as well as a defining gene expression program for tissue residency (169, 170). Under the current paradigm, both populations belong to the NK lineages and are related in a linear developmental pathway, namely, $CD56^{bright}CD16^-$ cells give rise to $CD56^{dim}CD16^+$ in a process of differentiation (171, 172). However, it is also possible that the two populations are in fact of disparate lineages, a distinction not unlike the one seen between mouse NK cells and ILC1s/ILCks. While this debate awaits, if possible, a resolution, some clinical evidence suggest a potential anti-tumor role for type 1 innate lymphocytes. For example, in clear cell renal carcinoma, enrichment of type 1 innate lymphocyte-associated transcripts in the tumor mass correlates with favorable prognosis (173). Similarly, for gastrointestinal stroma tumors, the number of CD56-expressing infiltrating lymphocytes is associated with better overall survival (174). For patients with non-small cell lung carcinoma however, the presence of CD56-expressing lymphocytes does not correlate with clinical outcomes, presumably because their cytokine production and cytotoxicity are inhibited by the tumor microenvironment (175). Overcoming immunosuppression strategies deployed by tumor cells may re-invigorate these innate lymphocytes (176–178). A recent study devised an antibody that stabilizes the expression of a stress-induced ligand for the NK activating receptor, NKG2D on the tumor cell surface (179). Administration of this therapeutic agent enhances innate lymphocyte-dependent anti-tumor responses (179). Collectively, tumor-resident cytotoxic innate lymphocytes present a promising target for therapeutic intervention in addition to conventional CD8 T cells, for which a plethora of checkpoint blockade modalities are already in place.

CONCLUDING REMARKS

Originally defined in the T cell field, the tissue residency program has now been found to be used by nearly all known lymphocyte lineages across the hematopoietic tree. Intriguingly, the vast majority of innate and innate-like lymphocytes (with the exception of NK cells) are inherently tissue-resident whereas the more recently evolved adaptive lymphocytes are not, suggesting an ancient origin of the tissue residency program. Since strong self-reactivity during lymphocyte development appears to be a key selection factor for gaining tissue-homing capacity, it is reasonable to assume that the most primordial function of tissue-resident lymphocytes is in fact to detect stress in host cells rather than to sense pathogen or its derivatives. Further extrapolation of this idea would provocatively suggest that the MHC-based selection mechanisms originally served to generate self-reactive T cells. Positive selection, templated on the extant agonist selection mechanisms, evolved later in vertebrate evolution.

AUTHOR CONTRIBUTIONS

CC and ML conceived the ideas. CC wrote the manuscript, and ML edited it.

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REVIEW
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Armed and Ready: Transcriptional Regulation of Tissue-Resident Memory CD8 T Cells

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A fundamental benefit of immunological memory is the ability to respond in an enhanced manner upon secondary encounter with the same pathogen. Tissue-resident memory CD8 T (T_{RM}) cells contribute to improved protection against reinfection through the generation of immediate effector responses at the site of pathogen entry. Key to the potential of T_{RM} cells to develop rapid recall responses is their location within the epithelia of the skin, lungs, and intestines at prime entry sites of pathogens. T_{RM} cells are among the first immune cells to respond to pathogens that have been previously encountered in an antigen-specific manner. Upon recognition of invading pathogens, T_{RM} cells release IFN-γ and other pro-inflammatory cytokines and chemokines. These effector molecules activate the surrounding epithelial tissue and recruit other immune cells including natural killer (NK) cells, B cells, and circulating memory CD8 T cells to the site of infection. The repertoire of T_{RM} effector functions also includes the direct lysis of infected cells through the release of cytotoxic molecules such as perforin and granzymes. The mechanisms enabling T_{RM} cells to respond in such a rapid manner are gradually being uncovered. In this review, we will address the signals that instruct T_{RM} generation and maintenance as well as the underlying transcriptional network that keeps T_{RM} cells in a deployment-ready modus. Furthermore, we will discuss how T_{RM} cells respond to reinfection of the tissue and how transcription factors may control immediate and proliferative T_{RM} responses.

Keywords: T cell diferentiation, tissue-resident memory T cells, transcription factors, homolog of Blimp-1 in T cells, BLIMP-1, Notch, RUNX3, secondary responses

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INTRODUCTION

CD8 T cell responses are an essential component of the adaptive immune system that serves to achieve sterile clearance after infection with intracellular pathogens as well as long-term protection against reinfection. To enable protective CD8 T cell responses against a wide spectrum of microbial threats, an extensive repertoire of naïve CD8 T cells is maintained. The diversity within the T cell repertoire is so large that, despite the millions of naïve CD8 T cells, each T cell specificity is only represented by a population in the order of 100–1,000 cells in mice (1–3). Strikingly, these few precursor cells are able to mount robust T cell responses that eliminate virally infected cells to completion within about 1–2 weeks. The efficiency of CD8 T cell responses depends on the highly effective recruitment of naïve CD8 T cells (4), their rapid proliferation resulting in a more than 1,000-fold expansion in about a week (5), and in the acquisition of effector functions

by the differentiation into effector CD8 T cells (6). Important effector functions of CD8 T cells include the production of the pro-inflammatory cytokine IFN-γ and the cytotoxic mediators perforin and granzyme B. These effector molecules assist in the activation and recruitment of other immune cells as well as in the elimination of infected cells, respectively. After resolution of infection, most of the effector CD8 T cells undergo apoptosis, resulting in contraction of the CD8 T cell response into an about 10-fold reduced population of memory cells (7, 8) that can be maintained for decades in men. Specific memory CD8 T cells are maintained at a higher frequency than naïve CD8 T cells, which enables them to establish secondary CD8 T cell responses with faster kinetics and of larger magnitude. In this manner, memory CD8 T cells can provide up to life-long protection against re-encounter with the same pathogen (6). Memory CD8 T cells do not only have a numerical advantage, they also display superior qualitative characteristics to provide improved protective immunity compared to naïve T cells (9).

Subsets of Memory CD8 T Cells

Distinct subsets of memory CD8 T cells have been recognized that contribute to enhanced recall responses in different ways and at separate sites (10). Central memory CD8 T (T_{CM}) cells express lymph node (LN) homing molecules such as the CC-chemokine receptor 7 (CCR7) and adhesion molecules such as L-selectin (CD62L) that provide access to secondary lymphoid organs. Due to these properties, T_{CM} cells retain the capacity of naïve CD8 T cells to survey the secondary lymphoid organs for cognate antigens. In contrast, effector memory CD8 T (T_{EM}) cells express low levels of CCR7 and CD62L and gain access to the non-lymphoid tissues (11), which enables these memory CD8 T cells to directly patrol the peripheral tissues for immune surveillance. T_{CM} and T_{EM} cells continually recirculate through blood and lymph to survey LN and peripheral tissues, respectively. Recent evidence suggests further heterogeneity within the circulating memory CD8 T cell pool, where expression of the fractalkine receptor CX3CR1 identifies three subsets with distinct migratory properties (12). These include CX3CR1^{low} T_{CM} cells, CX3CR1^{int} peripheral memory T (T_{PM}) cells, which survey peripheral tissues, and CX3CR1^{high} T_{EM} cells, which are largely confined to the vasculature (12). Upon recognition of reinfection, T_{CM}, T_{PM}, and T_{EM} cells mount secondary responses, which involve proliferation and differentiation into secondary effector cells to target the re-invading pathogen.

Next to T_{CM}, T_{PM}, and T_{EM} cells, a fourth subset of memory CD8 T cells, tissue-resident memory CD8 T (T_{RM}) cells, has been identified. In contrast to the circulating memory populations, T_{RM} cells permanently reside within the peripheral tissues after infection without accessing the blood or the lymph (13, 14). The non-recirculating nature of T_{RM} cells has been experimentally demonstrated in different ways. Intravascular antibody injection does not label T_{RM} cells within skin, lungs, and small intestine in contrast to circulating memory CD8 T cells within the bloodstream (15, 16). However, intravascular labeling cannot distinguish circulating memory CD8 T cells transiently passing through the tissues from T_{RM} cells that permanently reside in these tissues. Another exception in this context are liver T_{RM} cells, which reside on the inside of the liver sinusoids in direct contact with the blood (17, 18). Further experiments employing parabiosis, in which the bloodstream of two mice is conjoined, demonstrated that, while circulating memory CD8 T cells rapidly establish equilibrium, T_{RM} cells are permanently retained in peripheral tissues within their host (14, 19–21). The inability of T_{RM} cells to exit donor tissue upon engraftment into recipients has also provided experimental evidence of tissue residency of memory CD8 T cells (13). Quantitative microscopy has shown that T_{RM} cells are more prevalent than circulating memory cells in the non-lymphoid tissues, suggesting that T_{RM} cells form a substantial fraction of the memory repertoire (21). T_{RM} cells do not contribute to systemic immune surveillance, but they establish residence at strategic locations, such as sites, where the primary infection has occurred, positioning them at the frontline of the antimicrobial defense. In this manner, T_{RM} cells are able to mediate border patrol for improved protection against reinfection within the peripheral tissues.

Phenotype of T_{RM} Cells

Tissue-resident memory CD8 T cells can be distinguished from their circulating counterparts through the expression of key cell surface molecules that include CD69 and the α_E integrin, CD103 (**Figure 1**). CD69 is ubiquitously expressed early after activation on T cells, but exclusively T_{RM} cells are able to constitutively maintain CD69 expression under steady state conditions. The majority of T_{RM} cells throughout different tissues express CD69, but parabiosis studies have demonstrated the existence of T_{RM} populations that lack CD69 expression (21, 22). CD69 contributes to the establishment of tissue residency by interfering with spingosine-1 phosphate receptor (S1PR1) function (23, 24). To maintain residency, T_{RM} cells limit expression of tissue exit receptors such as the S1PR1 (25, 26). S1PR1 responds to its ligand S1P that is released by endothelial cells in blood and lymph to attract circulating memory T cells from the tissues into the circulation. In T_{RM} cells, CD69 mediates the internalization and degradation of S1PR1, which results in removal of S1PR1 from the surface and limits the migratory capacities of these memory cells (**Figure 1**). T_{RM} cells do not form upon forced expression of S1PR1, demonstrating the incompatibility of this pathway with establishment of tissue residency in memory CD8 T cells (26). Expression of CD103 appears to be enriched in T_{RM} cells within mucosal compartments, including the skin, lungs, reproductive tract, salivary glands, and small intestine (25, 27–29). A large fraction of CD103$^+$ T_{RM} cells within these tissues locates near or within the epithelium. Epithelial cells express the adhesion molecule E-cadherin, and interaction between CD103 (as part of the αEβ7 integrin) and E-cadherin has been shown to mediate the adhesion between T lymphocytes and epithelial cells (30, 31), suggesting an important role in the retention of T_{RM} cells within epithelial tissues (**Figure 1**). T_{RM} cells are present outside of the epithelia within a wide array of tissues, including the lamina propria of the small intestine, parenchyma of internal organs, such as the brain, kidney, liver, and within the secondary lymphoid organs (32–34). T_{RM} cells within these tissues largely lack expression of CD103 and may employ other adhesion molecules for retention within the tissues. For instance, T_{RM} cells within the

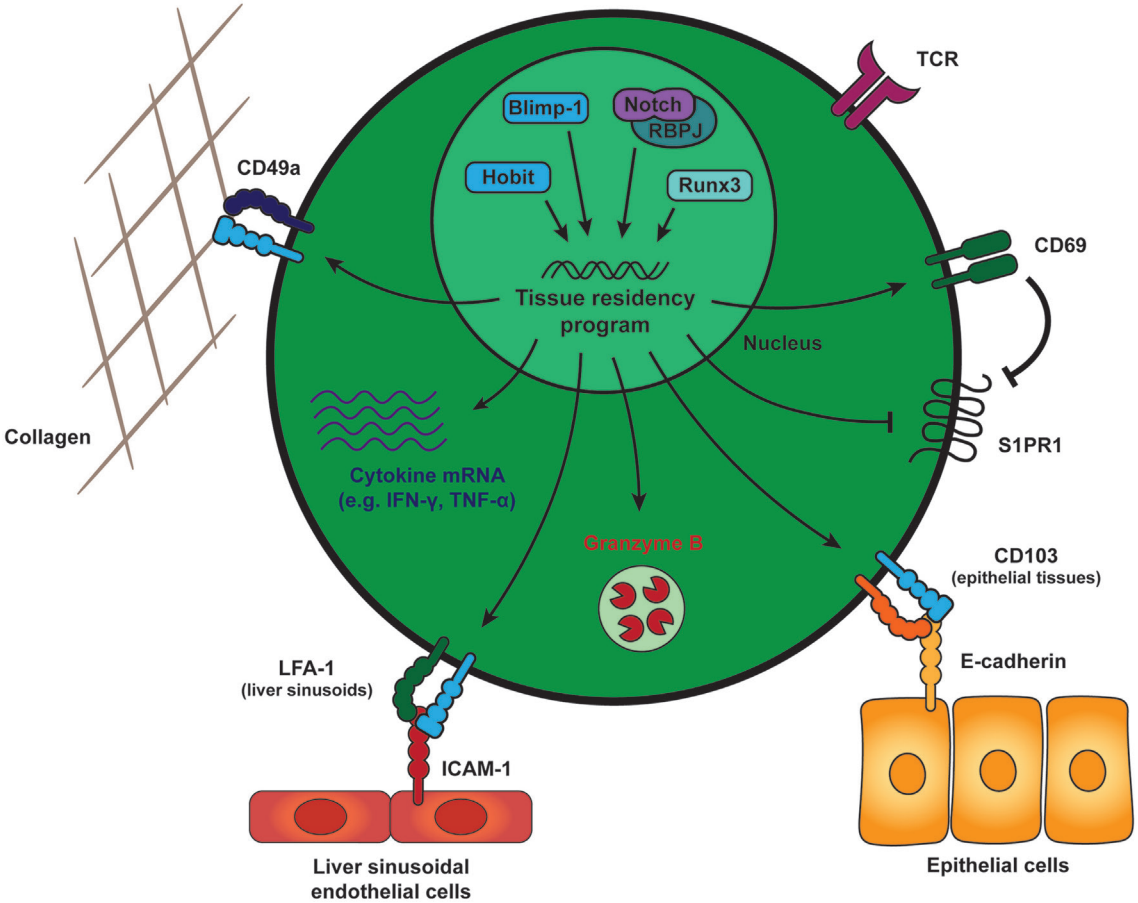

FIGURE 1 | General features of tissue-resident memory CD8 T (T_{RM}) cells. Generation and maintenance of T_{RM} cells is regulated by a distinct set of transcription factors, including Runx3, Blimp-1, and its homolog of Blimp-1 in T cells as well as the transcriptional activator Notch. These transcription factors instruct a tissue-residency program that allows for the long-term retention and maintenance of T_{RM} cells within peripheral tissues. T_{RM} cells across tissues maintain expression of CD69, which promotes tissue residency by interfering with spingosine-1 phosphate receptor (S1PR1) function. S1PR1 mediates egress of T cells into the circulation and its downregulation is a core characteristic of T_{RM} cells. In many tissues, T_{RM} cells also express high levels of CD49a, an adhesion molecule binding to collagen (in complex with β1 integrin) to establish tissue residency. The α_E integrin CD103 is expressed by mucosal T_{RM} cells and may contribute to tissue retention by interaction with E-cadherin on the surrounding epithelial cells. In liver sinusoids, local T_{RM} cells upregulate LFA-1, which supports their tissue residence by binding to ICAM-1 on liver sinusoidal endothelial cells. In addition to these adhesion molecules, T_{RM} cells in many tissues are characterized by elevated transcript levels encoding for pro-inflammatory cytokines, e.g., IFN-γ and TNF-α, and protein expression of the cytotoxic serine protease granzyme B. Abbreviations: Runx3, Runt-related transcription factor 3; Blimp-1, B lymphocyte-induced maturation protein-1; Hobit, homolog of Blimp-1 in T cells; RBPJ, recombining binding protein suppressor of hairless; LFA-1, lymphocyte function-associated antigen-1; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α.

liver express lymphocyte function-associated antigen-1 (LFA-1), which is essential for these cells to mediate interactions with intercellular adhesion molecules on liver sinusoidal endothelial cells (18) (**Figure 1**). Many T_{RM} cells throughout tissues also express high levels of CD49a, which, in complex with β1 integrin, binds collagen within the extracellular matrix to establish tissue residency (35) (**Figure 1**). Therefore, elevated expression of adhesion molecules, such as CD103, LFA-1, and CD49a characterizes populations of T_{RM} cells and distinguishes them from circulating memory CD8 T cells.

The identification of human T_{RM} cells largely relies on phenotypic markers, due to difficulties in experimentally addressing the migratory behavior of human memory T cells *in vivo*. Considerable numbers of T_{RM}-type memory CD8 T cells co-expressing CD69 and CD103 have been found within human tissues, including skin, lung, liver, and intestines (33, 36–38), suggesting that humans also contain a resident compartment of memory CD8 T cells. These human T_{RM} cells share characteristics with their murine counterparts (33, 39, 40), as determined by transcriptional and phenotypic profiling. Similar to the transcriptional profile of murine T_{RM} cells, the core signature of human T_{RM} cells includes upregulated genes associated with the establishment of tissue residency such as CD49a and downregulated genes associated with tissue egress, e.g., S1PR1 and CCR7 (40).

Tissue-resident memory CD8 T cells are essential and sufficient to establish immediate protection against reinfection with pathogens (20, 41, 42). The remarkable effectiveness of T_{RM} cells to achieve clearance of infection and their potential protective

capacities in anti-tumor responses have spurred investigation into the regulatory mechanisms underlying the differentiation, maintenance, and effector functions of these memory CD8 T cells. Transcription factors play important roles in the regulation of memory T cells through their ability to modulate gene expression. Recently, we have identified homolog of Blimp-1 in T cells (Hobit) as a T_{RM}-specific transcription factor that together with related Blimp-1 essentially contributes to the differentiation and/or maintenance of T_{RM} cells (43). Besides Hobit and Blimp-1, other factors, including Runx3, Notch, aryl hydrocarbon receptor (Ahr), and NR4A1 are involved in the regulation of T_{RM} cells (**Figure 1**), suggesting that these cells are under the control of a network of transcription factors (37, 44–46). In this review, we will focus on the role of transcription factors during the different stages of T_{RM} differentiation and during the reactivation of T_{RM} cells upon pathogen re-challenge.

FROM NAÏVE TO MEMORY CELL—DIFFERENTIATION OF T_{RM} CELLS

The development of naïve CD8 T cells into effector T cells and subsequently into T_{RM} cells involves priming in the LN, migration from the LN to the peripheral tissues and the acquisition of a T_{RM} phenotype to establish local retention. Here, we will discuss the cell intrinsic signals and tissue-derived cues that instruct the generation and maintenance of T_{RM} cells.

Heterogeneity in Effector CD8 T Cells—T_{RM} Precursors

The "one cell, multiple fates" hypothesis describes the potential of a single naive CD8 T cell to generate diverse subsets of effector and memory CD8 T cells (47, 48). Studies using genetic barcoding and adoptive transfers of single naïve T cells have demonstrated that T_{CM} and T_{EM} cells can differentiate from the same naïve CD8 T cell. However, it was not addressed whether T_{RM} cells originate from the same naïve T cells as T_{CM} and T_{EM} cells. More recent studies using deep sequencing of the T cell receptor (TCR) β repertoire have revealed substantial overlap in TCR usage between T_{CM} and T_{RM} populations in a skin immunization model (49), suggesting that T_{CM} and T_{RM} cells may develop from a common progenitor. However, given that the naive CD8 T cell population may contain multiple clones bearing identical TCRs, the development of T_{CM} and T_{RM} cells from different precursors cannot be completely excluded.

After recognition of cognate antigen, naïve CD8 T cells first differentiate into effector CD8 T cells. Effector cells diversify into different subsets that include terminal effector cells (TECs) and memory precursor effector cells (MPECs). TECs are characterized by surface expression of killer cell lectin-like receptor G1 (KLRG1) (50). In contrast, memory precursors express very low amounts of KLRG1, but maintain expression of IL-7Rα (CD127) (51). The IL-7Rα^{hi} MPECs differentiate into long-lived memory CD8 T cell populations, whereas the majority of TECs undergoes apoptosis after clearance of the infection. While these studies showed that circulating memory cells develop from MPECs, it was not addressed whether this is the case for T_{RM} cells. Similar to the spleen, peripheral organs such as the skin and small intestine contain KLRG1$^+$ and KLRG1$^-$ fractions within the virus-specific effector CD8 T cell population after infection (25, 29). The cells that remain within the skin and small intestine at the memory stage lack expression of KLRG1, suggesting that tissue-residing T_{RM} cells develop from MPECs. Indeed, adoptive transfer of the KLRG1$^+$ and KLRG1$^-$ fractions confirmed that T_{RM} cells preferentially arise from KLRG1$^-$ MPECs (25). A regulatory role has been reported for transforming growth factor (TGF) β in controlling TEC cell numbers under acute inflammatory conditions (52). Therefore, local TGF-β signaling may drive the preferential development of MPECs in the small intestine, by selectively inducing apoptosis of the TEC fraction during clonal expansion. Recently, *Klrg1* lineage reporter mice have been developed to track the memory offspring of KLRG1$^+$ cells after *Listeria* infection. Fate mapping using the KLRG1 reporter mice showed that approximately half of the T_{RM} cells in the liver and small intestine originate from KLRG1$^+$ precursors (53). These findings suggest that the T_{RM} precursor population may contain MPECs that transiently expressed KLRG1 besides MPECs that never expressed KLRG1.

While T_{CM}, T_{EM}, and T_{RM} cells all appear to develop from MPECs, the timing of branching into the different memory subsets remains unclear. Single cell sequencing data of effector CD8 T cells after the first cell division have revealed only two separate populations that correspond to TECs and MPECs (54), suggesting that at this early stage MPECs form a uniform population. It is conceivable that heterogeneity within MPECs arises at later stages. Adoptive transfer experiments have shown that as early as 7 days after viral infection, effector cells within the spleen have lost the potential to contribute to T_{RM} formation in the intestinal epithelium, while these cells retain the potential to form circulating memory cells (14). These experiments suggest separation between the T_{CM}, T_{EM}, and T_{RM} lineages at the peak of the effector response. Consistent with this time frame of T_{RM} commitment, kinetic analysis of the upregulation of T_{RM}-associated molecules, e.g., CD69 and CD103, during CD8 T cell responses demonstrated that pathogen-specific CD8 T cells within the small intestine and skin acquire a T_{RM} phenotype between 1 and 2 weeks after infection (25, 29, 44, 55). In fact, transcriptional profiling of effector CD8 T cells in the small intestine after lymphocytic choriomeningitis virus (LCMV) infection has shown that the T_{RM}-associated program is largely established within 1 week (44).

Signals Driving T_{RM} Differentiation

Sensing of inflammation and tissue damage during priming of T cells provide important cofactors for the generation of T_{RM} cells. Activated CD8 T cells home to inflamed tissues and can subsequently form T_{RM} cells at these locations, even when antigen is not present locally (41). *In vitro* experiments suggest that inflammatory stimuli may also induce T_{RM} differentiation in the peripheral tissues. Inflammatory cytokines, including type I IFN, IL-33, and tumor necrosis factor-α (TNF-α), downregulate expression of the transcription factor Krüppel-like factor 2 (KLF2) and the tissue exit receptor S1PR1 and upregulate expression of CD69 on CD8 T cells (26, 56).

In vivo evidence supports such a role for pro-inflammatory cytokines including type I IFN and IL-12 in T_{RM} differentiation (57). Local inflammatory cues might contribute differently to the generation and persistence of mucosal and non-mucosal T_{RM} cells. Inflammatory cytokines such as IFN-β and IL-12 counter-regulate the induction of CD103 by TGF-β during CD8 T cell priming and support the formation and persistence of $CD103^{-}$ $CD69^{+}$ T_{RM} cells in the small intestine (58). Binding of pSTAT4, which can be induced by IL-12 or type I IFN, to the CD103 encoding gene suggests that sensing of inflammation might directly affect CD103 expression (58).

These inflammatory signals might guide T_{RM} generation at different stages of CD8 T cell differentiation, with initial cues for commitment to the T_{RM} lineage already being provided in the lymph node. A specialized population of lymph node residing and crosspresenting $CD8\alpha^{+}$ DCs can provide signals, including IL-12, IL-15, and co-stimulation *via* CD24, which contribute to optimal generation of T_{RM} cells (59). Circulating memory CD8 T cells do not share this requirement for $CD8\alpha^{+}$ DCs in the early stages, suggesting that these DCs specifically drive the formation of T_{RM} cells. Following these early events during priming, effector T cells are recruited to the infected tissue. The inflammatory chemokine receptors CXCR3 and CCR5 have been shown to contribute to the recruitment of T_{RM} precursors. CXCR3 enables T_{RM} precursor cells to respond to the IFN-γ inducible chemokines CXCL9 and CXCL10, which is critical for differentiation of T_{RM} cells in the skin (25). CCR5 ligands provided by pro-inflammatory macrophages are important to instruct recruitment of T_{RM} precursors into the vaginal mucosa (60). These pro-inflammatory signals can be provided by a local network of macrophages (57, 60, 61). Thus, it appears that inflammatory stimuli within the LN and from the local environment contribute to T_{RM} differentiation.

The presence of local antigen is not required to attract activated CD8 T cells into the inflamed tissue (41, 62). In the skin, these activated CD8 T cells can subsequently develop into T_{RM} cells in the absence of local antigen (41). However, T_{RM} cell formation after local skin infection is greatly enhanced in the presence of cognate antigen in the tissue microenvironment (63–65). In other tissues, such as the lung and central nervous system, establishment of T_{RM} cells requires cognate antigen recognition in the tissue (28, 62). In the salivary glands, T_{RM} cell formation depends on antigen in the CD4 T cell compartment, but not in the CD8 T cell compartment (66). The presence of local antigen may, therefore, not impact the size of the effector response in the tissue, but rather promote local retention and the formation of T_{RM} cells. The role of antigen after establishment of T_{RM} cells is less clear, but the long-term maintenance of the T_{RM} cell pool in the lung and small intestine appears to be independent of local antigen (56, 67). Next to antigen, costimulatory signals might contribute to the differentiation of T_{RM} cells. Recent work has demonstrated the requirement of intrinsic signals *via* the tumor necrosis factor (TNF) receptor family member 4-1BB for the generation of influenza-specific CD8 T cells in the lung, in contrast to secondary lymphoid tissues (68).

Next to inflammation and local antigen, the accompanying tissue damage might also contribute to T_{RM} generation. Immunization *via* skin scarification generates highly protective T_{RM} cells, compared to subcutaneous or intradermal injection (69) and lung-resident T cells localize at spots that show signs of recovery from previous tissue damage (70). The factors contributing to these effects are still unknown. Inflammation accompanying tissue damage could be partly responsible for the accumulation of T_{RM} cells at sites of tissue damage. Additionally, competition for survival factors during the reorganization of the tissue after injury might influence T_{RM} persistence (71). Data on the local composition of skin-resident T cells support this view. Pre-existing tissue-resident dendritic epidermal γδ T cells are depleted at sites of infection and are replaced by virus-specific CD8αβ T cells (72). To cope with the inflection-related changes in their microenvironment, T_{RM} cells might have developed tissue-specific adaptations. For example, lung T_{RM} cells constitutively express the interferon-induced transmembrane protein 3 (IFITM3), which facilitates their survival during secondary challenges with influenza (73).

Maintenance of T_{RM} Cells

Tissue-resident memory CD8 T cells can persist in tissues for long periods of time (13, 20, 57). Their location at distinct sites throughout the body suggests different requirements for their maintenance and specific adaptions to the local environments. The local presence of antigen, cytokines, chemokines, and tissue-specific metabolites are factors that contribute to T_{RM} maintenance.

Similar to recently and chronically activated T cells, T_{RM} cells demonstrate increased expression of activation-associated molecules, such as PD-1 and importantly CD69 (40, 43). However, persistent stimulation by antigen is not required for T_{RM} maintenance. In fact, the development of T_{RM} cells in the intestine is compromised after chronic viral infection compared to acute viral infection (56). In addition, T_{RM} cells can be formed and maintained by recruiting activated T cells into tissues *via* sterile inflammation (41), suggesting that T_{RM} cell persistence does not require local antigen in the peripheral tissues after infection.

Similar to circulating memory cells, T_{RM} cells upregulate receptors for IL-7 and IL-15 (39, 74), suggesting that these homeostatic cytokines contribute to antigen-independent maintenance of T_{RM} cells. Indeed, IL-7 and IL-15 produced within hair follicles maintain T_{RM} cells near these structures within the skin (75). IL-15 already plays a role during lodgment of T_{RM} cells, but the continued presence of IL-15 is essential for long-term T_{RM} maintenance within the skin (74). IL-15 may not be crucial for T_{RM} cells at other sites, as virus-specific T_{RM} cells within the intestines, pancreas, and female reproductive tract (FRT) are maintained independently of IL-15, in contrast to those in the salivary glands and kidneys (76). The involvement of other homeostatic cytokines in the maintenance of these IL-15-independent T_{RM} populations is currently unclear. T_{RM} cells require TGF-β for maintenance in the mucosa (25, 56, 77). TGF-β instructs the upregulation of CD103 that allows retention of T_{RM} cells in the epithelium, potentially through interactions with E-cadherin on epithelial cells (25, 56, 77). TGF-β is produced as part of an inactive complex together with latency associated protein

(LAP). Integrins such as $\alpha_V\beta6$ and $\alpha_V\beta8$, which are expressed on keratinocytes, are required to release LAP and activate TGF-β in the epithelium (78). These integrins may restrict the action radius of TGF-β close to the epithelial layer. T_{RM} populations underneath the epithelium such as those within the lamina propria of the intestine are independent of TGF-β and largely do not express CD103 (57). T_{RM} populations within internal organs such as the liver and the kidney also largely lack CD103 expression (17, 43), suggesting TGF-β-independent maintenance. Thus, with notable exceptions, T_{RM} populations are maintained on homeostatic cytokines similar to other memory cells and epithelial T_{RM} cells uniquely require TGF-β.

After development, T_{RM} cells form stable populations in many tissues, including skin, liver, and the small intestine, and provide long-term protection against reinfection (13, 17, 20, 41, 57). Maintenance of T_{RM} cells in these tissues appears to be independent of the recruitment of circulating cells, as adoptive transfer experiments have shown that circulating memory CD8 T cells do not convert into T_{RM} cells under steady state conditions (14). In contrast, influenza-specific T_{RM} cells in the murine lungs fail to survive long-term (67, 79). These T_{RM} cells appear to be continuously replenished *via* recruitment from the circulating memory CD8 T cell pool (67).

Tissue-resident memory CD8 T cells are present throughout the body at distinct sites in highly diverse environments that differ in oxygen and nutrient levels, exposure to microbiota, and the regenerative ability of the tissue. Given that T_{RM} cells are permanently residing within the peripheral tissues, they are strictly dependent on the resources within the local environment in contrast to circulating memory cells. Therefore, T_{RM} cells may require tissue-specific adaptations to cope with different conditions posed by the local microenvironment. Transcriptional profiling has revealed a T_{RM}-specific core signature shared between T_{RM} cells at different locations, including the lungs, liver, intestine, and skin (25, 43). In addition to this core signature, T_{RM} cells at different sites are characterized by tissue-specific gene expression profiles (25, 43). The distinct gene programs of T_{RM} cells include chemokine receptors and adhesion molecules that are required to address T_{RM} cells to different tissues. The chemokine receptors CCR8 and CCR10 and the adhesion molecule cutaneous lymphocyte antigen (CLA) are specifically upregulated on skin T_{RM}. CCR10 and CLA have also been functionally implicated in the localization of T_{RM} in the skin (25, 80). In contrast, CCR9 is specifically expressed on intestine-derived T_{RM} cells and may, together with the α4β7 integrin, drive localization of T_{RM} cells in the small intestine (14). Skin-resident T_{RM} cells have been described to rely on the uptake of exogenous fatty acids *via* the fatty acid binding protein (FABP) 4 and FABP5 in contrast to circulating memory CD8 T cells (81). The metabolic requirements of T_{RM} cells at other locations are not yet clear. Members of the FABP family are expressed in a tissue-specific manner (82), suggesting that populations within brain, liver, and intestine may take advantage of local opportunities to meet metabolic demands. Thus, the heterogeneity within T_{RM} populations at different locations may reflect strategies to optimally adapt to the local circumstances.

EFFECTOR RESPONSES OF T_{RM} CELLS UPON REACTIVATION

Numerous studies have highlighted the essential role of T_{RM} cells in providing efficient protection against local reinfections at barrier sites (20, 41, 42). Being situated at the front lines of the immune defense, T_{RM} cells are poised for early detection of recurring pathogens. Here, we will discuss the mechanisms by which T_{RM} cells protect against local infections and the fate of T_{RM} cells after antigen re-encounter.

Border Patrol

Despite their inability to recirculate throughout the body, T_{RM} cells retain the ability to migrate within their local environment. This has been most extensively studied for T_{RM} cells in the skin. These T_{RM} cells localize to the basal layer of the epidermis, where they migrate in the two-dimensional plane of the tissue. Skin T_{RM} cells display a dynamic morphology and continually project dendritic extensions in multiple directions (72, 83, 84) (**Figure 2**). In contrast, T cells in the underlying dermis exhibit an amoeboid shape, which resembles that of migrating lymphocytes in the secondary lymphoid organs. The migration of T_{RM} cells within the epidermis appears to be constrained by the local environment upon resolution of inflammation (72). These constraints only permit relatively slow migration of skin T_{RM} cells, thus promoting their long-term persistence at sites of prior infection (72), and enhancing their ability to scan the local environment for recurring pathogens. This local border patrol requires a density of T_{RM} cells of approximately 100 or more cells per mm^2 for complete coverage of the local area and to ensure early detection of cognate antigens (84).

Patrol of the local tissue environment by T_{RM} cells has also been demonstrated in other organs, e.g., in the FRT and in the liver (17, 18, 85). T_{RM} cells in these tissues show a higher motility compared to the epidermis, which may be related to the more relaxed constraints posed by the tissue architecture. In fact, the speed of T_{RM} cell migration in the FRT is dependent on the local collagen density (85). Local encounter of T_{RM} cells with their cognate antigen in the skin and FRT results in motility arrest and loss of their dendritic morphology (85, 86) (**Figure 2**). The immobilization is transient and T_{RM} cells resume their migratory behavior within 48 h after antigen re-encounter. Motility arrest upon antigen encounter is important for T cell activation. The transient stop allows for the formation of an immunological synapse between T cells and antigen-presenting cells, and enables T cells to acquire of signals for activation (87). Given that most non-lymphoid tissues are primarily surveyed by T_{RM} cells (21), border patrol by these memory cells likely plays an essential role for the local protection throughout the body. This property as motile sentinels places T_{RM} cells in the front lines of defense, enabling rapid responses to reinfection.

Early Effector Response of T_{RM} Cells Upon Reactivation

Tissue-resident memory CD8 T cells are among the first immune cells to act in response to pathogens that have been

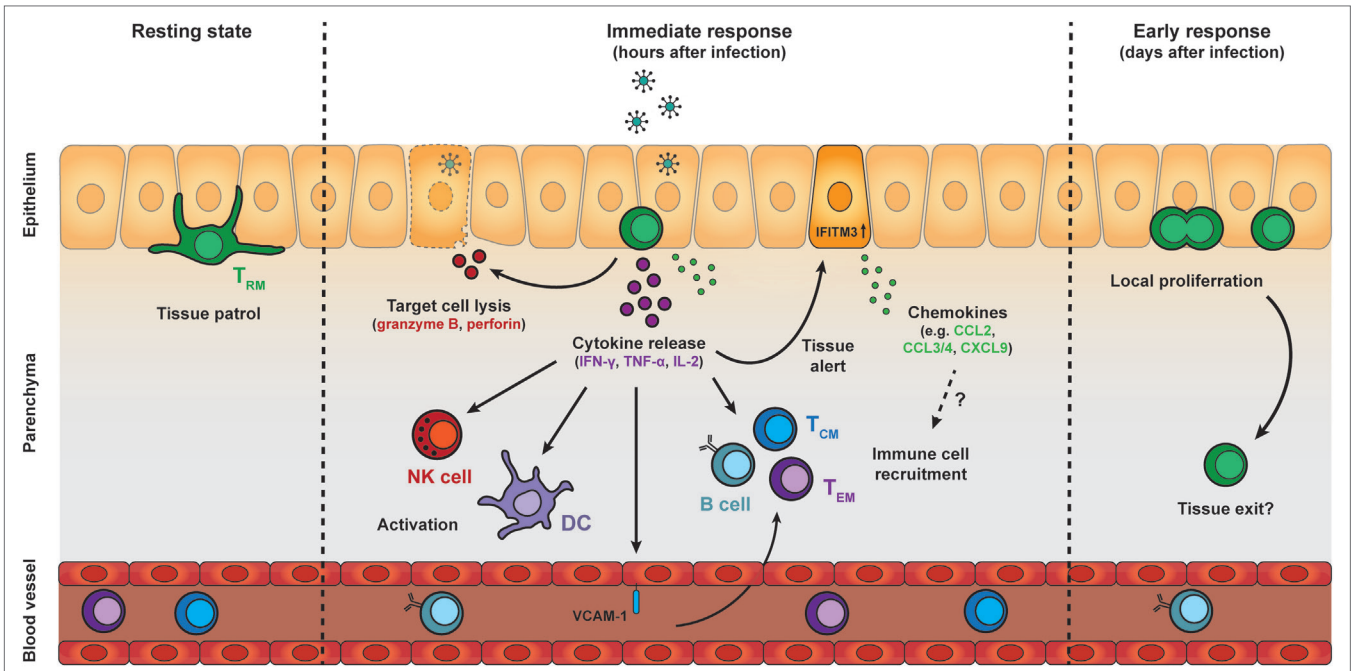

FIGURE 2 | Protective effector responses of epithelial T_{RM} cells upon secondary infection. T_{RM} cells in the epithelia continually patrol their local environment, projecting dendritic extensions in multiple directions. Upon pathogen challenge and antigen re-encounter, T_{RM} cells rapidly release pro-inflammatory cytokines, including IFN-γ, TNF-α, and IL-2, which induce several immune cell- and tissue-specific effects. Local cytokine release by T_{RM} cells results in recruitment and activation of natural killer (NK) cells and dendritic cells (DCs), as well as upregulation of VCAM-1 on endothelial cells in local blood vessels, which may enhance the recruitment of T_{CM}, T_{EM}, and B cells from the circulation. T_{RM} cell reactivation and cytokine release also induces a tissue-wide state of alert, resulting in upregulation of many innate immune response genes, including interferon-induced transmembrane protein 3 (IFITM3), and the increased local expression of inflammatory chemokines. The protective capacity of T_{RM} cells may also rely on perforin-mediated killing of target cells. One to two days after antigen re-encounter, T_{RM} cells undergo local proliferation. Further investigation is required to determine whether T_{RM} cells exit their local environment after reactivation. Abbreviations: IFN-γ, interferon γ; TNF-α, tumor necrosis factor α; IL-2, interleukin 2; ICAM-1, vascular cell adhesion molecule 1; T_{CM} cell, central memory T cell; T_{EM} cell, effector memory T cell; CCL, C-C motif chemokine; CXCL9, C-X-C motif chemokine 9.

previously encountered in an antigen-specific manner. Upon activation, T_{RM} cells rapidly respond by the production of pro-inflammatory cytokines, including IFN-γ (**Figure 2**). In both mice and men, T_{RM} cells across different tissues express high transcript levels of these pro-inflammatory cytokines compared to their circulating counterparts (37, 40, 43, 88). These elevated transcript levels may endow T_{RM} cells with the potential to rapidly produce cytokines upon activation. In addition, post-transcriptional mechanisms have been shown to control cytokine production in CD8 T cells (89, 90), and may contribute to the fast responsiveness of T_{RM} cells. IFN-γ has direct antiviral properties, but is also important for the recruitment and activation of immune cells. The early release of IFN-γ by T_{RM} cells has been demonstrated to stimulate immune cells including DCs and NK cells (91). T_{RM}-derived IFN-γ also elevates expression of the homing molecule vascular cell adhesion molecule 1 on endothelial cells, and enhances the recruitment of circulating B cells and memory T cells from the bloodstream (60, 91, 92) (**Figure 2**). Furthermore, antigen recognition by T_{RM} cells potentiates the local expression of inflammatory chemokines in the tissue, including CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10 (60, 91). High transcript levels of CCL3, CCL4, and XCL1 in quiescent T_{RM} cells suggest that T_{RM} cells participate themselves in the production of these chemokines (43, 88). T_{RM}-derived IFN-γ may also contribute to the release of IFN-γ-dependent chemokines, such as CXCL9 and CXCL10, from the surrounding tissue. These chemokines may trigger the attraction of innate myeloid cells, e.g., neutrophils and monocytes, to the site of infection, thereby further enhancing the immune response (93, 94). In addition, IFN-γ release by reactivated T_{RM} cells has been shown to induce a tissue-wide state of alert in the skin, resulting in elevated expression of many innate immune response genes, including IFITM3, in the tissue (95) (**Figure 2**). Under certain conditions, T_{RM} cells may even induce a body-wide state of alert to prevent viral spread (96). Interestingly, while the local activation of T_{RM} cells is pathogen-specific, the triggering of downstream immune responses can ultimately lead to near-sterile protection of the tissue against antigenically unrelated pathogens (92, 95). The importance of cytokine production by T_{RM} cells for tissue protection has also been demonstrated in the lung, where airway T_{RM} cells protect against respiratory influenza virus through production of IFN-γ (42). Similarly, IFN-γ production by brain T_{RM} cells is crucial for protection against intracerebral infections (97). Tissue-specific adaptations may exist in the secreted factors of T_{RM} cells at different locations (96). For example, lung-resident T_{RM} cells release IL-22 next to IFN-γ, while T_{RM} cells in the liver co-produce granulocyte-macrophage colony-stimulating factor and IFN-γ (96). These

differences in local cytokine repertoires may allow T_{RM} cells to tailor responses to their local microenvironment.

Protection against intracellular pathogens by effector CD8 T cells is partly mediated by the removal of infected cells through the targeted release of cytotoxic molecules, including perforin and granzyme B. After clearance of infection, the expression of cytotoxic molecules is strongly downregulated in circulating memory CD8 T cells. In contrast, T_{RM} cells in several tissues maintain high levels of granzyme B in the memory phase (17, 56, 97) (**Figure 1**). The constitutive expression of granzyme B suggests that T_{RM} cells can rapidly employ cytotoxic mechanisms to eliminate infected cells early after pathogen re-encounter. Indeed, T_{RM} cells in the brain can kill target cells and their protective capacity is dependent on perforin (28, 97). Granzyme B has furthermore been implicated in the remodeling of extracellular matrices (98, 99), suggesting that the serine protease may also contribute to the local migration of T_{RM} cells within tissues. Granzyme B-driven cytotoxicity may not be essential for T_{RM}-mediated protection at other sites, given that, for example, airway T_{RM} cells do not maintain expression of granzyme B and other cytotoxic mediators (42). The selective killing of infected cells by T_{RM} cells minimizes off-target immunopathology, but this protective mechanism may be overwhelmed by rapidly replicating pathogens. Under these conditions, the potential of T_{RM} cells to amplify immune responses through the release of pro-inflammatory cytokines and chemokines may be essential and offset the increased risk for collateral damage.

Proliferation and Maintenance of the Local T_{RM} Repertoire

The protective capacity of memory CD8 T cells depends on their robust proliferation upon recall to establish an army of secondary effector cells. The large number of effector cells can be crucial to counter rapidly replicating and spreading pathogens. In particular, T_{CM} cells have a robust proliferative capacity (100–102). These memory cells patrol secondary lymphoid organs and are, therefore, ideally positioned at these distal sites to the infection to mount secondary responses. T_{EM} cells, which survey peripheral tissues and have limited access to the LN, undergo less pronounced proliferation upon re-challenge (100–102). Using intravital imaging, it has been demonstrated that T_{RM} cells in the skin and FRT undergo local proliferation *in situ* within the first days after antigen re-encounter (85, 86). Potential changes in phenotypic markers on reactivated T_{RM} cells and timespan limitations for intravital imaging pose challenges for long-term follow-up of secondary T_{RM} responses. Despite these technical difficulties, it appears that pre-existing T_{RM} cells within peripheral tissues are the main origin of local proliferative recall responses (**Figure 2**). In line with this, the secondary T_{RM} population arising after pathogen clearance primarily develops from pre-existing T_{RM} cells (85, 86). Recruited circulating memory CD8 T cells also contribute to secondary effector responses (68) and the formation of secondary T_{RM} cells, albeit to a lesser extent (85, 86). However, these memory cells appear to have a limited potential to form T_{RM} cells, at least compared to naïve CD8 T cells (103). The importance of the recruitment of circulating memory cells into the secondary T_{RM} pool may reside in the introduction of new specificities to the local repertoire. Despite local proliferation, reinfection does not numerically increase the pool of local T_{RM} cells (86), suggesting that limits exist in the number of T_{RM} cells that can populate the peripheral tissues. If that is indeed the case, then secondary T_{RM} cells may compete for available niches, which may re-shape the local repertoire after reinfection (71). Previously, it has been demonstrated that circulating memory T cells undergo qualitative changes after successive infections (104, 105). In this context, it will be interesting to investigate the quality, function, and longevity of these secondary T_{RM} cells compared to primary T_{RM} cells.

Tissue Exit and Contribution to Systemic Responses

While local reinfection results in the recruitment of circulating memory T cells to the tissue, locally proliferating T_{RM} cells may in turn downregulate their tissue residency program and egress from the peripheral tissues. Secondary lymphoid organs, including lymph nodes (LN) that drain tissues, are mainly populated by circulating naïve and memory T cells, but also harbor T_{RM} cells (34). Recent work has shown that the T_{RM} cell population in the draining LN increases after a secondary challenge in the skin or the FRT and that these secondary T_{RM} cells are derived from reactivated T_{RM} cells in the non-lymphoid tissue (22). This demonstrates that, upon antigen exposure, T_{RM} cells possess the ability to leave their local environment and enter other tissues, where they can form secondary T_{RM} cells. It remains to be determined whether T_{RM} cells can also disseminate beyond the local draining LN and form secondary memory cells in anatomically distinct tissues (**Figure 2**). Consistent with a contribution of T_{RM} cells to systemic secondary responses, adoptively transferred intestinal T_{RM} cells can acquire properties of circulating memory CD8 T cells upon re-stimulation (55). Further work is required to address whether *in situ* reactivated T_{RM} cells also differentiate into circulating effector and memory cells during secondary responses. After tissue exit, reactivated T_{RM} cells may return to their tissue of origin. Previous work has demonstrated that re-stimulated memory CD8 T cells have a homing bias to their tissue of origin (27, 106), suggesting that reactivated T_{RM} cells may retain an imprint that permits re-entry into their former tissue of residence.

TRANSCRIPTIONAL CONTROL OF T_{RM} DIFFERENTIATION AND FUNCTION

The transition of naïve CD8 T cells into effector and memory cells is a tightly coordinated differentiation process under the control of transcription factors. Upon activation, naïve CD8 T cells upregulate a transcriptional program that drives their differentiation into effector CD8 T cells, thus enabling the establishment of immune responses against pathogens. After clearance of infection, T_{CM} and T_{EM} cells downregulate the effector program and partially re-acquire transcriptional regulators of naïve CD8 T cells to assist in the long-term maintenance of these memory

CD8 T cells. In contrast to circulating memory T cells, T_{RM} cells retain immediate potential to exert effector functions and do not re-establish body-wide immune-surveillance. Therefore, growing evidence suggests that T_{RM} cells require a specific program of transcriptional regulation. Here, we summarize data on the role of T_{RM} cell-specific transcription factors as well as on how transcription factors with a crucial role for effector CD8 T cell differentiation regulate T_{RM} cell generation and maintenance. Finally, we will discuss the transcriptional regulation of T_{RM} effector function and T_{RM} differentiation upon activation in secondary responses.

Transcription Factors Regulating Tissue Residency

Gene expression analysis of circulating memory CD8 T cells and T_{RM} cells has revealed transcription factors with T_{RM}-restricted expression profiles (**Figure 3**). One of these T_{RM}-specific transcription factors is Hobit. Hobit is upregulated in murine T_{RM} cells within skin, lungs, liver, kidney, small intestine, and brain, suggesting that Hobit is widely expressed throughout T_{RM} populations (25, 43, 88). These Hobit$^+$ T_{RM} populations include CD103$^+$ T_{RM} cells within epithelial tissues and CD103$^-$ T_{RM} cells within internal organs, underlining that the transcription

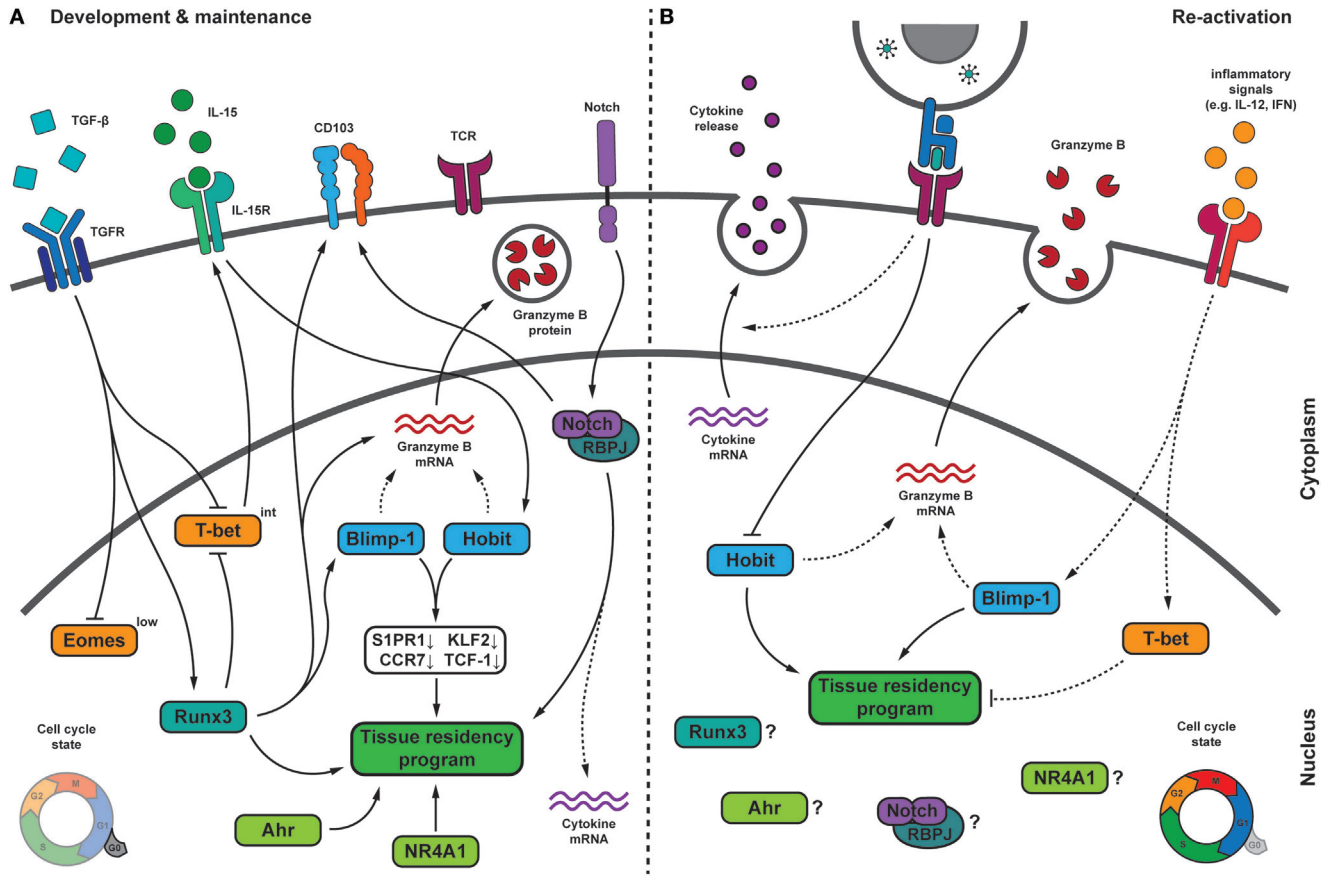

FIGURE 3 | Transcriptional regulation of T_{RM} cells during development, maintenance, and upon pathogen re-challenge. **(A)** During their formation, T_{RM} cells receive multiple signals from the tissue microenvironment that integrate into a transcriptional program, which drives T_{RM} differentiation and maintenance. In several tissues, transforming growth factor β (TGF-β) signaling drives the downregulation of the T-box transcription factors Eomesodermin (Eomes) and T-bet. Residual T-bet expression is required for IL-15Rβ expression. The transcription factor homolog of Blimp-1 in T cells (Hobit) can be induced in an IL-15-dependent manner and, together with its homolog Blimp-1, represses the expression of S1PR1, CCR7, Krüpple-like factor 2 (KLF2), and TCF-1, which is crucial for tissue residency. Blimp-1 and Hobit may also contribute to granzyme B maintenance in T_{RM} cells. The transcription factor Runx3, which can be induced by TGF-β signaling, is crucial for the establishment and maintenance of many aspects of T_{RM} cells, including granzyme B and CD103 expression. Runx3 has been shown to induce Blimp-1. Notch may regulate expression of the adhesion molecule CD103, is essential for maintenance of T_{RM} cells and might contribute to the elevated transcript levels encoding for pro-inflammatory cytokines in T_{RM} cells. Other factors regulating T_{RM} cells include the nuclear receptor NR4A1, and the aryl hydrocarbon receptor (AhR). During quiescence, T_{RM} cells show low proliferative activity. **(B)** Following pathogen re-encounter, T_{RM} cells are exposed to antigen-dependent T cell receptor (TCR) triggering and a variety of inflammatory signals. TCR triggering in T_{RM} cells may result in downregulation of Hobit, thereby weakening its contribution to maintenance of tissue residency. Inflammatory signals, such as IL-12 and type I interferons (IFN), can induce expression of Blimp-1 and T-bet. While increased Blimp-1 expression might fortify T_{RM} features, elevated levels of T-bet could interfere with tissue residency. T_{RM} cells rapidly release pro-inflammatory cytokines upon reactivation in an antigen-dependent manner. Upon re-infection, T_{RM} cells undergo local proliferation. Dashed lines indicate relations that require further investigation. Abbreviations: Blimp-1, homolog of B lymphocyte-induced maturation protein 1; IL, interleukin; S1PR1, sphingosine-1-phosphate receptor 1; CCR7, C-C chemokine receptor type 7; TCF-1, T cell factor 1; Runx3, Runt-related transcription factor 3; RBPJ, recombining binding protein suppressor of hairless; NR4A1, nuclear receptor subfamily 4 group A 1.

factor is ubiquitously expressed in murine T_{RM} subsets. In addition, other tissue-resident lymphocytes such as natural killer T (NKT) cells and innate lymphoid cells 1 express Hobit, suggesting that Hobit is a central regulator of the tissue-residency program of lymphocytes (43). Due to limitations in access to peripheral tissues, analyses of Hobit expression in human T_{RM} cells have not been as extensive as in mice. In line with findings in mice, a substantial proportion of $CD69^+$ CD8 T cells within the human liver expresses Hobit at the protein level (38, 107). Transcriptional profiling also revealed that $CD69^+$ CD8 T cells in human lungs express Hobit in contrast to their $CD69^-$ counterparts, although expression levels are low compared to murine T_{RM} cells (40). We have previously described that $CD45RA^+$ $CD27^-$ effector and $CD45RA^-$ $CD27^-$ effector memory CD8 T cells in human peripheral blood also express Hobit (108). Therefore, despite the presence of Hobit in subpopulations of human T_{RM} cells, no strict association of Hobit with tissue residency exists in human CD8 T cells.

In mice, Hobit specifically instructs the differentiation and/or maintenance of T_{RM} cells, but the transcription factor does not operate alone. Hobit is highly homologous to Blimp-1 and both factors co-operate in the transcriptional regulation of T_{RM} cells. Hobit and Blimp-1 both recognize a "GAAAG" containing binding motif and share the majority of their DNA-binding sites, suggesting that the related factors collaborate through competitive regulation at overlapping target genes. Hobit and Blimp-1 lock T_{RM} cells into the tissues, as these transcription factors instruct shutdown of exit pathways through CCR7 and S1PR1, thus preventing T_{RM} cells from re-entering the circulation (43). In circulating memory cells, the transcription factor KLF2 drives the expression of S1PR1 to provide access to the blood or lymph (109–111). Downregulation of S1PR1 and KLF2 is essential for T_{RM} differentiation, as evidenced by forced expression of S1PR1 that completely prevents the generation of T_{RM} cells (26). The Wnt signaling associated transcription factor TCF1 is involved in maintenance of the distinct phenotype of T_{CM} cells, including upregulation of CD62L and CCR7 (112). Hobit and Blimp-1 directly bind within the *Klf2* and the TCF1 encoding *Tcf7* locus and within the loci of the downstream targets S1PR1 and CCR7, suggesting that these transcription factors efficiently downregulate tissue exit pathways at multiple levels (26, 43) (**Figure 3**). The expression of Hobit in circulating human effector-type and effector memory-type CD8 T cells is enigmatic, given that Hobit in mice directly suppresses expression of tissue exit receptors. Although S1PR1 and CCR7 are nearly absent in quiescent human effector CD8 T cells (113), the putative repressive actions of Hobit on these pathways in long-lived human effector CD8 T cells appear insufficient to retain these cells within the peripheral tissues.

Other T_{RM}-specific transcription factors contribute to the regulation of T_{RM} cells. Expression of the Ahr has been identified in T_{RM} populations of the lungs, skin, and small intestine, but not in circulating memory CD8 T cells (25). In line with its expression pattern, Ahr specifically regulates the persistence of T_{RM} in the skin after HSV infection (72). Ahr is a ligand-operated transcription factor that responds to the presence of dietary components (45), but its ligands in virus-specific T_{RM} cells within the skin are unknown. The transcription factor NR4A1 is also expressed in T_{RM} cells in contrast to circulating memory CD8 T cells (46). NR4A1 is specifically involved in the development and/or maintenance of T_{RM} populations, in particular those in the epithelium and lamina propria of the small intestine (46). The downstream targets of Ahr and NR4A1 in T_{RM} cells have not been identified. Therefore, it remains unclear which aspects of T_{RM} differentiation are regulated by these transcription factors.

Regulation of T_{RM} Cells by Transcription Factors of Effector CD8 T Cells

Runx3, T-bet, Blimp-1, and Notch are each individually important in driving terminal differentiation of effector CD8 T cells and in the acquisition of important effector functions including the production of IFN-γ and/or cytotoxicity (50, 114–117). T_{RM} cells maintain direct effector function into the memory phase, suggesting a requirement for the persistent activity of these transcription factors. Indeed, Runx3, T-bet, Blimp-1, and Notch have also been implicated in the development and/or in the maintenance of T_{RM} cells (37, 43, 44, 74) (**Figure 3**).

Runx3 drives the generation of the CD8 T cell lineage in the thymus and is broadly expressed in peripheral naïve, effector, and memory CD8 T cells (118, 119). Runx3 pairs with the obligatory factor core binding factor of the Runx family that stabilizes binding of Runx proteins, including Runx3, to DNA (120). Functional profiling of CD8 T cell responses demonstrated that Runx3 expression is more relevant in T_{RM} cells than in circulating memory CD8 T cells (44). The transcriptional activity of Runx3 is already apparent at the effector stage in putative T_{RM} precursors, suggesting that Runx3 drives the formation of T_{RM} cells. Runx3 remains essential during the memory phase, implicating a continued role for Runx3 in the maintenance of T_{RM} cells (44). Virus-specific and tumor-specific T_{RM} cells in different tissues and settings require Runx3 for development, exemplifying Runx3 as an important transcriptional regulator of T_{RM} cells. Overexpression of Runx3 is sufficient to repress the expression of signature genes of circulating memory CD8 T cells and to promote the expression of residency signature genes including that of CD103 (44, 121). Collectively, these observations suggest that Runx3 has a primary role in the transcriptional regulation of T_{RM} differentiation. Runx3 may act upstream of Hobit and Blimp-1 in T_{RM} cells, given that the transcription factor induces expression of Blimp-1 and enhances accessibility to motifs shared by Hobit and Blimp-1 (122).

Notch is a surface receptor that interacts with the membrane-bound ligands Jagged and Delta-like on antigen-presenting cells (123). After ligand-induced activation, Notch is cleaved by γ-secretase, which enables its intracellular domain to translocate to the nucleus. Following translocation, Notch associates with the DNA-binding factor recombining binding protein suppressor of hairless (RBPJ) to form a transcriptional activator (124). Notch signaling orchestrates the maintenance of $CD103^+$ T_{RM} cells in the lungs after influenza infection (37). TGF-β-driven upregulation of Notch ligands within the epithelium may provide a mechanism to activate Notch specifically at these sites (125, 126). Notch appears to directly regulate expression of CD103 (37), thus

facilitating binding of T_{RM} cells within the epithelium. In addition, downstream targets of Notch include the glycerol transporter aquaporin-3, solute carriers for amino acids and other nutrients, suggesting that Notch contributes to the maintenance of T_{RM} cells through regulation of their metabolism (37).

T-bet is a T-box factor family member, which drives expression of the IL-15 receptor in circulating memory CD8 T cells (127) and appears to have a comparable role in T_{RM} cells (74). Similar to circulating memory CD8 T cells, T_{RM} populations in several, but not all tissues, require the homeostatic cytokine IL-15 for long-term maintenance (74, 76). Underlining its subordinate role in T_{RM} cells, it has been reported that T-bet acts at a lower level of expression in T_{RM} cells than in effector or circulating memory CD8 T cells. T_{RM} cells also completely lack the T-bet-related T-box factor Eomesodermin (Eomes) that, similarly to T-bet, can support IL15 receptor expression in circulating memory CD8 T cells (74). Overexpression of T-bet or Eomes abrogates differentiation of T_{RM} cells in skin and lungs, suggesting that high-level expression of these transcription factors is incompatible with long-term survival of T_{RM} cells (74, 128). The expression of T-bet is suppressed in T_{RM} cells in a TGF-β- and Runx3-dependent manner (44, 74). Downregulation of T-bet may dampen its suppressive impact on the CD103 encoding *Itgae* locus, where T-bet is able to bind at sites that overlap with the TGF-β-driven Smad proteins (128). Therefore, reduction of T-bet expression may limit interference with TGF-β-driven induction of CD103 expression in T_{RM} cells, while the residual T-bet expression may be sufficient to upregulate IL-15 receptor in T_{RM} cells and to receive IL-15-dependent survival signals for homeostasis (128).

Taken together, transcriptional regulation of T_{RM} cells includes the up-regulation of T_{RM}-specific transcription factors, suppression of transcription factors important for circulating memory T cells, and the maintenance of transcription factors involved in effector differentiation.

Transcriptional Regulation of Direct Effector Functions of T_{RM} Cells

Tissue-resident memory CD8 T cells rapidly exert effector functions upon activation, suggesting that transcription factors that regulate the expression of cytotoxic and pro-inflammatory molecules may also be active in T_{RM} cells. Interestingly, transcription factors that are important for T_{RM} development also play crucial roles in the regulation of effector functions.

In contrast to circulating memory T cells, T_{RM} cells maintain expression of the cytotoxic mediator granzyme B at the protein level, which provides them with the potential to contain infection at early stages through the elimination of infected cells. Runx3 has been shown to induce expression of granzyme B in T_{RM} cells, directly implicating the transcription factor in the regulation of cytotoxicity in these memory T cells (44). A role for Runx3 in the instruction of lytic activity through the upregulation of granzyme B and perforin expression has been previously established in effector CD8 T cells (114, 129). Runx3 directly binds at the granzyme and perforin loci, but also recruits Eomes for synergistic activity at the perforin locus in effector CD8 T cells (114). Mucosal T_{RM} cells do not express Eomes (74), suggesting that in these cells the activity of Runx3 is Eomes-independent. The Runx3-driven program of cytotoxicity in effector CD8 T cells may also involve the upregulation of Blimp-1 expression (129). Blimp-1 and its homolog Hobit have been directly implicated in the regulation of cytotoxicity in effector CD8 T cells (115, 116) and in NKT cells (130), respectively. Blimp-1 drives the acquisition of granzyme B in effector CD8 T cells after acute infection with LCMV and influenza (115, 116). Hobit is required for NKT cells to upregulate granzyme B after stimulation with pro-inflammatory cytokines such as type I IFN and after infection with mCMV (130). The role of Hobit and Blimp-1 in the regulation of cytotoxicity in T_{RM} cells remains to be investigated. The transcriptional regulation of cytotoxicity in T_{RM} cells involves the long-term maintenance of cytotoxic molecules during steady state. Currently, it is not clear how the transcriptional network of T_{RM} cells achieves the retention of cytotoxic molecules into the memory phase. Constitutive expression of Runx3, Blimp-1, and Hobit in T_{RM} cells may be required for persistent expression of granzyme B and other cytotoxic molecules in these memory T cells (**Figure 3**).

Tissue-resident memory CD8 T cells are able to mount rapid cytokine responses upon reactivation, which at least in part resides in their superior capacity to retain mRNA molecules encoding pro-inflammatory cytokines, including IFN-γ (43, 88). The transcriptional network underlying the persistence of mRNA of pro-inflammatory cytokines has not yet been established. Important transcriptional regulators of IFN-γ include T-bet and Eomes (127, 131), but these T-box transcription factors are downregulated in T_{RM} cells in mice and humans (37, 74, 128), suggesting that they do not play a dominant role in T_{RM} cells. Runx3 has been described to regulate IFN-γ, TNF-α, and IL-2 in effector CD8 T cells (114), but is not essential for the regulation of cytokine production by T_{RM} cells (44). Although Notch ligands induce IFN-γ expression in human T_{RM} cells, Notch deficiency only marginally reduces the expression of IFN-γ in murine T_{RM} cells (37). It is possible that the absence of an essential role in the regulation of IFN-γ production for any of these transcription factors relates to redundancy between the IFN-γ-driving molecules.

Taken together, the overlap in the transcriptional programs of effector CD8 T cells and T_{RM} cells suggest a high degree of conservation in the regulation of their effector capacities. Understanding the interplay between the different transcriptional programs in the maintenance of the poised effector state of T_{RM} cells is crucial to further unravel the underlying transcriptional network.

Transcriptional Regulation of T_{RM} Cells Upon Re-Stimulation

While the transcriptional program of T_{RM} generation and maintenance is starting to become clear, it is currently not known how transcription factors regulate T_{RM} functions after reactivation during reinfection. Based on the available information in circulating CD8 T cells, we can speculate on how the signals received by T_{RM} cells during infection may influence their transcriptional program (**Figure 3**).

The transcription factor Hobit is specifically expressed by T_{RM} cells and other tissue-resident lymphocytes including NKT cells during quiescence. Antigen recognition by NKT cells leads to immediate downregulation of Hobit (130). Hobit expression might be similarly regulated in T_{RM} cells. Downregulation of Hobit after TCR activation might allow T_{RM} cells to release effector molecules and undergo proliferation. Additionally, the loss of the tissue-residence transcription factor Hobit might enable T_{RM} cells to leave the tissue, enter the circulation, and migrate to secondary lymphoid organs. In memory CD8 T cells, the sensing of inflammation alone without cognate antigen recognition is sufficient to induce upregulation of effector molecules such as granzyme B (132). IFN-α receptor 1 and signal transducer and activator of transcription 1 are critical in this bystander cytotoxicity of circulating memory CD8 T cells. In NKT cells, Hobit is crucial for the ability to respond to inflammatory cytokines and type I interferon-driven granzyme B upregulation (130). Similarly, Hobit expression may also drive the innate functions of T_{RM} cells after recognition of inflammation.

As pointed out above, many of the transcription factors, which are induced during priming of naïve CD8 T cells and upregulated in effector cells, are also critical for T_{RM} formation and maintenance. Blimp-1 and T-bet are highly expressed in effector T cells and maintained at a lower level in memory CD8 T cells (50, 115, 116). Upon reinfection, reactivated memory cells form secondary effector cells that phenotypically and transcriptionally resemble primary effector cells, e.g., high expression of T-bet. Recognition of IL-12 by memory CD8 T cells during recall responses is one of the main drivers of T-bet upregulation (133). Blimp-1 expression may be similarly regulated, as Blimp-1 is induced by pro-inflammatory cytokines including IL-12 *in vitro* (134). The data suggest that Blimp-1 and T-bet are upregulated in T_{RM} cells in response to inflammation and/or TCR triggering. Given its crucial role in T_{RM} differentiation, increased expression of Blimp-1 may manifest tissue-resident features upon reinfection. At the same time, concurrent inflammation-induced upregulation of T-bet may interfere with maintenance of tissue residency, as elevated levels of T-bet are incompatible with T_{RM} formation (74). The role of the transcription factors Ahr and NR4A1 during activation of memory CD8 T cells is less clear. The expression of Ahr is increased upon activation of memory T cells (135). Also NR4A1 expression is upregulated after TCR triggering (136), but appears to exert a regulatory role after activation, as the transcription factor can maintain T cells in a quiescent state *via* the suppression of IRF4 (137). These data suggest that changes in the transcriptional programming of T_{RM} cells likely occur upon reactivation. Further research is required to determine how the transcriptional network of T_{RM} cells controls their function and differentiation upon re-challenge with antigen and/or inflammation during infection.

CONCLUDING REMARKS

The unique properties of T_{RM} cells compared to circulating memory CD8 T cells have sparked interest in the development of therapeutic approaches that induce T_{RM} formation, especially in the context of future vaccination strategies (138, 139). Given their superior protective capacity at barrier sites, local establishment of T_{RM} cells constitutes an attractive approach to confer long-lasting tissue immunity. Recent work has demonstrated the potency of vaccine-induced T_{RM} cells in providing protection against heterotypic viral challenges (140) and local tumor development (141, 142). In line with this, the improved survival rates of patients with tumors containing large quantities of T_{RM}-type cells highlights T_{RM} cells as a potential target in the treatment of cancer (143–145). A better understanding of the transcriptional network underlying the differentiation and function of T_{RM} cells may assist in unlocking these potent memory cells for therapeutic purposes.

AUTHOR CONTRIBUTIONS

FB, AC, RS and KG drafted and edited the manuscript. FB drafted and edited the figures and figure legends. All authors approved the work for publication.

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REVIEW
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Metabolic Reprogramming and Longevity of Tissue-Resident Memory T Cells

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Tissue-resident memory T cells (T_{RM}) persist in peripheral tissues for long periods of time in the absence of antigenic stimulation. Upon re-encounter with cognate antigen, T_{RM} trigger an immediate immune response at the local tissue microenvironment and provide the first line of host defense. T_{RM} have been reported to play significant roles in host antimicrobial infection, cancer immunotherapy, and pathogenesis of a number of human autoimmune diseases, such as psoriasis, vitiligo, and atopic dermatitis. T_{RM} display a distinct gene transcriptome with unique gene expression profiles related to cellular metabolism that is different from naive T cells (T_N), central memory T cells (T_{CM}), and effector memory T cells (T_{EM}). Skin $CD8^+$ T_{RM} upregulate expression of genes associated with lipid uptake and metabolism and utilize mitochondria fatty acid β-oxidation to support their long-term survival (longevity) and function. In this review, we will summarize the recent progresses in the metabolic programming of T_{RM} and will also discuss the potential to target the unique metabolic pathways of T_{RM} to treat T_{RM}-mediated diseases.

Keywords: metabolism, longevity, TRM cells, viral vaccines, cancer immunotherapy

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Memory T cells mediate immunosurveillance and protect the host through rapid recall responses upon re-exposure to previously encountered pathogens (1). In addition to the two previously identified circulating memory T cells, central memory T cells (T_{CM}), and effector memory T cells (T_{EM}), a new subtype of memory T cells—tissue-resident memory T cells (T_{RM})—has been identified and characterized (2–4). Unlike T_{CM} and T_{EM} that circulate within blood, T_{RM} reside and remain within epithelial barrier tissues for long periods of time without trafficking back into lymph or blood (5). Upon antigen re-exposure, T_{RM} trigger an immediate immune response and provide the first line of protection against the antigen/pathogen they are specific for (4, 6–11). In addition, T_{RM} create a general antiviral microenvironment at the local tissue site and provide cross-protection against antigenically unrelated pathogens (7, 9). Activation of T_{RM} alters tissue-wide gene expression profiles, induces B cell and circulating memory T cell recruitment through IFN-γ-dependent vascular cell adhesion molecule 1 upregulation, and leads to maturation of local dendritic cells and activation of natural killer cells. These activities support the idea that T_{RM} function as a bridge between the adaptive and innate immune system (7, 9). As many viruses show tissue tropism, T_{RM} also provide protective immune responses for the tissue through which it was previously encountered. T_{RM} specific for HSV are in skin (12–14), T_{RM} specific for rotavirus are in gut (6, 15), and T_{RM} specific for influenza are in lung (16–18). Collectively, we propose that sensitization of relatively small numbers of T_{RM} may lead to an amplified signal to more abundant elements of the innate immune system and trigger an organ-wide antiviral state. The placing of adaptive immune memory cells at the body's interfaces with the environment, and moreover

those specific for a given pathogen, speaks to the elegance of adaptive immune memory.

Upon cognate recognition of antigen *via* T-cell receptor, naive T cells (T_N) undergo extensive clonal expansion and differentiate into several T cell subtypes, including effector T cells (T_{eff}) for immediate pathogen elimination and memory T cells for long-term protection (19). Recent studies showed that T cell activation and differentiation are accompanied with and regulated tightly by metabolic reprogramming, presumably to provide the divergent energetic and functional needs for their development, maintenance, and function (20–23). T_N primarily depend on glucose catabolism and oxidative phosphorylation (OXPHOS) to derive energy to support the maintenance of their relatively quiescent state. T_{eff} reprogram their metabolic state to anabolism to enable rapid cell division and cytokine production (24). T_{eff} increase glucose acquisition from blood through upregulating gene expression of glucose transporter-1 (Glut 1) and conduct glycolysis (converting glucose into pyruvate with the production of two molecules of ATP) to meet their energy demand (25). Although glycolysis is less efficient in generating ATP compared to OXPHOS, it is faster and thus rapidly accommodates the increased demand for the energy and biomass formation of T_{eff}. Unlike T_N and T_{eff}, T_{CM} utilize endogenously synthesized fatty acids and OXPHOS to support their long-time survival (longevity) and function (26–28). T_{CM} maintain substantial mitochondrial spare respiratory capacity and display increased mitochondrial mass, thus providing metabolic advantage and equipping them for both longevity and the ability of rapid recall upon antigen re-challenge (26). O'Sullivan et al. showed that rather than importing extracellular fatty acids, T_{CM} utilize endogenous fatty acid synthesis and subsequently conduct mitochondrial fatty acid oxidation (FAO) and OXPHOS for their differentiation and maintenance (27). T_{CM} take up extracellular glucose from blood to synthesize fatty acids in the endoplasmic reticulum, a process dependent on lysosomal acid lipase, which is critical in hydrolyzing cholesteryl esters and triglycerides within LDL particles into free cholesterol and free fatty acids (FFAs) (29, 30). Cui et al. additionally showed that interleukin-7, a cytokine critical for T_{CM} differentiation and survival, induced glycerol transport and triacylglycerol synthesis *via* enhanced gene expression of glycerol channel aquaporin 9, thus providing substrates for mitochondria FAO (28). However, compared to the well-defined metabolic reprogramming of circulating memory T cells, the metabolic programs utilized by T_{RM} to dictate their fate differentiation and sustain their longevity and function, are only beginning to be understood.

T_{RM} METABOLISM IN SKIN

Skin, as the primary interface between the body and outer environment, provides a first line of defense against microbial pathogens, physical damage, and chemical insults. In addition to the role of barrier maintenance and sensing, skin functions as a hotbed of immunological activity (31). It has been shown that healthy skin of an adult human being contains about twice T cells as many as are present in the entire blood volume (1×10^6 T cells/cm^2 and an estimated 2×10^{10} T cells in the entire skin surface) (32, 33). T cells contained in human skin are all CD45RO$^+$ memory T cells, co-express skin-homing addressin cutaneous lymphocyte-associated antigen and the chemokine receptor CCR4, and more than half of human skin T cells are resident under resting conditions and do not re-circulate (T_{RM}) (34). A recent study revealed that pathogenic T cell clones persist in "healed" psoriatic lesions as T_{RM} after complete remission using TNFα blocker (35). Studies on vitiligo showed that vitiligo perilesional skin is enriched with a population of CD8$^+$ T_{RM} expressing both CD69 and CD103, in both stable and active disease stages (36, 37). Residing in a nutrient-restricted (particularly glucose) but lipid-rich environments (38, 39), the mechanisms by which skin T_{RM} sustain their longevity and function remained elusive. Using a well-established model of generating CD8$^+$ T_{RM} in skin after cutaneous immunization with Vaccinia virus, we showed that skin CD8$^+$ T_{RM} adapt to utilize lipid metabolism of exogenous FFAs internalized from the surrounding microenvironment to support both their longevity and protective function (**Figure 1**) (40). CD8$^+$ T_{RM} develop a transcriptional program that features marked overexpression of molecules facilitating exogenous FFAs acquisition and metabolism. Specifically, fatty acid binding proteins 4 and 5 (Fabp4/5), CD36, and lipoprotein lipase (lpl) were in the top 35 most highly overexpressed genes in T_{RM}, as compared to T_N, T_{CM}, and T_{EM}. Fabp's are conventionally thought to function as intracellular chaperones for FFAs, shuttling FFAs from cytoplasm to mitochondria for β-oxidation (41). CD36 is a lipid scavenger receptor that binds to and internalizes FFAs and other lipids (42), and lpl is a lipoprotein lipase that cleaves triglycerides to yield a FFA and diacylglycerol (43). This collection of overexpressed genes involved in lipid uptake and metabolism suggested a special relationship between T_{RM} and lipid metabolism. Further study showed that skin CD8$^+$ T_{RM} upregulated the gene expression of Fabp4/5 in a peroxisome proliferator-activated receptor gamma (PPAR-γ)-dependent manner. When incubated under the presence of exogenous fluorescently conjugated FFAs, skin CD8$^+$ T_{RM} internalized extracellular FFAs much more efficiently compared to other counterparts. Addition of exogenous FFAs induced a significantly higher basal and FCCP-stimulated maximal oxygen-consumption rate in skin CD8$^+$ T_{RM}, which could be blocked by pretreatment with etomoxir, an irreversible inhibitor of mitochondrial carnitine palmitoyltransferase 1, an enzyme that is the rate limiting step for mitochondrial fatty acid β-oxidation and ATP generation (44). Skin CD8$^+$ T_{RM} rendered unable to metabolize exogenous FFAs through mitochondrial β-oxidation, whether through deficiency of Fabp4/5 or pretreatment with etomoxir, cannot persist in skin. By contrast, T_{CM} generated from Fabp4$^{-/-}$Fabp5$^{-/-}$ mice in parallel have no survival disadvantage. Referring to functionality, skin CD8$^+$ T_{RM} deficient in Fabp4/5 were inferior in clearing viral infection and insufficient to protect host against lethal viral re-challenge. Consistent with data from mice, CD8$^+$ T_{RM} from human skin tissue display higher level of Fabp4/5 expression and internalize more exogenous FFAs compared to other human counterparts, indicating that acquisition of exogenous FFAs for metabolism might represent a conserved feature of T_{RM} across species. Given the dependence of skin CD8$^+$ T_{RM} on lipid metabolism and the increasingly uncovered roles of T_{RM} in skin autoimmune diseases such as psoriasis and vitiligo, it is tempting to speculate a novel and promising treatment strategy

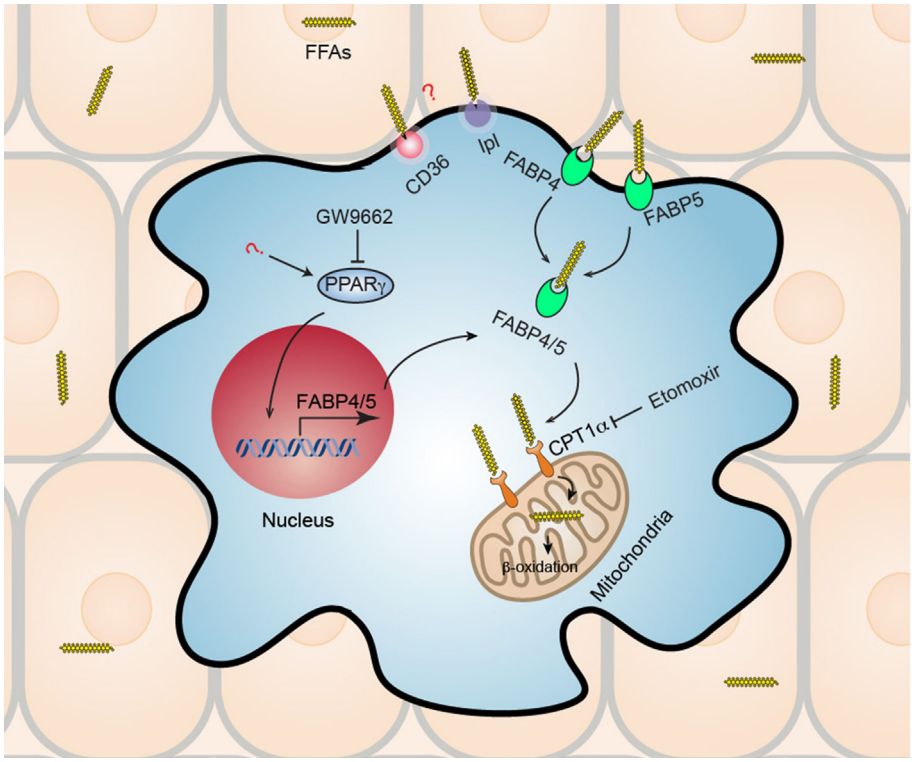

FIGURE 1 | Metabolic reprogramming of skin CD8$^+$ tissue-resident memory T cells (T_{RM}). Skin CD8$^+$ T_{RM} depend on increased uptake of exogenous fatty acid and mitochondrial β-oxidation for their long-term survival (longevity) and function. Skin CD8$^+$ T_{RM} upregulate gene expression of transcription factor peroxisome proliferator-activated receptor gamma (PPAR-γ) and its downstream molecules fatty acid binding proteins 4 and 5 (Fabp4/5), which accounts for the increased uptake of free fatty acids (FFAs) from surrounding tissue environment. Subsequently, Skin CD8$^+$ T_{RM} utilize mitochondrial fatty acid β-oxidation to generate ATP to support their survival and function. Skin CD8$^+$ T_{RM} loss of Fabp4/5 is more prone to cell apoptosis, deficient in long-term survival, and could not protect host efficiently upon viral re-challenge. Treatment with either PPAR-γ inhibitor (GW9662) or with fatty acid mitochondrial β-oxidation inhibitor (etomoxir), results in impaired long-term maintenance of CD8$^+$ T_{RM} in skin. In addition, the roles of CD36 and lipoprotein lipase (lpl), both of which are also upregulated in skin CD8$^+$ T_{RM} and are involved in lipid metabolism, remain unknown and await to be elucidated by future studies.

for skin immune disorders by blocking critical lipid metabolic pathways in T_{RM}. Still, much remains to be elucidated further for the mechanism of T_{RM} metabolic maintenance and survival. The precise roles of gene upregulation of PPAR-γ in skin CD8$^+$ T_{RM} and the contribution of other lipid metabolism-related genes to the survival and function of skin CD8$^+$ T_{RM}, such as CD36 and lpl, both of which were also upregulated as part of the T_{RM} transcriptional program, remain to be investigated. Overall, a detailed signaling pathway of T_{RM} metabolism, as well as the cross talk among skin tissue microenvironment, T_{RM} metabolism and effector function, will be of great interest and may facilitate the development of efficient treatment strategy for T_{RM}-mediated skin diseases.

T_{RM} METABOLISM IN TUMOR MICROENVIRONMENT (TME)

Solid tumors are infiltrated by heterogeneous immune cell types that work in a coordinated fashion to effect antitumor immunity (45). The presence and abundance of tumor-infiltrating lymphocytes (TILs) in tumors is associated with better clinical outcomes after tumor immunotherapy (46–49). TILs differ from their blood counterparts both in terms of upregulated gene expression of immune checkpoint molecules (PD-1, LAG3, TIGIT, and CTLA-4) and reduced effector functions (tumor immunosuppression) (50). Phenotypic analysis of TILs from melanoma revealed that nearly 60% of CD8$^+$ T cells and 50% of CD4$^+$ T cells are CD45RO$^+$CD69$^+$CCR7$^-$, characteristic of a T_{RM} phenotype (51). Melanoma antigen-specific T_{RM} cells resided predominantly in melanocyte-depleted hair follicles and mediate durable immunity to melanoma (36). Other studies showed that the number of T_{RM} within tumors associates with cytotoxic T cell responses and correlates with a better overall survival in lung cancer, head and neck cancer, and breast cancer (52–54). Local T_{RM} induced *via* immunization through mucosal vaccine inhibited tumor growth (52, 55). Thus, a TIL T_{RM} phenotype is considered as a new surrogate biomarker for the efficacy of cancer vaccines, and development of vaccine strategies designed to generate T_{RM} against tumor cells has attracted great interest as a potentially significant therapeutic application.

Inside tumors, both tumor cells and TILs compete for the oxygen and nutrients supplied *via* infiltrating blood vessels. Rapidly growing tumor cells utilize more glucose and glutamine to conduct glycolysis, resulting in a TME of hypoxia and glucose

deprivation for TILs (56, 57). This leads to enhanced expression of immune checkpoint molecules and loss of effector functions in TILs, a process that results in T cell exhaustion (51). On the other hand, TILs conduct metabolic reprogramming to adapt to the metabolic constrains within the TME and sustain their maintenance and antitumor function. Using mouse melanoma models, Ying et al. reported that under short-term hypoxia and hypoglycemia, $CD8^+$ T cells decreased transcripts for genes important in glycolysis while increasing transcripts of PPAR-α and downstream molecules involved in FA uptake and mitochondrial FA catabolism (58). Enhanced FA uptake and increased expression of Cpt1a were observed in vaccine-induced $CD8^+$ TILs from late-stage tumors, implying the increasing dependence of TILs on fatty acid metabolism for energy maintenance. Promoting fatty acid catabolism with fenofibrate, a PPAR-α agonist, markedly improves the capacity of $CD8^+$ TILs to delay tumor growth. This enhancement synergizes with PD-1 blockade to efficiently enhance the efficacy of melanoma immunotherapy. With regard to human tumor biology, the same study reported that TILs isolated from human melanoma metastases show evidence of enhanced FA catabolism, which could be fueled by increased level of FAs within tumor intestinal fluid (58). Collectively, these data suggest that TILs in the TME engage in metabolic reprogramming to utilize FAO for their survival and function. The mechanism by which the TME influences TILs metabolic reprogramming, as well as the nutrient source of FAs for fatty acid catabolism, remains to be further investigated. Also, further studies are required to elucidate the mechanisms by which TILs reprogram their metabolism to cope with the TME and how this metabolic switch affect their survival and antitumor function. Since a growing body of studies support the idea that enhancing already present immune responses against tumors leads to considerably long-lasting tumor remissions and delayed tumor metastasis, a better understanding of TIL T_{RM}'s metabolic switch and how to manipulate this process to increase their maintenance and antitumor effector function, may increase the efficacy and improve the outcome of tumor immunotherapy.

T_{RM} METABOLISM IN WHITE ADIPOSE TISSUE (WAT)

White adipose tissue is a storage depot for fat and an endocrine organ that secretes adipokines to regulate whole-body energy homeostasis and metabolism (59). It connects body barrier surfaces and the internal organs, thereby forming a bridge between tissues that are constantly challenged with surrounding microbes and the inner sterile environments. WAT constitutively regulates glucose and lipid homeostasis by sorting and releasing FFAs *via* lipolysis for usage by other organs (60). Previous work has shed light on cross talks between WAT and immune system in a series of metabolic disorders and inflammatory diseases (61–63). WAT infiltrating lymphocytes are predominantly localized within organized structures referred to as fat-associated lymphoid clusters or milky spots (in the omentum), which can rapidly expand in response to local inflammatory cues (64, 65). Recent studies by Han et al. reveal the residency and occupancy of T_{RM} in WAT and their contribution to immune surveillance and long-term protective memory responses to infection (66). WAT functions as a major hub for adaptive immune memory T cells, predominantly T_{RM}. These adipose T_{RM} express a well-established T_{RM} cell surface marker (CD69) and do not equilibrate between the adipose tissue of conjoined naïve and previously infected mice, confirming the residency of these cells. Transplantation of adipose tissue from previously infected mice was sufficient to protect uninfected mice from lethal pathogen challenge, whereas depletion of T cells abrogated this protective effect, indicating a functional protective role of adipose T_{RM} in systemic pathogen challenge. Following gene expression analysis revealed that adipose T_{RM} cells upregulated genes involved in effector functionalities and lipid metabolism. When incubated *ex vivo* with fluorescently labeled long chain fatty acid palmitate (Bodipy FL C16), adipose T_{RM} cells displayed high rates of lipid uptake and mitochondrial respiration compared to their counterparts from spleen and small intestine lamina propria (siLP), while no difference could be observed in FFA uptake between siLP T_{RM} and spleen T_{EM}. These data suggest that T_{RM} in WAT might also utilize fatty acid metabolism for their survival and function. To what extent do adipose T_{RM} depend on fatty acid metabolism and the contribution of fatty acid metabolism to their longevity and function remain to be evaluated further. The same study showed that induction of WAT memory responses results in the remodeling of WAT physiology (66). Thus, it would be interesting to investigate the cross-regulation between adipocytes and T_{RM} metabolism within WAT, as well as how to manipulate the regulation of pathways to increase host protection or treat individuals with obesity and metabolic disorders.

TARGETING T_{RM} METABOLIC PATHWAYS TO TREAT ASSOCIATED AUTOIMMUNE DISEASES

Targeted therapies are increasingly successful at inducing temporary and partial remissions in organ-specific immune mediated autoinflammatory diseases, but it remains nearly impossible to induce durable remission or cure (6). These autoimmune disorders, including diseases of skin (psoriasis, vitiligo, graft vs host disease), GI tract (Crohn's disease, ulcerative colitis), lung (asthma), joint (rheumatoid arthritis, spondyloarthropathies), CNS (multiple sclerosis), and endocrine system (Type I diabetes), are increasing in incidence and prevalence. Over the past decade, a line of investigation central to the understanding of diseases pathogenesis leads to the discovery of T_{RM}. Increasing evidences from various experimental models and clinical data support a theory that these autoimmune diseases are driven, at least partially, by inappropriate and chronic activation of pathogenic T_{RM} (6). This provides a plausible explanation for the T cell pathogenesis of these diseases and their organ specificity, something that prior explanations of pathogenesis could not adequately explain. This also provides an explanation for the chronicity of these diseases, as T_{RM} are nearly impossible to dislodge from their tissue sites of residence once established. Currently, in clinic these diseases of regional immune hyperactivation (*via* T_{RM}) are usually being treated with systemic immunomodulation and immunosuppression. After successful

therapy is withdrawn, T_{RM} remain *in situ* and can become reactivated by pathogenic stimuli, thus resulting in disease relapse. Therefore, a therapy that could not only suppress the activity of pathogenic T_{RM} but also dislodge them from their tissue niches has the potential to induce remissions that are much longer and ideally indefinite. The uniqueness of T_{RM} in their dependence on lipid metabolism of FFAs from the external environment makes it a previously unappreciated "Achilles Heel," and one that could be exploited therapeutically. Indeed, administration *in vivo* with a pharmacologic mitochondrial β-oxidation, trimetazidine [blocks the long chain 3-ketoacyl CoA thiolase activity (67)], decreased the survival and maintenance of T_{RM} in skin (40). Thus, the likelihood exists that pharmacologic approach targeting the lipid metabolic pathway in T_{RM} could reduce, and theoretically eliminate, the pathogenic T_{RM} that are causative in autoinflammatory disorders of multiple tissues.

CONCLUSION AND FUTURE PERSPECTIVES

It has recently become clear that control of metabolism and the adaptive immune system are tightly linked (21, 22, 68). Nutrient availability and cellular metabolism closely regulate the differentiation, survival, and function of immune cells (23). T_{RM} are not simply memory T cells residing in an unexpected location; rather, they are a specific group of memory T cells with unique lineage (40, 69–71). As revealed from gene transcriptional profiling, T_{RM} display a quite distinct transcriptome from those of T_{CM} and T_{EM}, both of which were more similar to that of T_N (69, 70, 72). Recent findings have shed light on the role of cellular metabolism in regulating differentiation and memory formation of T_{CM} (26–28). However, it remains unknown how cellular metabolism controls T_{RM} fate decision. Moreover, the focus of previous studies on T_{RM} metabolism is primarily on $CD8^+$ T_{RM}, and little is known about the metabolic reprogramming of $CD4^+$ T_{RM} and their roles in $CD4^+$ T_{RM} differentiation, survival, and function. In addition, attributed to the restricted nutrient availability at specialized tissue sites, more studies will be required to elucidate the metabolic pathways of T_{RM} at other tissue sites such as lung, intestine, and brain. Finally given that generation of long-lived T_{RM} are a goal of efficient vaccination, and considering the dual role of T_{RM} in tumor and autoimmune tissue disorders, a more detailed understanding of the unique metabolic programs intrinsic to T_{RM}, and how these programs might be manipulated to enhance or decrease T_{RM} longevity and function, will be a subject of future study with high clinical relevance and therapeutic significance.

AUTHOR CONTRIBUTIONS

YP drafted and edited the manuscript; TK edited and approved the final version of the manuscript.

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REVIEW
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Genealogy, Dendritic Cell Priming, and Differentiation of Tissue-Resident Memory CD8$^+$ T Cells

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Tissue-resident memory CD8$^+$ T (Trm) cells define a distinct non-recirculating subset. Trm cells constitute a first line of defense against local infections in barrier tissues, but they are also found in non-barrier tissues and play a role in antitumor immunity. Their differentiation in tissues and their phenotypical, transcriptional, and functional characteristics are the object of active research. Herein, we will discuss the potential existence of committed CD8$^+$ Trm precursors and the genealogy of memory CD8$^+$ T cell subsets. In addition to the priming of naive T cells, there is some plasticity of antigen-experienced effector and memory T cell subsets to generate Trm precursors. Local inflammation, antigen presentation, and cytokines drive Trm differentiation. It is of prime interest how specific dendritic cell subsets modulate priming and differentiation of Trm cells, as well as their reactivation within tissues. Research on how we can manipulate generation of memory T cells subsets is key for improved vaccination strategies.

Keywords: memory CD8$^+$ T cell, circulating memory, tissue-resident memory, infection, plasticity, priming, differentiation, dendritic cells

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ARE THERE COMMITTED Trm PRECURSORS?

Dendritic cells (DCs) prime naive T cells in secondary lymphoid organs generating both a short-term effector response and a memory response. Memory T cells are further subdivided based on their distribution and trafficking properties. Circulating memory T cells can be further subdivided as central memory T (Tcm) cells that re-circulate between secondary lymphoid organs, blood and lymph, and effector memory T (Tem) cells that can also access the tissues (1, 2). Conversely, a distinct subset of sessile tissue-resident memory T (Trm) cells has been defined in the last years. Trm cells are long-lived and confined in a wide variety of tissues, including barrier tissues, such as the skin and lung, where they comprise the first line of defense against local re-infections and provide superior protective immunity compared with circulating memory cells (3–7). However, Trm cells are also found in non-barrier tissues like brain (8), heart (9), and play a role in tumor immunity (**Box 1**) (10–12). Trm cells are phenotypically, transcriptionally, and functionally distinct from their circulating counterparts. Trm cells do not express the lymph node homing receptors CCR7 or CD62L, and expression of CD69 and the integrin CD103 is often used to define T cells as tissue resident (7, 13). However, CD103$^-$ CD69$^+$ cells make up almost half of the dermal Trm population (3, 14), while the intestine and the lung contain subsets of T cells that lack CD103 and/or CD69 expression but are nonetheless capable of maintaining tissue residence (15–17). This phenotypic heterogeneity among Trm populations is dependent not only on the tissue of residence but also on how Trm cells are

BOX 1 | Trm in immunity against tumors.

The relative contribution of different memory CD8+ T cell subsets to antitumor immunity is starting to be explored. Data in human tumors show that the number of cells with a Trm phenotype infiltrating tumors correlates with a better overall survival in different cancers, including early stage non-small-cell lung carcinoma, pulmonary squamous cell carcinoma, and high-grade serous epithelial ovarian cancer (11, 28–31). Immunotherapy of cancer using vaccination routes that generate Trm may be superior in generation of antitumor immunity (32, 33). In addition, reprogramming of infiltrating DCs in the tumor with curdlan induce a Trm phenotype in tumor-infiltrating T cells that can reject tumors (34). The contribution of Trm to cancer immunity has been explored in several mouse cancer models. Using a mouse model of melanoma-associated vitiligo induced by depletion of regulatory T cells and surgical excision of a primary dermal B16 melanoma, functional melanoma antigen-specific Trm cells develop in the skin of mice with vitiligo and are critical for protection against melanoma rechallenge (10). Intranasal vaccination with a mucosal vector targeting DCs fused to an HSV-derived peptide leads to generation of Trm that are protective against an orthotopic head and neck TC1 tumor (11). Following skin scarification with rVACV-OVA, both circulating memory CD8+ T cells and Trm cells are sufficient to mediate immunity against B16-OVA melanoma (12). Surgical parabiosis of rVACV-OVA skin-scarified mice with naive mice leads to share circulating memory T cells while antigen-specific Trm cells are restricted to the immunized parabiont. Challenge with melanoma of separate parabionts shows that circulating memory cells transfer antitumor immunity but this response is improved in the presence of Trm cells (12). In addition, Tcm cell infiltration in the tumor also induce the generation of cells with a Trm phenotype expressing PD-1, showing that anti-PD1 therapy can improve the effectiveness of Trm cells within the tumor (12). These results suggest that strategies aimed to enhance Trm generation or infiltration within tumors, in cooperation with circulating memory T cells, may result in improved cancer immunotherapy.

generated by local tissue infection. In addition, Trm cells exhibit a unique transcriptional signature that comprises modulation of chemokine receptors like CXCR3 (4), upregulation of genes associated to tissue residency including *Cdh1* (E-cadherin) (18), *Itgae* (CD103) (8, 19), and *Itga1* (CD49a) (13, 20–22), and downregulation of genes related to tissue egress, such as *Klf2*, *S1pr1* (23), and *Ccr7* (4, 24) among others. They also show augmented effector function compared with circulating memory cells, with elevated expression of *Granzyme B* and *Tnf-a*, and genes encoding immunoregulatory molecules such as ICOS and CTLA-4, indicating tight modulation of the robust effector function of Trm cells (4, 24). Importantly, this transcription core is shared between human and mouse Trm cells (25–27).

Trm cells can be generated from KLRG1^{lo} memory precursors (4). These KLRG1^{lo} memory precursors are either KLRG1^{-} IL-7Rα^{+} memory precursor CD8+ effector T cells or KLRG1^{+} effector cells that have lost KLRG1 expression (ExKLRG1) (35). These cells seed in non-lymphoid tissue where differential expression of transcription factors and tissue-derived signals instruct the tissue residency program of this T cell lineage. Trm formation requires partial downregulation of T-bet and complete shutdown of eomesodermin (Eomes), being both events controlled by TGF-β derived from the tissue. Remaining T-bet is critical for IL-15R expression, which allows responsiveness to tissue-derived IL-15 necessary for their long-term survival (36). T-bet along with IL-15 signaling are also critical for the expression of the transcription factor Hobit, that is essential for establishment of Trm cells in the tissue. Hobit cooperates with the transcription factor Blimp1 to control the transcriptional program of residency of Trm cells and concomitantly blocks the differentiation to alternative T cell memory lineages (37) and regulates effector functions in quiescent human effector-type CD8+ T cells (38). In addition, the transcription factor Runx3 is also a key regulator of Trm generation and modulates tissue residency (39).

Recent studies have revealed important contributors to Trm cell establishment and differentiation in the tissue (4–7, 23). However, less is known about the early priming signals in secondary lymphoid organs that precede entry into peripheral tissues (14). While both resident and circulating memory T cells have a common naive precursor (40), there are evidences suggesting the existence of a committed Trm precursor. Modulation of T cell metabolic reprogramming affects Trm generation acting early after activation and determining T cell fate and function (41). Specifically, inhibition of mTOR by rapamycin during priming and expansion of CD8+ T cells upon viral infection impairs the formation of Trm cells by blocking migration into the tissue, despite increasing the number of circulating memory T cells (41–43). This is consistent with data demonstrating that inhibition of mTOR induces Eomes and blocks persistent T-bet expression, favoring circulating memory T cell generation (44). These results suggest that differential modulation of mTOR-dependent early signals received during T cell activation can instruct circulating and memory compartments before tissue entry and differentiation. Moreover, cross-priming by type 1 classical DCs (cDC1s) is required for optimal generation of Trm but not circulating memory cells, supporting the notion that priming signals can imprint acquisition of a committed Trm cell fate (14). However, Trm precursors are not only derived from naive T cells, since antigen-experienced circulating memory T cells are also able to produce Trm cells after infection or in a tumor context (**Box 1**), highlighting the plasticity of the memory T cell subsets, as explained below. We will thus discuss the genealogy of CD8+ Trm cell generation and the differential role of DCs during priming, differentiation, and reactivation of Trm cells, highlighting them as a strategy in vaccination and tumor immunotherapies.

GENERATION OF MEMORY CD8+ T CELL PRECURSORS

The traditional definition of memory T cells is based on the survival time after infection, once antigen-specific T cell numbers stabilize, which normally occurs several weeks to months after priming. However, this survival-based definition does not take into account some key functional aspects of memory T cells that, on the other hand, define diverse memory subsets. These characteristics comprise the capacity of memory cells to develop rapid recall responses, the high proliferative capacity or stemness, and the homeostatic turnover. We could hypothesize that CD8+ T cells do not acquire these memory-related functional features until infection has been controlled, meaning that effector cytotoxic T lymphocytes only become Trm-committed cells once they have been established in their destination tissue.

Alternatively, divergent differentiation fates of T cell progeny could be specified when a naive T cell is activated during the acute phase of the immune response. Several evidences suggest that the fate of memory versus effector CD8$^+$ T cells is determined early after priming or gradually during their development, meaning that memory cells are derived from early committed precursors (44–47). Notwithstanding, it is still not well understood whether this paradigm can be applied to Trm differentiation. The existence of an imprinted Trm precursor generated in secondary lymphoid organs is supported by the reconstitution of mature Trm cells upon KLRG1$^-$ adoptive transfer (4). In this study, CD8$^+$ effector cells isolated from spleen of gBT-I.1 transgenic mice expressing a TCR specific for the MHC class I-restricted immunodominant peptide from HSV glycoprotein B (gB498-505) were sorted 6 days after infection with HSV based on KLRG1 expression. The authors showed that, upon adoptive transfer, only the KLRG1$^-$ population generated matured CD103$^+$ Trm cells in the skin of HSV-infected recipient mice. Moreover, Trm differentiation requires a distinct program that combines effector and memory cell transcriptional programs, sharing some features with early effector CD8$^+$ T cells or Tem cells but also some of the Tcm cell properties (39, 48).

There are several models that explain generation of committed precursors for Tcm, Tem, and Trm cells (**Figure 1**). The "one cell, one fate" model (**Figure 1A**) proposes that distinct fates emerge from different naive T cells, with one single activated T cell giving rise to daughters of only one fate. In other words, this "one cell, one fate" model suggests that naive T cells are predetermined during thymic development to give rise to effector or memory T cells. Therefore, we can speculate that specific TCR-bearing cells will give rise to circulating (Tcm or Tem) memory cells, while other CD8$^+$ T clones expressing a different TCR will generate Trm cells. Nevertheless, Trm cell clones generated in the skin and Tcm cell clones in the draining lymph nodes (dLN) show a similar abundance of particular TCR clones tracked by CDR3 sequences, suggesting that a common naive T cell precursor is able to give rise to both Trm and Tcm cells after skin immunization (40). However, there may also be some pre-determination to become Trm or Tcm cells based on TCR-MHC interaction strength. For example, Trm cells in brain and kidney express TCRs with higher affinity to MHC-I tetramers (up to 20-fold) than their splenic memory T cells counterparts, whereas effector cells express similar high-affinity TCRs in all organs (49). Conversely, low-affinity T cells, with reduced T-bet expression during priming, preferentially differentiate into Tcm precursors (50). Similarly, different CD8 T cell clones have a distinct and fixed hierarchy in terms of effector function in response to the same *Toxoplasma* antigen measured as proliferation capacity, trafficking, T cell maintenance, and memory formation. Homing to the brain was directly related to TCR affinity. The highest affinity clone

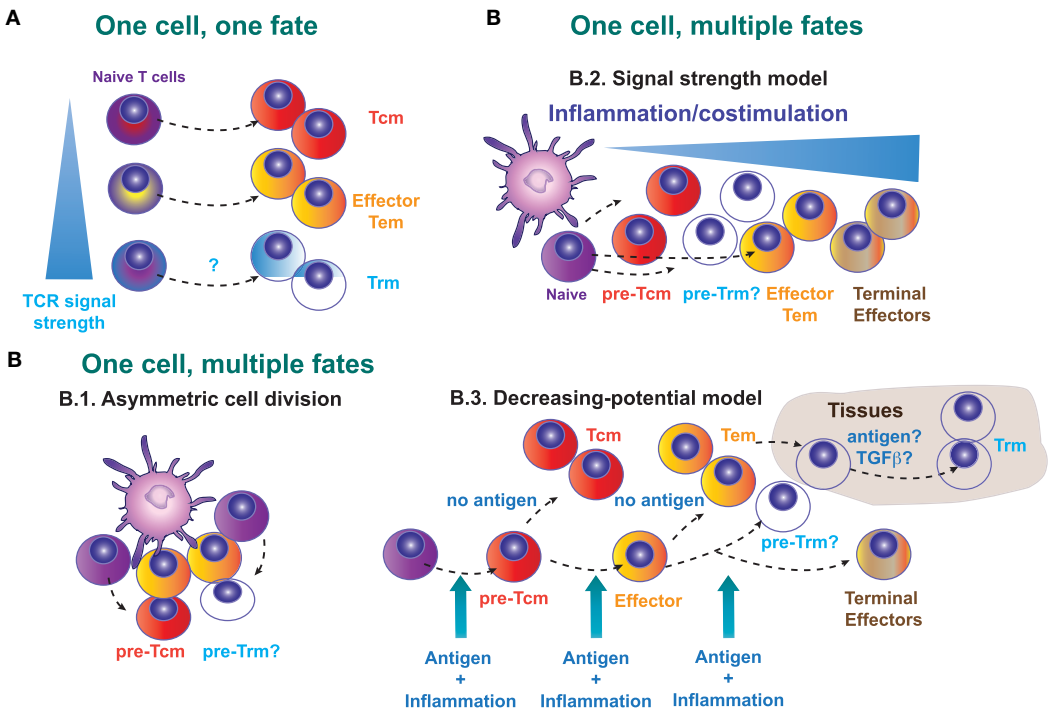

FIGURE 1 | Possible models that explain the generation of a committed Trm precursor in secondary lymphoid organs. **(A)** One cell, one fate model. Distinct naive T cells will exhibit a different lineage decision determined by the quality (intensity of signal) of their TCR. **(B)** One cell, multiple fates model. B.1., Asymmetric cell division in T lymphocytes may determine fate diversification. B.2., Signal strength model. The strength of the signals 1, 2, and 3 determines the fate of the activated CD8$^+$ T cells, with low strength signals generating central memory T (Tcm) precursors and high strength supporting the generation of terminal differentiated effectors. B.3., Decreasing potential model. This model proposes that a short duration of antigenic stimulation favors development of activated cells that will give rise to greater numbers of Tcm cells, while longer duration of stimulation promotes terminal effector cell differentiation and death.

persisted longer in the host during chronic infection as a resident memory population ($CD103^+$) in the brain (51). These data suggest that the non-lymphoid microenvironment may facilitate the retention of T cells with high-affinity TCRs, particularly in persistent infections, which would facilitate detection of infected cells expressing low levels of antigen. We can thus conclude that although the "one cell, one fate" model does not always explain how a naive $CD8^+$ T cell become a Trm or a circulating memory cell, the clonal TCR affinity may influence on this Trm cell fate or their persistence, depending on the nature of the infectious pathogen, or the infected target tissue where Trm cells establish.

Alternatively, it is possible that effector T cells and different memory T cell subsets can derive from a single naive T cell clone (**Figure 1B**). This "one cell, multiple fates" model, proposes that the fate decision is taken during T cell priming or even in later stages during the T cell response. Several possible mechanisms may explain how different memory and effector subsets emerge from one single cell. During the immunological synapse between the antigen-presenting cell and the T cell, asymmetric cell division (**Figure 1**B.1) allows the generation of two different daughter cells. Accordingly, the generation of effector and memory T cells from naive T cells in primary responses could depend on the asymmetric inheritance of intracellular fate determinants (52). However, the relevance of this asymmetric cell division in the generation of different memory precursors has not been determined yet.

In vivo cell tracking of individual OT-I cells demonstrated that, even for T cells with the same TCR, there are heterogeneous patterns of clonal expansion and differentiation. Therefore, the dynamics of the single-cell response are not uniform, as demonstrated by the differential participation of their progeny during primary versus recall infections. Therefore, individual naive T lymphocytes contributed differentially to short- and long-term protection (53, 54). In addition, the progeny of naive clonal $CD8^+$ T cells displayed unique profiles of differentiation based on extrinsic antiviral- or antibacterial-induced environmental cues. A single naive $CD8^+$ T cell exhibited distinct fates that were controlled by tissue-specific events (55, 56). Following oral infection with *Listeria monocytogenes*, an antigen-specific CD8 T cell population can be separated into cells with a memory precursor phenotype in the intestine, whereas in the spleen and lung, *L. monocytogenes*-specific CD8 T cells maintained a prolonged short-lived effector phenotype. This intestinal $CD127^+$ $KLRG1^-$ CD8 T cell population resembling memory precursor formed in response to TGF-β following oral *L. monocytogenes* infection. This subset rapidly upregulated CD103 needed for association to the epithelium and survived long-term, identifying mucosal Trm precursors (56). In either case, these observations exclude models in which each naïve T cell exclusively yields progeny with the same distribution of either short- or long-term potential phenotype, arguing against asymmetric division as a singular driver of $CD8^+$ T cell heterogeneity.

During priming, T cells receive three key signals: antigen recognition (signal 1), co-stimulation (signal 2), and cytokines that modulate T cell differentiation (signal 3). According to the "Signal strength model" (**Figure 1**B.2), the strength of the three signals will determine the expansion amplitude and the fate of the primed T cell (57). Generation of short-lived or terminally differentiated $CD8^+$ T cells is favored by a strong pro-inflammatory signal (58), whereas precursors for Tcm cells are increased by the deficiency in type I interferon signaling (59), or deficiency in IFN-γ or IL-12 (60). Contrary to Tcm generation, inflammation drives Trm differentiation in several non-lymphoid tissues (9). Many tissue-specific cytokines including IL-15, TGF-β, IL-12, and type I IFN are produced upon infection and inflammation and regulate differentiation and persistence of Trm cells in non-lymphoid tissues, with differential requirements that may be tissue specific (4, 61, 62).

The "decreasing potential model" (**Figure 1**B.3) states that the history and accumulative duration of signals that a $CD8^+$ T cell has encountered during infection impacts on its differentiation state. Repetitive antigen encounter and/or exposure to inflammatory cytokines, differentiates T cells toward terminal effector T cells that retain their cytolytic capacity but lose features owned by Tcm cells, such as longevity, proliferative potential, and IL-7Rα expression. In this sense, and contrary to Tcm cells, local antigen presentation may favor the expansion of Trm cells in the skin (14, 63). The composition of the local Trm cell pool is shaped by antigen-dependent competition between $CD8^+$ T cells of different specificities in the infected tissue (64). Therefore, Trm cells development seems to be favored by antigen encounter and/or specific inflammatory signals in the tissue that favor, or are even needed for their retention (4, 9, 65). Regardless of the apparently contradictory different mechanisms proposed by these models, they are not necessarily mutually exclusive and multiple models may simultaneously contribute to *in vivo* induction of memory T cells.

PLASTICITY AMONG DIFFERENT T CELL SUBSETS

Independently of the existence or not of a committed Trm precursor, it is well documented that naive ($CD8^+CD44^-CD62L^+$) T cells differentiate into Trm cells in multiple scenarios: skin infection with VACV (3), or HSV (66), intranasal infection with influenza (67) or in non-infectious disorders, such as chemical hapten inflammation (40). In several cases, optimal generation of committed Trm precursors requires further antigen presentation in the inflamed tissue (**Figure 2A**). However, Trm differentiation and maintenance is dependent on tissue-specific signals that may be antigen independent. Inflammation drives Trm differentiation in many non-lymphoid tissues (9) (**Figure 2B**). Many tissue-specific cytokines including IL-15, TGF-β, IL-12, and type I IFN are produced upon infection and inflammation and regulate differentiation and persistence of Trm cells in non-lymphoid tissue, with differential requirements that may be tissue specific (4, 61, 62). Effector $CD8^+$ T cells can also differentiate into nasal Trm cells independently of local antigen (68) (**Figure 2C**). However, it is difficult to know if the conversion of effector T cells into Trm occurs in all effector cells infiltrating the tissues, or whether there are specific features in the effector T cells that commit them to Trm differentiation under the right tissue environment, as we have discussed in the former section.

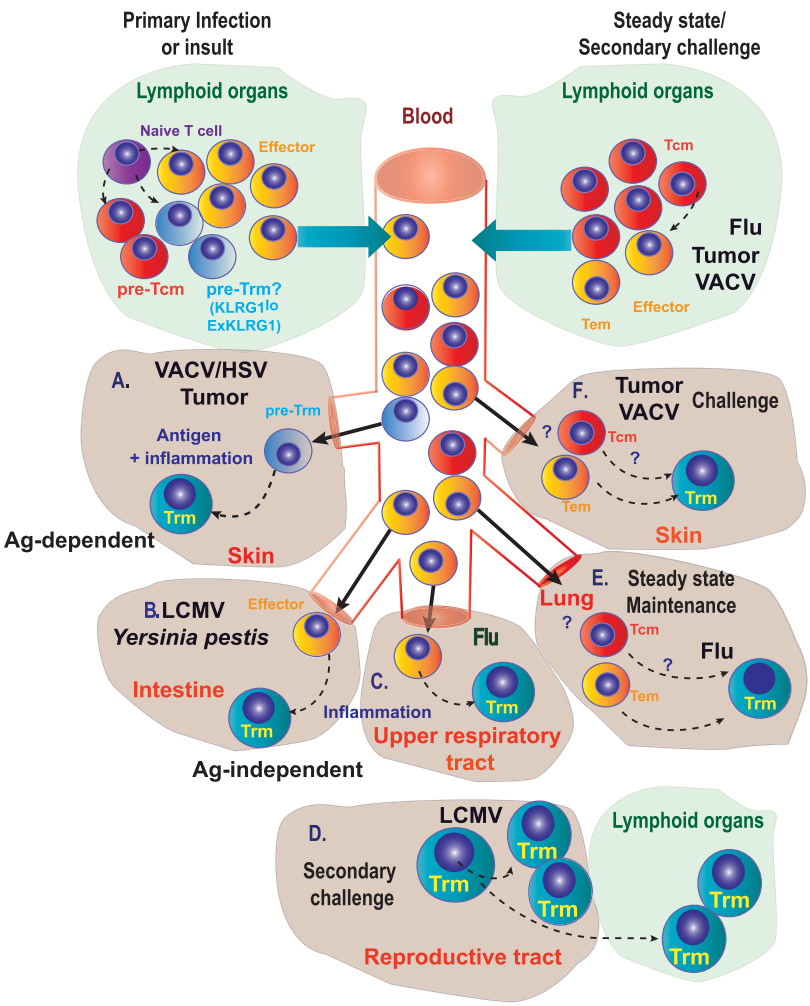

FIGURE 2 | Genealogy of Trm. **(A)** During primary infection, immunization, or other insults, naive T lymphocytes differentiate into precursors of circulating memory cells, effectors, and putative precursors of Trm (pre-Trm) cells that can differentiate into Trm cells in the skin, in response to viruses (VACV/HSV) or tumors. **(B,C)** Inflammation in the intestine **(B)** or in the upper respiratory tract **(C)** is able to promote Trm generation. **(D)** In the female reproductive tract, proliferating pre-existing Trm cells contribute substantially to the boosted secondary Trm population and can exit non-lymphoid tissues to convert into new Trm cells in lymphoid tissues. **(E)** Under steady-state conditions, circulating memory T cells can differentiate into Trm cells in the lung of mice previously infected with influenza A virus. **(F)** Circulating memory [effector memory T (Tem) or central memory T (Tcm)] cells can differentiate into Trm cells in the skin upon secondary challenge with viruses or tumors.

In steady state or upon challenge, Trm cells may also be generated from antigen-experienced cells: Tcm, Tem, and Trm cells themselves (self-maintenance) (65, 69–71). Local antigen reactivation of pre-existing Trm in the female reproductive tract (70) or the skin (69) results in their arrest and *in situ* division (**Figure 2D**). These proliferating Trm also exhibit some plasticity and can exit non-lymphoid tissues to convert into new Trm in the draining lymphoid tissue (71, 72) (**Figure 2D**). Although local mucosal recall response is dominated by proliferating pre-existing Trm that contribute most substantially to the boosted secondary Trm population, Trm reactivation also induces the antigen-independent recruitment of Tcm that differentiate into Trm *in situ* (69, 70). Maintenance of a Trm pool in the lung by conversion of incoming circulating memory $CD8^+$ T cells is critical for protection after influenza A virus infection (73). Lung Trm cells are replenished mainly from circulating $CD8^+CD69^-CD103^-$ Tem rather than Tcm cells, even in the absence of persisting antigen (**Figure 2E**). However, this lung Trm pool declines with time as circulating memory $CD8^+$ T cells lose migratory capacity to the lung, together with an enrichment of Tcm versus Tem among circulating population of memory cells, thus reducing the efficiency of conversion to Trm cells. These findings support a model where gradual loss of protection to influenza is linked to a decline of Trm cells in the lungs caused by apoptosis and decreased input from the circulating memory $CD8^+$ T cell population (73).

Transfer of CD8$^+$CD44$^+$CD62L$^+$ Tcm cells specific for ovalbumin (OT-I) followed by epicutaneous VACV-OVA infection also induced Trm cells in the skin (12) (**Figure 2F**). The efficiency of Trm generation is, however, not equal depending on the different T cell source. For example, although both Tcm and naive T cells induce Trm cells that persist at least 2 months after infection, Tcm cells are less efficient at producing Trm cells (12). Most Trm cells generated from adoptively transferred Tcm cells showed hallmark CD69 expression, with half of them co-expressing CD103. Trm cells derived from adoptively transferred Tcm cells were unable to migrate *via* blood or lymph (12), supporting that they are *bona fide* Trm cells without recirculating capacity (74). Plasticity of transferred Tcm to become Trm cells does not only occur upon infection but also in the context of tumor challenge. Mice transferred with OVA-specific Tcm cells and challenged with intradermal inoculation of B16-OVA melanoma developed cells with a Trm cell phenotype (CD69$^+$ CD103$^+$) within the tumor mass. Furthermore, when mice transferred with Tcm cells were challenged with MC38-OVA colon adenocarcinoma, CD69$^+$CD103$^+$ OVA-specific CD8$^+$ T cells were found in the skin proximal to rejected MC38-OVA tumors 45 days after inoculation (**Box 1**) (12). However, whether the conversion of Tcm into Trm occurs directly or is mediated by Tcm conversion into effector or Tem needs to be further studied.

DCs DRIVE Trm CELL PRIMING AND REACTIVATION

While most of the studies in Trm generation and development have focused on differentiation and maintenance dependent on specific tissue-derived signals, priming of committed precursors in the secondary lymphoid organs has been less explored. The analysis of mice deficient in DNGR-1 or Batf3 (75, 76) has shown the relevance of cDC1 in priming of CD8$^+$ T cell memory subsets. High expression of DNGR-1 is restricted to the cDC1 subset, where DNGR-1 plays an essential role in cross-presentation to VACV antigens (77, 78). In addition, the cDC1 subset depends on the Batf3 transcription factor for their development and/or function (76, 79). Deficient cross-presentation by cDC1 results in a threefold reduction in the numbers of Trm cells in a model of skin VACV infection, while circulating memory CD8$^+$ T cells are not affected (14) (**Figure 3A**). The cDC1 subset provides the antigen for priming by cross-presentation in this context of infection, but also provide specific signals 2 (CD24) and 3 (IL-12 and

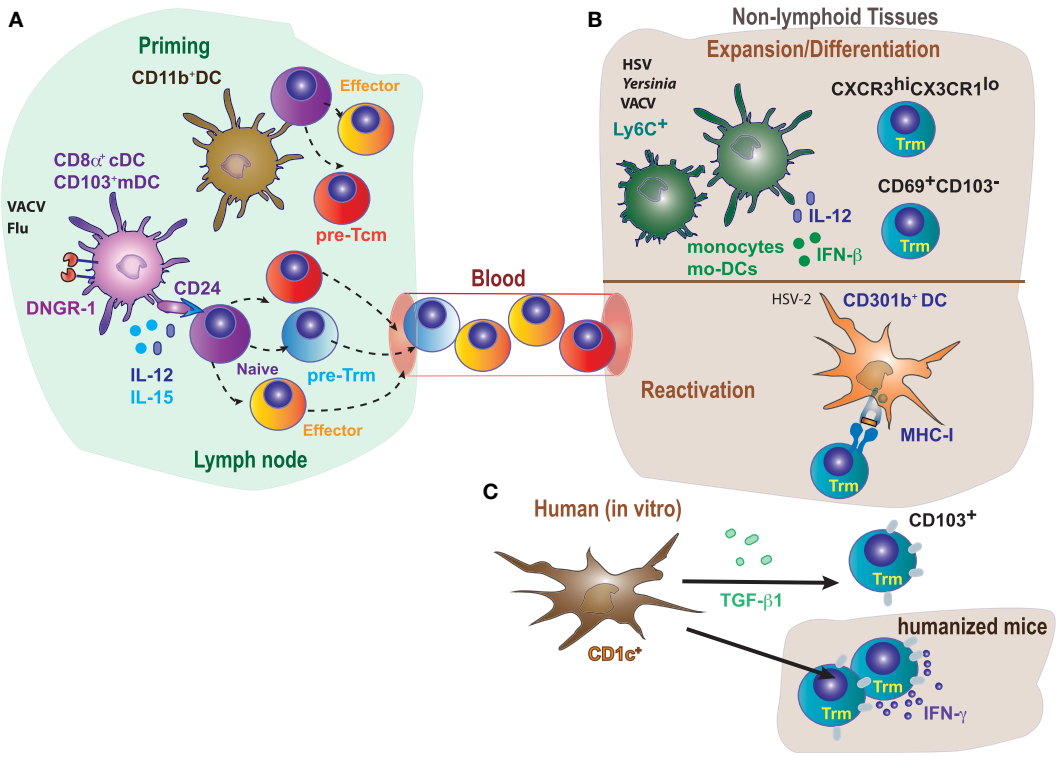

FIGURE 3 | Differential role of antigen-presenting cells in priming, expansion/differentiation, and reactivation of tissue-resident memory T cells. **(A)** In mice, optimal generation of Trm in response to VACV or Flu requires CD8$^+$ T cells cross-priming by DNGR-1$^+$ dendritic cells (cDC1, CD8α$^+$, and CD103$^+$), while circulating memory T cells could be primed by both CD11b^{hi} (cDC2) or cDC1. Naive T cells cross-primed by cDC1 receive CD24 co-stimulation, IL-15 and IL-12 specifically produced by this dendritic cell (DC) subset, contributing to the generation of committed Trm precursors. **(B)** Monocytes (Ly6C$^+$) and monocyte-derived DC (Mo-DCs) contribute to expansion of Trm in response to HSV or *Yersinia* by secretion of inflammatory cytokines, and also modulate the generation/differentiation of specific Trm subpopulations (CXCR3^{hi}CX3CR1^{lo}; CD69$^+$CD103$^-$). In addition, reactivation of Trm cells in response to HSV-2, requires MHC-I expression in CD301b$^+$ DC. **(C)** Notably, human CD1c$^+$, but not CD141$^+$, induce CD103 expression on CD8$^+$ T cells and their accumulation in the lung, in a process dependent on TGF-β1.

IL-15) (47, 80–83). These specific priming signals from cDC1s are also essential for optimal priming of Trm precursors (14), suggesting that priming by cDC1s is key for optimal Trm cell priming in this context of VACV infection, and cross-priming is the operational manner in which the antigen is presented in this setting. The key role of cDC1 for priming of Trm cells could be extended to additional infection models: for example, targeting malaria antigen to DNGR-1-expressing cDC1s in the presence of adjuvant generates Trm cells in the liver upon trapping primed T cells with a recombinant adeno-associated virus that targets hepatocytes to express the same malaria antigen (84).

Following viral infection, cross-priming transiently induces T-bet and its target CXCR3 in $CD8^+$ T lymphocytes in the dLN, correlating with the generation of Trm precursors (14). T-bet induction at priming may contribute to longer retention in the LN of T cells that eventually egress to the tissue with low expression of T-bet and KLRG1. Consistent with the notion that high expression of T-bet inhibits Trm differentiation in the skin (36, 85), cross-priming ultimately favors T cells with delayed egress and lower expression of T-bet and KLRG1 in the skin (14). In addition, cross-priming transiently phosphorylates Foxo1 in $CD8^+$ T cells (14), resulting in its degradation that favors retention of $CD8^+$ T cells in the LN. However, cross-priming deficiency does not affect expression of the transcription factor Eomes, involved in Tcm generation (44, 58). Thus, this early transcriptional regulation by cross-priming does not affect effector or circulating memory $CD8^+$ T cell development, IFN-γ production, or viral clearance mediated by $CD8^+$ T cells. However, the analysis of $CD103^+$ Trm cell differentiation in the skin revealed that formation of $CD103^+CD8^+$ T cells was slower between 7 and 14 days in the absence of cross-priming, suggesting a lower number of Trm cell precursors seeding the skin. Impaired Trm but not Tcm cell generation in vaccinated DNGR-1-deficient mice results in defective viral clearance (14).

Cross-priming through cDC1 also results in more prolonged downregulation of KLF2 and S1P (14). Downregulation of the KLF2-dependent S1P receptor leads to retention during priming (86). Weak priming in the absence of cross-presentation by cDC1s leads to early upregulation of KLF2 and S1P, leading to early egress of $KLRG1^+$ cells that are not Trm precursors (4, 14) and migrate to the skin to generate terminal effector $CD8^+$ T cells (58, 87). Once in the skin, inflammatory signals downregulate again KLF2 and S1P contributing to retention (23). Consistently, the inhibition of T cell egress with FTY720 treatment increases generation of both circulating memory and Trm cells in WT mice, partially rescuing the defect in Trm cell generation in mice deficient in cross-priming by cDC1s (14). These data highlight that retention of $CD8^+$ T cells during priming in the LN favors Trm cell generation. However, it is not sufficient to compensate the specific signals provided by *Batf3*-dependent $DNGR\text{-}1^+$ DCs. *In vitro* co-culture of $CD8^+$ T cell with different DC subsets shows that $CD103^+$ and $CD8\alpha^+$ DC (cDC1s) but not $CD11b^{hi}$ $CD8\alpha^-$ (cDC2s) induce generation of Trm cells, in a DNGR-1-dependent fashion. The blockade of specific priming signals provided by cDC1s such as CD24, IL-12, and IL-15 reduces T-bet induction and generation of Trm precursors; however, cDC1 priming blockade does not affect the generation of circulating memory T cells (14).

It is debated to which extent antigen presentation (signal 1), co-stimulation (signal 2), or cytokines (signal 3) derived from different DC subsets are required for differentiation and for reactivation upon rechallenge. The requirement of antigen for Trm cell differentiation in tissues has been described (3, 14, 66, 67). Antigen recognition within the tissue drives expression of CD103 by brain Trm cells (8). The restimulation of Trm cells and induction of IFN-γ is dependent on MHC-I expression on $CD301b^+$ DC (**Figure 3B**), while inflammatory cytokines alone are likely not sufficient by themselves for full activation of Trm cells responding to genital HSV-2 infection (88). However, antigen presentation is dispensable for Trm generation in other systems (4, 9, 89), supporting the notion that the particular pathogen or inflammatory insult triggers a distinct response that determines the requirements for Trm differentiation (40). Inflammatory signals derived from myeloid cells can also impact in the Trm cell phenotype (**Figure 3B**). Recruitment of monocyte-derived DCs in the LNs is required for the activation of HSV-specific $CD8^+$ Trm cells (66). $Ly6C^+$ inflammatory monocytes contribute to the persistence, but not generation, of lung memory $CD8^+$ Trm cells, affecting selectively to a $CXCR3^{hi}CX3CR1^{lo}$ subset upon VACV intranasal challenge (90). Moreover, IFN-β and IL-12 derived from monocyte-derived intestinal macrophages during *Yersinia* infection, favors the differentiation of $CD69^+CD103^-$ Trm cells (**Figure 3B**) (62).

While cDC1s are essential for optimal priming, they are dispensable for differentiation in the skin, which also requires antigen presentation in the VACV infection model (14). Thus, different DC subsets may work cooperatively in the LN priming of Trm precursors and differentiation in the skin in an antigen-cognate fashion. However, the requirement of antigen presentation by different DC subsets may be model dependent. $XCR1^+$ cDC1 seem to be necessary to promote recall of circulating memory $CD8^+$ T cells upon secondary infections with pathogens such as *L. monocytogenes* or certain viruses (91), or in response to tumors (12). But this particular DC subset does not seem to play a role in the maintenance of Trm cells upon viral infection (14). In a mouse model of HSV-2 intravaginal infection, depletion of $CD301b^+$ cDC2 results in significantly worse clinical symptoms, higher weight loss, and mortality after viral rechallenge (88). However, $CD301b^+$ cDC2 depletion does not affect circulating memory, while stimulates the differentiation and antiviral function of vaginal $CD8^+$ Trm cells (**Figure 3B**). Accordingly, $CD301b^+$ cDC2 depletion has minimal impact on disease severity and weight loss when protection is exclusively dependent on circulating memory $CD8^+$ T cells (88).

In comparison to murine DCs, less is known about the function of human DCs in tissues. By using lung tissues from humans and humanized mice, it has been found that both lung DC subsets ($CD1c^+$ and $CD141^+$) acquire antigens from live-attenuated influenza virus *in vivo* and expanded specific cytotoxic $CD8^+$ T cells *in vitro* (**Figure 3C**). However, lung tissue-resident $CD1c^+$ DC but not $CD141^+$ DC induce CD103 expression on $CD8^+$ T cells and promoted $CD8^+$ T cell accumulation in lung. Induction of CD103 expression mediated by $CD1c^+$ DCs was dependent on TGF-β1. Thus, $CD1c^+$ and $CD141^+$ DCs generate $CD8^+$ T cells with different properties (92). The results discussed

above are consistent with the notion of division of tasks among DC subsets during the priming and differentiation of Trm cells, although the particular role of a DC subset or even the dependence on antigen presentation or priming by DC-derived cytokines may depend on the particular settings in which Trm cells are generated.

CONCLUDING REMARKS

Following immunization, DCs in the secondary lymphoid organs prime $CD8^+$ T cells for generation of effector and memory responses, but there are different flavors of memory T cells and the specific requirements for priming, differentiation, and reactivation of each subset are different. Tissue-resident memory $CD8^+$ T (Trm) cells represent the newest layer of complexity in memory subsets. By virtue of their location, they act as sensor and effector cells, triggering both innate and adaptive responses, therefore providing a superior immunity against reinfection in the tissue (3, 4, 93).

Current evidences support the idea that Tcm, Tem, and Trm cells are generated from common precursors that are committed upon differential priming in secondary lymphoid organs (14, 40). Asymmetric inheritance of intracellular fate determinants could explain generation of effector and memory T cells from clonal naive T cells (52). TCR affinity and duration of signals during priming can also determine the T cell fate, with Trm cells linked to high-affinity TCR and Tcm cells to low affinity (49, 94).

Naive T cells differentiate into Trm in many scenarios: infectious and even non-infectious, such as chemical hapten inflammation (3, 12, 40, 66, 67). However, independently of the generation of Trm cells from naive cells primed in the LN, there is some degree of plasticity among T cell subsets. Trm cells can be generated from antigen-experienced cells such as effector $CD8^+$ T cells, Tcm, Tem, or even Trm cells (self-maintenance). Several factors may condition the relative efficiency of Trm generation from difference sources, including the type of challenge (infection, inflammation), the presence of specific antigen driving reactivation and tissue-specific signals that can promote Trm generation in an antigen-independent fashion (12, 70, 71, 73).

Different subsets of DCs may affect differentially the priming of Trm precursors. cDC1s drive priming of Trm precursors in the LN, but not Trm tissue differentiation, in a VACV skin infection model, and targeting malaria antigen to cDC1s generates antigen-specific Trm in the liver, requiring both models antigen presence in the target tissue (14, 84). Antigen presentation and inflammatory cytokines produced by other myeloid cell subsets contribute to Trm differentiation (62, 88, 90, 92), suggesting a division of tasks among DC subsets in the priming and differentiation of memory T cell subsets that can be model dependent. Further dissection of how DC prime and generate different memory T cell subsets, what are the requirements for differentiation and effector function of each subset, and how these memory T cell subsets act in concert to induce optimal immunity will be important to improve current immunotherapy strategies against pathogens or cancer.

AUTHOR CONTRIBUTIONS

ME, SK, SI, and DS conceived and wrote the manuscript. ME and SI did the figures.

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REVIEW
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Niches for the Long-Term Maintenance of Tissue-Resident Memory T Cells

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Tissue-resident memory T cells (T_{RM} cells) are a population of immune cells that reside in the lymphoid and non-lymphoid organs without recirculation through the blood. These important cells occupy and utilize unique anatomical and physiological niches that are distinct from those for other memory T cell populations, such as central memory T cells in the secondary lymphoid organs and effector memory T cells that circulate through the tissues. $CD8^+$ T_{RM} cells typically localize in the epithelial layers of barrier tissues where they are optimally positioned to act as sentinels to trigger antigen-specific protection against reinfection. $CD4^+$ T_{RM} cells typically localize below the epithelial layers, such as below the basement membrane, and cluster in lymphoid structures designed to optimize interactions with antigen-presenting cells upon reinfection. A key feature of T_{RM} populations is their ability to be maintained in barrier tissues for prolonged periods of time. For example, skin $CD8^+$ T_{RM} cells displace epidermal niches originally occupied by γδ T cells, thereby enabling their stable persistence for years. It is also clear that the long-term maintenance of T_{RM} cells in different microenvironments is dependent on multiple tissue-specific survival cues, although the specific details are poorly understood. However, not all T_{RM} persist over the long term. Recently, we identified a new spatial niche for the maintenance of $CD8^+$ T_{RM} cells in the lung, which is created at the site of tissue regeneration after injury [termed repair-associated memory depots (RAMD)]. The short-lived nature of RAMD potentially explains the short lifespans of $CD8^+$ T_{RM} cells in this particular tissue. Clearly, a better understanding of the niche-dependent maintenance of T_{RM} cells will be important for the development of vaccines designed to promote barrier immunity. In this review, we discuss recent advances in our understanding of the properties and nature of tissue-specific niches that maintain T_{RM} cells in different tissues.

Keywords: distribution of memory T cells, maintenance of memory T cells, mucosal immunity, infectious immunity, vaccine

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INTRODUCTION

When naïve T cells encounter cognate antigen in the draining lymph node (LN), the cells are activated, initiate a proliferative program, and differentiate into a heterogeneous population of effector T cells. These effector T cells then home back to the site of infection and eliminate pathogen-infected cells. While most effector cells die after clearance of the pathogens, some cells subsequently differentiate into memory T cells. During the course of a T cell response, each T cell

receives spatially and temporally distinct instructive signals that impact their ultimate fate; either death or differentiation into different types of memory cells with distinct functional and migratory properties (1, 2). For example, T cells primed by antigen-presenting cells (APC) with weak stimulatory potential preferentially remain in the LN and differentiate into central memory T cells (T_{CM} cells) where they survey lymph and blood (3, 4). On the other hand, T cells primed by APC with high stimulatory potential (e.g., strong costimulation) differentiate into potent effector cells that migrate to inflamed tissues and subsequently die (3). Effector cells that additionally receive tissue-specific instructive signaling differentiate into tissue-resident memory T cells (T_{RM} cells) and establish permanent residency within the tissues (1, 5). Effector T cells that fail to receive optimal tissue-instructive signals may differentiate into effector memory T cells (T_{EM} cells) that circulate between blood and certain peripheral tissues.

It is now appreciated that T_{RM} cells comprise the majority of memory T cells in the non-lymphoid tissues (NLT) and confer immediate protection against infection of barrier tissues (6). These cells are part of a comprehensive memory response that also include the T_{CM} and T_{EM} populations. T_{CM} cells exhibit high proliferative potential upon reactivation in the LN, thereby providing a major source of secondary effector cells that ultimately facilitate pathogen clearance (7). T_{EM} cells play a supportive role to T_{RM} by virtue of their immediate effector functions and their ability to rapidly traffic sites of infection (8). While the maintenance of circulatory memory T cell populations (T_{CM} and T_{EM}) has been shown to depend on the homeostatic cytokines IL-7 and IL-15, the factors that regulate the maintenance of T_{RM} cells are ill defined. Furthermore, since T_{RM} cells in each tissue are maintained in distinct microenvironments, these cells must adapt to local cues for their long-term survival.

The external or internal surfaces of the body such as the skin and the mucosal linings of the gastrointestinal, respiratory, and urogenital tracts are a major gateway for infectious pathogens to access to the body. The surfaces of these barrier tissues are covered by different types of epithelial layers: from single layers of flattened or columnar cells to multiple layers of different types of epithelial cells. Each of these epithelial layers, along with the connective tissues that underlie the epithelium in each tissue, provide distinct microenvironments depending on their particular physiological and functional needs. The different types of immune cells that reside in these distinct microenvironments, such as macrophages, dendritic cells (DC), γδ T cells, and innate lymphoid cells (ILC), each adapt to these unique environments and play important roles in maintaining the integrity of these epithelial barriers (9–12). Accumulating evidence has revealed that the relationship between T_{RM} cells in these tissues and the original resident cell populations is dynamic and complex. For example, some tissue-resident immune cells interact with T_{RM} cells and provide niche factors for their maintenance (13–15). In other cases, tissue-resident immune cells and T_{RM} cells share local signals necessary for their long-term survival or compete with one another for access to niches that enable them to persist in the tissue (16). Furthermore, it is becoming clear that T_{RM} cells are also established in non-barrier tissues (such as the brain, liver, and kidney) as well as the primary lymphoid organs and secondary lymphoid organs (SLOs) and protect tissues from infectious pathogens disseminated by hematogenous or cellular (e.g., neural) pathways (17). The niches and factors that enable the maintenance of T_{RM} cells in these tissues differ significantly from those in the epithelial tissues. In this review, we discuss the distribution of T_{RM} cells in each tissue and the factors that influence the establishment and maintenance of T_{RM} cells.

NON-LYMPHOID ORGANS

Barrier Tissues

Skin

The skin is comprised of three main layers: the epidermis, dermis, and subcutaneous fatty region. The epidermis and dermis are separated by a basement membrane and harbor numerous unique populations of innate and adaptive immune cells. Many of these cells are resident populations and form a sophisticated immune network that provides a biological barrier against invading pathogens (18).

The epidermis is an avascular tissue composed primarily of keratinocytes (19). Dead keratinocytes comprise the outmost layer of the epidermis, known as the stratum corneum, and serve as a physiological barrier (20). Keratinocytes in the deeper layers, such as the stratum granulosum and stratum spinosum, provide integrity to the skin and play multiple roles in the initiation of local immunity by recognizing pathogens through pattern recognition receptors and by secreting a wide variety of cytokines and chemokines (21). These cells also secrete various factors necessary for the development and homeostasis of immune cells residing in the epidermis (21). The bottom layer, the stratum basale, consists primarily of a single layer of basal cells—precursors of the keratinocytes that comprise the upper layers of the skin (22). The hair follicles also consist of keratinocytes and provide unique niches for immune cells including T_{RM} cells (23).

At least three immune cell types are maintained in the epidermis: Langerhans cells (LC), dendritic epidermal T cells (DETC) expressing γδ T cell receptors (TCR), and memory T cells expressing αβ TCR. These cells do not recirculate under steady-state conditions, exhibit a dendritic morphology, and inhabit several anatomical as well as physiological niches for their development and maintenance (20).

Langerhans cells are present in all layers of the epidermis, especially in the stratum spinosum, and are the only APC in the epidermis under steady-state conditions (24). The development and maturation of LC depends on transforming growth factor-β (TGF-β), which is secreted by keratinocytes, DETC (paracrine), and the LC themselves (autocrine) (24). Although TGF-β1 is secreted as a latent (inactive) form, it is trans-activated by integrin $\alpha_v\beta_6$ and $\alpha_v\beta_8$ expressed on keratinocytes in the interfollicular regions and near the hair follicles (25, 26). TGF-β has also been shown to be required for the retention of LC within the epidermis since the loss of TGF-β1 signaling leads to the spontaneous migration of LC to the regional LN (25). In addition to initiating adaptive immune responses, LC are also involved in the induction

of tolerance by promoting the proliferation of regulatory T (Treg) cells in the epidermis under steady-state conditions (27).

In mice, DETC comprise a large proportion of immune cells in the epidermis (20). DETC are distributed throughout the epidermis, secrete a variety of cytokines, chemokines, and growth factors, and play key roles in the wound repair, tumor surveillance, and inflammation (28). They persist in the epidermis for life and are maintained by homeostatic turnover. Common γ-chain signaling through IL-7 and IL-15, as well as signaling *via* the aryl hydrocarbon receptor (AhR) are known to be required for the development and maintenance of DETC (29–32). This is consistent with the fact that AhR ligands are abundant in the skin since they are formed from tryptophan *via* ultraviolet radiation (33). In contrast to LC, the maintenance of DETC is independent of TGF-β (34).

The majority of αβ T cells that reside in the epidermis are $CD8^+$ T_{RM} cells (35) (**Figure 1**). These cells express canonical T_{RM} makers such as the activation marker CD69, the E-cadherin-binding integrin CD103, and the collagen-binding integrin CD49a, in the absence of cognate antigen signaling (36, 37). Although $CD8^+$ T_{RM} cells are widely found throughout the body (38), their numbers are generally elevated at sites of infection and/or inflammation (37, 39, 40). Several chemokines are known to be involved in the recruitment of $CD8^+$ T_{RM} precursors ($KLRG1^{lo}$) into the epidermis, including cutaneous T cell-attracting chemokine (CTACK), CXCL9 and CXCL10. CTACK is constitutively expressed by epidermal keratinocytes and attracts CCR10 expressing T cells (41). Since memory T cells do not express CCR10, it is likely that CTACK primarily drives the recruitment of effector T cells to the epidermis, but not the retention of memory T cells at that site (42). Other inflammatory chemokines, such as CXCL9 and CXCL10, are highly expressed by keratinocytes in response to infection, and facilitate the recruitment of $CXCR3^+$ memory precursor effector $CD8^+$ T cells to the epidermis (43). Like LC, these cells subsequently receive TGF-β signals upon arrival, which is a critical factor for the upregulation of the E-cadherin binding integrin, CD103 (43) (**Figure 1**). Since E-cadherin is expressed on epithelial cells, including keratinocytes, it is likely that the upregulation of CD103 facilitates the retention of T cells in the epidermis (44). TGF-β signaling also downregulates the T-box family protein T-bet and eomesodermin, a process of which facilitates T_{RM} cell development (45). CCR8 expression is also upregulated following the migration of T cells into the epidermis by yet unidentified factors derived from keratinocytes. It appears likely that this chemokine receptor also facilitates the maintenance of cells within the epidermis (46, 47). Finally, there may also be a role for CXCR6 in the maintenance of T_{RM}

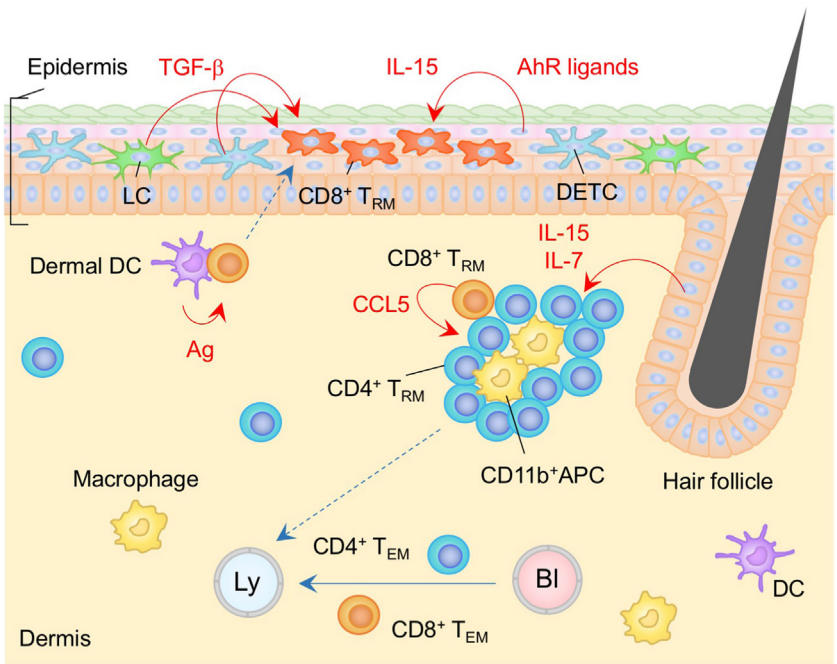

FIGURE 1 | T_{RM} niches in the skin. Langerhans cells (LC), dendritic epidermal T cells (DETC) expressing γδ T cell receptors, and $CD8^+$ T_{RM} cells are maintained in the epidermis. $CD8^+$ T_{RM} cells displace epidermal niches originally occupied by DETC at the site of infection. Transforming growth factor (TGF)-β secreted from LC and DETC, IL-15, and aryl hydrocarbon receptor (AhR) ligands play a role in the generation and maintenance of epidermal $CD8^+$ T_{RM} cells. Memory $CD4^+$ T cells in the dermis form clusters with $CD11b^+$ APC around the hair follicles. CCL5 secreted from peri-collicular $CD8^+$ T cells promotes formation of clusters. Although most memory $CD4^+$ T cells in the cluster exhibit canonical T_{RM} phenotypes, long-period parabiosis experiments revealed that this population is slowly replenished by cells from the circulation. IL-7 and IL-15 secreted from keratinocytes in the hair follicles promote T cell persistence in the cluster. T_{EM} cells are passing through the dermis. Orange and blue cells indicate $CD8^+$ and $CD4^+$ T_{RM} cells, respectively, unless otherwise stated. Red lines indicate the representative niche factors that influence the maintenance of T_{RM} cells. Blue lines indicate the migratory routes. Dashed lines indicate potential impact of niche factors (red) or migratory routes (blue). Abbreviations: Ly, lymph vessel; Bl, blood vessel; Ag, antigen; APC, antigen-presenting cell; T_{RM}, tissue-resident memory T cells; T_{EM}, effector memory T cells.

in the epidermis since its absence results in a marked reduction in the number of skin $CD8^+$ T_{RM} (42).

$CD8^+$ T_{RM} cells in the epidermis display a unique dendritic morphology (16, 35, 48), which is distinct from that of LC and DETC (20, 48). Epidermal $CD8^+$ T_{RM} cells are located in the basal layers of the epidermis and slowly but continuously migrate between keratinocytes, while LC and DETC are mostly immotile (16, 48). Importantly, Zaid et al. have demonstrated a substantial decrease in the numbers of DETC and a concomitant increase in the numbers of $CD8^+$ T_{RM} cells at the site of infection, indicating the strict competition between DETC and $CD8^+$ T_{RM} cells for the epidermal niches (16) (**Figure 1**). Furthermore, both of these populations also depend on locally produced homeostatic signals, such as IL-15 and AhR ligands, for their long-term maintenance (16, 30, 32, 43). These common features may explain the stable persistence of $CD8^+$ T_{RM} cells within the epidermal niches for many years without repopulation by DETC (16). Furthermore, the relatively higher numbers of αβ T cells, as compared to γδ T cells, in the human epidermis might be a consequence of the persistent occupation of epidermal niches by $CD8^+$ T_{RM} cells generated by prior infection and/or inflammation (20). It is important to note here that the capacity of epidermal T_{RM} niches are extremely large (approximately 7×10^3 T cells/cm^2) (49). The high capacity of epidermal niches allows the *de novo* establishment of T_{RM} cells with different specificities without displacement of pre-existing T_{RM} cells after rechallenge. Importantly, this allows T_{RM} cells with multiple specificities to be stably maintained in the epidermis (49). By contrast, γδ T cells are displaced by $CD8^+$ T_{RM} cells even when the number of T_{RM} cells relatively low, suggesting an occupational advantage for $CD8^+$ T_{RM} cells over γδ T cells in the epidermal niches. Finally, since the environment in which epidermal $CD8^+$ T_{RM} cells persist has limited access to blood-derived signals as well as nutrients, these cells uniquely express fatty acid transporters, Fabp4 and Fabp5, and rely on extracellular fatty acid for their survival (50).

The dermis that underlies the basement membrane is composed mainly of fibroblasts and the extracellular matrix (a network of collagen and elastin fibers). Heterogeneous populations of immune cells, including αβ T cells, γδ T cells, subsets of DC, macrophages, mast cells, and ILC are all found in the dermis (21). The dermis also contains both lymphatic and blood vessels, providing a source of T_{EM} cells that are transiting through the tissues.

In contrast to the situation in the epidermis, most αβ T cells located in the dermis are $CD4^+$ T cells, including both conventional T cells and Treg (14, 35, 51, 52). These cells display an amoeboid morphology and traffic rapidly through the dermis (35). Long-period parabiosis experiments (12–16 weeks) using naïve animals has revealed that a large fraction of $CD4^+$ T cells recruited from the circulation acquire the expression of CD69 and CD103 following entry into the skin (14). Of note, T_{RM}-phenotype $CD4^+$ T cells in the dermis are tissue-circulating T_{EM} cells despite their relatively slow turnover rate, as the ratio of host and partner $CD4^+$ T cells was equilibrated in these parabiosis experiments (14). These $CD4^+$ T cells form clusters with $CD11b^+$ APC around hair follicles (14) (**Figure 1**). The numbers of hair follicle-associated clusters, as well as the numbers of $CD4^+$ T cells within each cluster, are increased following local infection and/or inflammation, indicating that tissue conditioning creates new dermal $CD4^+$ T cell niches (14). CCL5 secreted from peri-follicular $CD8^+$ T cells promotes the formation of the $CD4^+$ T cell clusters (14). In addition, IL-7 and IL-15 are predominantly secreted by unique population of keratinocytes in the hair follicles, helping to sustain T cell persistence within the cluster (23). Such unique structures are potentially identical to the classical inducible skin-associated lymphoid tissues that provide both spatial and physiological niches for the maintenance of memory T cells (53).

Although local tissue instructions promote the formation of T_{RM} in the absence of local antigen (37), recent studies have revealed that encounters with cognate antigen at the site of infection significantly enhance the establishment of $CD8^+$ T_{RM} cells in the skin, presumably in the epidermis (54). While several cell-intrinsic mechanisms of T_{RM} formation induced by an antigen-driven "second hit" are suggested (5), one certain outcome is the upregulation of CD69 (54). It has been established that T cells recruited to peripheral tissues upregulate sphingoshin-1-phosphate receptor 1 ($S1P_1$), and sense the gradient of sphingoshin-1-phosphate (S1P) (55), which guides T cells to the draining lymphatics of the tissue. Surface expression of CD69 antagonizes the expression of $S1P_1$ (56), thereby inhibiting the egress of T cells from the skin (57). Since lymphatic vessels are not found in the epidermis, it is likely that the second antigen hit and the resultant retention induced by CD69-mediated inhibition of $S1P_1$ occurs in the dermis, and subsequently promotes the establishment of $CD8^+$ T_{RM} in the epidermis. In support of this concept, APC in the skin function as a gatekeeper for the development of $CD8^+$ T_{RM} cells, such that $CD8^+$ T cells with distinct antigen specificities compete for APC as a source of second hit signaling, leading to the selection of dominant epitope-specific $CD8^+$ T cells (58). This leads to the reduced formation of $CD8^+$ T_{RM} cells specific for subdominant epitopes since these T cells presumably fail to receive second antigen hit signaling and rapidly egress from the dermis. Such antigenic selection may be the underlying mechanism driving the accumulation of highly functional, melanocyte antigen-specific $CD8^+$ T_{RM} cells in the vitiligo-affected skin (59, 60). It is important to note that transcriptional downregulation of *Klf2*, as well as its downstream target *S1pr1* (which encodes $S1P_1$), is also induced by several cytokines such as TGF-β, IL-33, and tumor necrosis factor (TNF), even in the absence of local antigen (61). However, certain factors that enable the acquisition of a unique transcription profile defining T_{RM} cells, including the upregulation of *Hobit* and *Blimp1*, have not been not fully elucidated (62, 63).

Gut, Intestine

The intestinal mucosa consists of a single layer of intestinal epithelial cells that overlies the lamina propria (LP), a thin layer of loose connective tissue. The epithelium and LP are separated by a basement membrane and each provides a distinct immunological niche for the maintenance of T_{RM} cells.

The diverse populations of immune cells embedded within the intestinal epithelium are referred to as intestinal intraepithelial

lymphocytes (IELs). The greatest concentration of IEL is located in the small intestine (SI) where there are approximately 10–15 IEL per 100 epithelial cells. This ratio of IEL to epithelial cells gradually decreases along the intestines, such that the colon hosts relatively few IEL (64). The differences in the relative numbers of IEL in each intestinal compartment likely reflects regional differences in the anatomy of the villi, the intestinal microenvironment (including microbiota), and the composition of epithelial cells (e.g., enterocytes, goblet cells, Paneth cells, enteroendocrine cells, and stem cells). Epithelial cells are a dynamic population and cells situated at the top of the villi typically die within 3–5 days and are continually replaced by new cells generated from the progenitor cells located in the crypt. Despite the short lifespan of epithelial cells, IEL are resident and do not recirculate (65).

Intraepithelial lymphocytes in the intestines are primarily T cells, although there is also a small population of cells that are negative for TCR, such as ILC-like cells (66). IEL T cells can be divided into two subsets, referred to as peripheral and thymic. Peripheral IEL (type a, induced or conventional) are derived from antigen-experienced $CD8^+$ or $CD4^+$ T cells that have homed to the epithelium. Thymic IEL (type b, natural or unconventional) express CD8α homodimers with either TCRαβ or TCRγδ, and migrate from the thymus to the epithelium shortly after birth (67). In mice, thymic IEL dominate in the SI while peripheral IEL dominate in the colon (64). The overall ratio of thymic to peripheral IEL declines with age, although the total number of IEL remains relatively constant (67, 68), suggesting that the two types of IEL share the same spatial niche in the epithelium. However, there is a severe reduction in the numbers of peripheral but not thymic IEL in germ-free animals (69), suggesting that the physiological niches that maintain peripheral and thymic IEL must differ in some way. This review will focus on peripheral IEL.

Significant numbers of antigen-specific T_{RM} cells are established in the intraepithelial compartment following intestinal infections (70–72) (**Figure 2**). The majority of these cells are $CD8^+$ T cells,

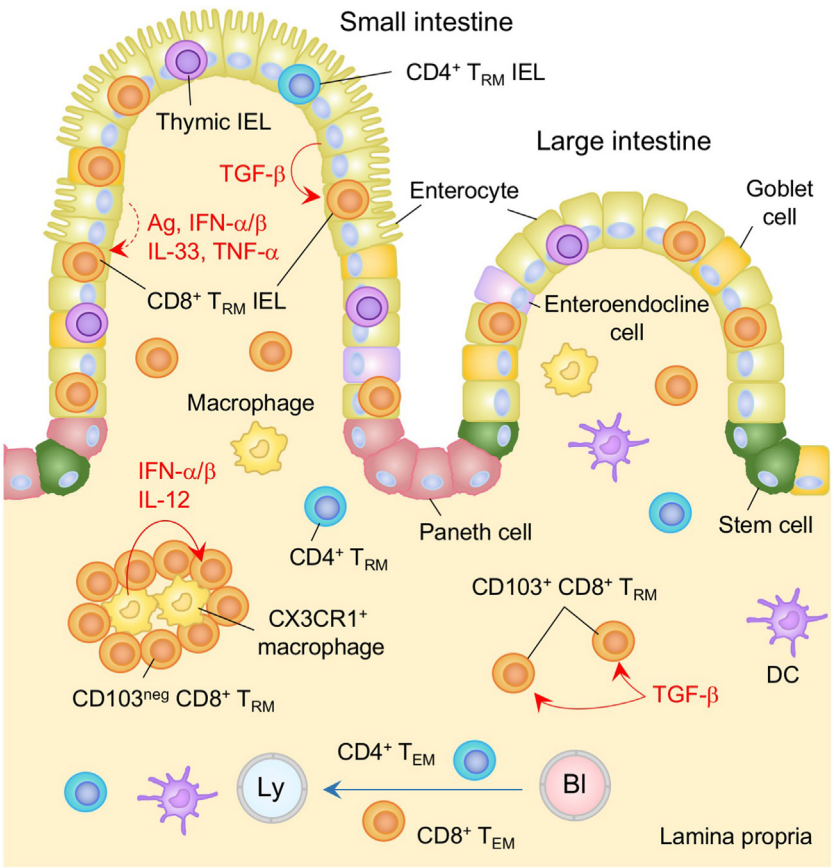

FIGURE 2 | T_{RM} niches in the in the intestine. Large numbers of $CD8^+$ T_{RM} cells and few numbers of $CD4^+$ T_{RM} cells are present in the intestinal intraepithelial lymphocyte (IEL) compartment. TGF-β is constitutively available in the intestinal epithelium and promotes the generation of T_{RM} cells in this compartment by upregulating CD103 as well as Runx3. Either cognate antigen or inflammatory cytokines is required for upregulation of CD69 on epithelial T_{RM} cells. Both TGF-β-dependent ($CD103^+$) and independent ($CD103^-$) populations of $CD8^+$ T_{RM} cells present in the lamina propria (LP). The latter form cluster with $CX3CR1^+$ macrophages. Interferon (IFN)-α/β and IL-12 secreted by macrophages control the size of the cluster. T_{EM} cells are passing through the LP. Orange and blue cells indicate $CD8^+$ and $CD4^+$ T_{RM} cells, respectively, unless otherwise stated. Red lines indicate the representative niche factors that influence the maintenance of T_{RM} cells. A blue line indicates the migratory route. A dashed line indicates potential impact of niche factors. Abbreviations: Ly, lymph vessel; Bl, blood vessel; Ag, antigen; neg, negative; T_{RM}, tissue-resident memory T cells; T_{EM}, effector memory T cells.

although smaller numbers of $CD4^+$ T cells are also observed (73). Interestingly, a large number of memory-like γδ T cells is also generated following intestinal infection. However, these cells are rarely found in the IEL compartment, suggesting that $CD8^+$ T_{RM} cells but not γδ T cells are preferentially lodged in the intraepithelial niches (74). Nearly all $CD8^+$ T_{RM} cells in this compartment express CD69 and CD103 (70, 71) and are scattered within the epithelium. Recruitment of effector cells to this site, including T_{RM} precursors, is governed by the α4β7 and CCR9 integrins, both of which are upregulated on T cells, mainly in response to retinoic acid (a vitamin A metabolite) which is present during priming in the intestinal inductive sites (75). The α4β7 integrin facilitates the extravasation of the cells from the venules in the LP (76, 77). CCR9 is required for T cell migration to the SI (78, 79), since its ligand, CCL25, is constitutively expressed by epithelial cells in the SI but not the colon (80).

As with thymic IEL, a process of tissue adaptation takes place following recruitment of peripheral $CD8^+$ T cells into the epithelium. Specifically, the local environment promotes the differentiation of effector T cells into T_{RM} and facilitates their subsequent retention at that site. In this regard, TGF-β, which is constitutively available at the intestinal epithelium (81, 82) (**Figure 2**), induces the upregulation of CD103 on recent immigrants. Consistent with this, the lack of CD103 or the TGF-β receptor on T cells is correlated with a significant defect in the accumulation of both peripheral and thymic IEL within the intestinal epithelium (71, 83–85). By contrast, overexpression of TGF-β results in increased proportion of thymic IEL in the SI (86), highlighting the non-redundant, regulatory role of TGF-β in the number of T_{RM} cells retained in the intestinal epithelium. TGF-β signaling also induces the expression of Runx3 (87–89), which has been identified as a master regulator of tissue residency (90). Although the precise role of Runx3 in retaining cells in the SI is not yet clear, it is known to promote the expression of CD8αα (88), which binds to the thymus leukemia antigen that is constitutively expressed on the intestinal epithelium (91). Interestingly, TGF-β-independent populations of T_{RM} cells also accumulate in the IEL compartment during chronic infection with lymphocytic choriomeningitis virus (LCMV) (85). These cells do not express CD103 and are thought to represent recent arrivals that are recruited continually from the circulation upon activation with persistent viral antigens (85).

While $CD8^+$ T_{RM} IEL are associated with gut infection, they are also established following systemic infections (6, 83, 85, 90, 92, 93), and their numbers are especially robust under lymphopenic conditions (e.g., $Rag^{-/-}$) (83, 93, 94). IEL generated through systemic immune responses exhibit canonical T_{RM} phenotypes ($CD69^+$ $CD103^+$) despite the absence of TCR signaling (as determined by the lack of Nur77 expression) (83), indicating that cognate antigen is not required for the upregulation of CD69 in the gut. In fact, some cytokines that can be secreted in the epithelium, such as IL-33, interferon-α/β (IFN-α/β), and TNF-α, are known to contribute to the antigen-independent upregulation of CD69 (83). Nevertheless, the number of $CD8^+$ T_{RM} cells established in the intestinal epithelium following systemic priming is significantly less than that generated by gut infection (71). This is largely due to the relatively poor accumulation of memory precursor cells into the intestinal epithelium following non-intestinal infection (71). While significant progress has been made in understanding gut T cell memory, the impact of infection-driven tissue conditioning on the spatial as well as the physiological niches (local antigen and cytokine milieu) on the maintenance of T_{RM} cells in the intestinal epithelium is largely unknown.

The homeostatic cytokine IL-15 is constitutively produced by intestinal epithelial cells in response to signaling through MyD88, suggesting that there is a background level of stimulation by intestinal microflora (95). As with DETC in the skin epidermis, the development and maintenance of thymic IEL depends on local signaling *via* IL-15, as lack of this signaling results in the loss of more than 90% of thymic IEL (96–98). Although it has been proposed that IL-15 produced by inflamed mucosal tissues accelerates the accumulation of circulating effector $CD8^+$ T cells in the SI through the upregulation of the mammalian target of rapamycin and T-bet (93), survival of $CD8^+$ T_{RM} cells in most peripheral tissues, including the SI (both in the epithelial compartment and LP), is independent of IL-15 (99). This suggests that the physiological niches inhabited by peripheral and thymic IEL exhibit different characteristics.

The LP harbors the vast majority of immune cells in the body. These cells are located in organized lymphoid structures, termed gut-associated lymphoid tissues, such as Peyer's patches (PP), cecal patches, colonic patches, cryptopatches, and solitary isolated lymphoid tissues (100). Large numbers of T cells are present throughout the LP. T-cell homing to small intestinal LP is mediated by integrin α4β7 and CCR9, whereas the orphan G-protein-coupled receptor 15 is required for migration of T cells to the large intestinal LP (101). Once in the relevant gut site, T cells receive instructive signals for their full differentiation into T_{RM} cells. Note that a stable population of memory-like γδ T cells is established in the LP, suggesting limited competition of anatomical niches between T_{RM} cells and γδ T cells in this compartment (74).

In contrast to memory T cells in the IEL compartment, memory cells located in the LP include both T_{EM} and T_{RM} (**Figure 2**). This is because the LP contains both lymphatic drainage and blood supplies (65) and suggests that T_{RM} cells in this tissue need to continually resist tissue egress signals for their long-term maintenance. CD69 is expressed on a large proportion of T cells in the LP (13, 65, 70, 71, 73, 83, 85), and plays a key role in antagonizing $S1P_1$-mediated tissue egress. As with the IEL compartment, T cells in the LP express CD69 despite the absence of cognate antigen (83). In support of this, parabiosis experiments have revealed that although partner-derived cells include sizable proportion of $CD69^-$ cells (which represent transients in the LP), nearly 80% of $CD8^+$ T cells recruited from the partner become $CD69^+$ following arrival (65), indicating the influence of constitutively secreted inflammatory cytokines in this tissue (83). However, the ratios of host and partner $CD8^+$ T cells in the LP as well as the epithelium never become fully equilibrated following parabiosis, indicating the limitation of local instructive signaling for the formation of T_{RM} cells in those tissues under steady-state conditions (65).

Following recruitment to the LP, T cells downregulate integrin α4β7, indicating that integrin α4β7 is not required for their retention (83). Instead, a proportion of $CD8^+$ T cells upregulate

CD103 in a TGF-β-dependent manner (70, 71, 83, 85). These cells form a resident population and are scattered throughout the LP (70) (**Figure 2**, shown as CD103$^+$ CD8$^+$ T_{RM}). Interestingly, CD103$^-$ cells are also found to be resident in the LP (these cells are refractory to depletion by a systemically introduced antibody) (70), suggesting the presence of CD103-independent retention signals. These cells form clusters with CX3CR1$^+$ macrophages primarily located under the crypts and the size of this population is independent of TGF-β, but is controlled by type I IFN and IL-12 (13) (**Figure 2**). Since these cytokines are provided mainly by monocyte-derived CCR2$^+$ macrophages that have been recruited in response to local infection, and *Cxcr3*-deficient CD8$^+$ T cells fail to form clusters (13), it is reasonable to conclude that infection-induced tissue conditioning facilitates the development of CD103$^-$ CD8$^+$ T_{RM} population. However, the accumulation of CD103$^-$ CD8$^+$ T_{RM} cells is also evident even in the absence of intestinal infection (83, 85), suggesting the presence of additional niches that sustain CD103$^-$ CD8$^+$ T_{RM} cells in the infection/inflammation-inexperienced LP.

Female Reproductive Tract (FRT)

The mucosal surfaces of FRT can be divided into two types, referred to as type I and type II. The upper FRT, such as endometrium and endocervix, expresses type I mucosal surfaces, which are covered by a single layer of columnar epithelial cells linked by tight junctions. The lower FRT, such as the vagina and ectocervix, expresses type II mucosal surfaces, which are covered by multiple layers of non-keratinized stratified squamous epithelium binding to a basement membrane (102). Mucosa-associated lymphoid tissues (MALT) are found in the stromal layer (lamina propria) and the submucosa of the upper but not the lower FRT (103) (**Figure 3**). Migration of effector, as well as memory, T cells into the mucosa of the FRT is significantly restricted in the absence of local infection and/or inflammation (104). Once recruited, however, T_{RM} cells are formed and maintained in both compartments under the control of local environmental cues.

The endometrium is a highly dynamic tissue in women. It undergoes remarkable cyclical changes of growth, differentiation, and degeneration under the control of the hormones estrogen and progesterone. The spontaneous decidualisation of the endometrial epithelium and stroma, which causes menstruation, and subsequent re-epithelization of endometrium periodically occurs (105), suggesting that limited, if any, anatomical niches are available for the long-term maintenance of T_{RM} cells. Yet, numerous immune cells, including memory T cells, are found along the stroma/submucosa of the upper FRT (106, 107). During the proliferative phase of the menstrual cycle, uterine immune cells become condensed, leading to a formation of lymphoid aggregates (107). These lymphoid aggregates, which are presumably identical to the MALT described above, mainly consist of a B cell core surrounded by memory CD8$^+$ T cells and macrophages (107, 108) (**Figure 3**). The size of the MALT varies with the phase of the menstrual cycle, rising to 3,000–4,000 cells during the secretory phase and declining to 300–400 cells

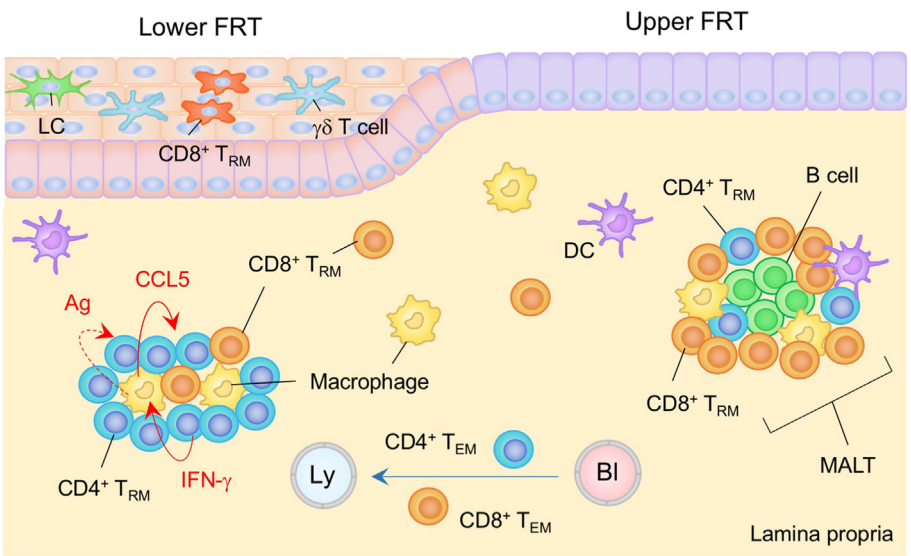

FIGURE 3 | T_{RM} niches in the in the female reproductive tract (FRT). The FRT consist of the upper (endometrium and endocervix) and lower (vagina and ectocervix) reproductive tract. The upper FRT is composed of type I epithelia while the lower FRT is composed of type II epithelia. Mucosa-associated lymphoid tissues (MALT) are found in the lamina propria (LP) of the upper FRT. Both CD4$^+$ and CD8$^+$ T_{RM} cells are involved in the MALT. The size of the MALT is regulated by the phase of the menstrual cycle. In the lower FRT, CD8$^+$ T_{RM} cells present mainly within the epithelial layers. Antigen is not required for the establishment of CD8$^+$ T_{RM} cells in this tissue. CD4$^+$ T_{RM} cells usually form clusters with macrophages in the LP. The structure of the cluster is sustained by a constitutively produced cytokine/chemokine network in which IFN-γ secreted by CD4$^+$ T_{RM} cells drives CCR5 production by macrophages, which attracts and retains CD4$^+$ T_{RM} cells within the cluster. Cognate antigen may be involved in driving CD4$^+$ T cell production of IFN-γ. T_{EM} pass through the LP. Orange and blue cells indicate CD8$^+$ and CD4$^+$ T_{RM} cells, respectively, unless otherwise stated. Red lines indicate the representative niche factors that influence the maintenance of T_{RM} cells. A blue line indicates the migratory route. A dashed line indicates potential impact of niche factor. Abbreviations: Ly, lymph vessel; Bl, blood vessel; Ag, antigen; LC, Langerhans cells; T_{RM}, tissue-resident memory T cells; T_{EM}, effector memory T cells.

during the proliferative phase (109). This implies that there must be endocrine regulation of the T_{RM} niches. It is also known that $CD8^+$ cytotoxic T lymphocyte (CTL) activity is suppressed during the secretory stage, presumably to minimize the recognition and rejection of allogenic sperm and the semi-allogenic fetus (107). Thus, the deployment of memory $CD8^+$ T cells within the MALT in the uterine stroma/submucosa but not epithelial layer is organized to maintain reproductive function.

Recently, intravital imaging of the perimetrium and myometrium of the fallopian tubes has demonstrated the establishment of antigen-specific $CD8^+$ T_{RM} cells in the upper FRT following resolution of virus infection at the uterus (110). The velocity of $CD8^+$ T_{RM} cells in the uterine stroma (~10 μm min^{-1}) is similar to that of $CD8^+$ T_{CM} cells in the LN and is significantly higher than that of $CD8^+$ T_{RM} in the skin epidermis (~2 μm min^{-1}) (35, 110, 111). Since uterine $CD8^+$ T_{RM} cells display poor dendritic morphology, as compared to skin $CD8^+$ T_{RM} cells, and are found in a site where immune cells are present at relatively high density (35, 110, 112), it is likely that the $CD8^+$ T_{RM} cell niches in the upper FRT exist within the MALT in the uterine stroma/submucosa. Furthermore, an experimental *Chlamydia* vaccine that promotes antigen presentation by immunogenic $CD11b^+$ $CD103^-$, but not tolerogenic $CD11b^-$ $CD103^+$ DC, elicits stable $CD4^+$ T_{RM} cell populations in the upper FRT. These cells provide significant protection against subsequent *Chlamydia* infection (113). The integrins α4β1 and α4β7 are involved in the migration of effector $CD4^+$ T cells to this site as blockade of integrin α4 blocks uterine T cell homing during the early phase of infection (113–115). Large numbers of $CD4^+$ T cells are recruited to the uterine stroma/submucosa after local infection with *Chlamydia* (116) and form clusters that also include small numbers of B cells and $CD8^+$ T cells (117, 118). This indicates that $CD4^+$ T_{RM} cells in the upper FRT are also maintained in MALT structures (**Figure 3**). B cells in the cluster also act as APC to $CD4^+$ T cells, leading to the selection and maintenance of highly protective $CD4^+$ T_{RM} cells (108, 119).

The immune cell composition of the lower FRT (type II epithelia) is basically similar to that of the skin: LC and γδ T cells survey the epithelium, while heterogeneous subsets of DC and macrophages survey the LP (103). Although the lower FRT does not contain MALT in the steady state, both $CD8^+$ and $CD4^+$ T_{RM} cells can be established in the lower FRT following intravaginal infections, such as those mediated by herpes simplex virus type 2 (HSV-2). Notably, after the clearance of the infection, memory $CD4^+$ T cells, B cells, DC, and macrophages form clusters beneath the epithelial layer of the vagina (120) (**Figure 3**). $CD4^+$ T_{RM} cells are predominantly distributed within the clusters, and their structures are sustained by a constitutively produced cytokine/chemokine network. IFN-γ secreted by $CD4^+$ T_{RM} cells drive CCL5 production by macrophages which attracts and retains $CD4^+$ T_{RM} cells within the cluster (15). Residual antigen may be involved in driving $CD4^+$ T_{RM} cell production of IFN-γ (15). Although $CD4^+$ T_{RM} cells are crucial for full protection against HSV-2 infection (15), establishment of $CD4^+$ T_{RM} cells in the vaginal mucosa increases susceptibility to subsequent human immunodeficiency virus infection due an increase in the number of susceptible target cells (121, 122).

As with the skin epidermis, antigen-specific $CD8^+$ T_{RM} cells reside within the epithelium and LP of the vaginal mucosa (123–125). T_{RM} cells in the vaginal LP are predominantly found in clusters (15). Migration of effector $CD8^+$ T cells to the vaginal epithelium largely depends on CXCR3, a receptor for inflammatory chemokines CXCL9 and CXCL10 (126). IFN-γ secreted by arriving $CD4^+$ T cells triggers production of those chemokines at the site of infection, demonstrating the importance of $CD4^+$ T cells in promoting anti-viral $CD8^+$ T cell responses in the FRT (126). Topical administration of these chemokines can effectively recruit circulating effector, but not memory, $CD8^+$ T cells primed at a remote site to the genital mucosa even in the absence of cognate antigen, a strategy known as "prime and pull." This leads to the establishment of long-term populations of $CD8^+$ T_{RM} cells in the vagina (127). Interestingly, although effector $CD4^+$ T cells are also recruited to the genital mucosa following prime and pull strategies, memory $CD4^+$ T cells are not retained for the long term within the vagina (127), implying that the maintenance of $CD4^+$ T_{RM} niches (the clusters in the vaginal LP) relies on local antigen. By contrast, and similar to the skin $CD8^+$ T_{RM} cells that populate epidermal niches for DETC (16), $CD8^+$ T cells recruited to the vaginal mucosa may occupy unique niches that were originally occupied by other resident cell types, such as γδ T cells in the epidermal layer of the vagina. Distinct from the skin $CD8^+$ T_{RM} cells, however, the development and maintenance of $CD8^+$ T_{RM} cells in the FRT is IL-15-independent (99). Currently, the factors that regulate the maintenance of T_{RM} cells in the FRT are largely unknown.

Upper Respiratory Tract (URT) and Lower Respiratory Tract (LRT)

The respiratory tract is divided into two compartments; the URT, comprised of the nasal cavities, pharynx, and larynx, and the LRT, comprised of the trachea, primary bronchi, and lungs. Although most studies have largely focused on T_{RM} cells in the LRT, most common airborne pathogens in the human primarily infect the URT. Thus, understanding the T_{RM} niches in both compartments is key for the development of vaccines that confer protection against respiratory pathogens.

The mucosal surface of the URT is comprised of pseudostratified ciliated columnar epithelial cells and an underlying LP. In mice, nasal-associated lymphoid tissues (NALT), the murine equivalent of the tonsils in human, are embedded directly in the submucosa at the base of the nasal cavities (128). NALT is considered to be a mucosal inductive site for humoral and cellular immune responses in the URT since it hosts B cell follicles surrounded by T cell areas (128, 129). In contrast to the LN, where naïve $CD4^+$ T cells predominate over memory T cells, the NALT is surveyed primarily by memory $CD4^+$ T cells, presumably resident type, suggesting that it is optimized to initiate memory recall responses, rather than initiate primary T cell responses (130). In contrast to memory $CD4^+$ T cells in the NALT, CD8 T_{RM} cells tend to be distributed throughout the nasal turbinate and septum, although some antigen-specific $CD8^+$ T_{RM} cells are also established in the NALT following recovery from a respiratory virus infection (131). In this regard, the distribution of T cells in the URT is similar to that in the skin and the FRT,

where $CD8^+$ T_{RM} cells are widely distributed in the epithelial tissues and $CD4^+$ T_{RM} cells form clusters in the LP.

While the majority of $CD8^+$ T_{RM} cells in the nasal tissues express CD103, a small fraction of the cells are CD103 negative (131). This differential expression of CD103 may reflect the localization of $CD8^+$ T_{RM} cells within the epithelium and LP (132). Despite the high proportion of $CD103^+$ cells in the URT, the differentiation of $CD8^+$ T_{RM} cells in the nasal tissues does not appear to be dependent on local signaling through TGF-β and cognate antigen (43, 131, 133). This is in stark contrast to the LRT where both of these factors are absolutely required for the establishment of $CD8^+$ T_{RM} cells (134, 135). Thus, the local instructions required for the differentiation of $CD8^+$ T_{RM} cells in the nasal mucosa are distinct from those in the LRT. Furthermore, the number of $CD8^+$ T_{RM} cells in the nasal tissues is relatively stable (there was no visible decline in number of these cells at least 3 months post-infection), whereas there is a significant decline in number of these cells in the LRT (lung) (131). This suggests that the nature of the anatomical niches that maintain $CD8^+$ T_{RM} cells differ between URT and LRT. Given the structural similarity between nasal mucosa and other mucosal tissues and the fact that the nasal tissues retain γδ T cells in the epithelium (136), it is tempting to speculate that $CD8^+$ T_{RM} cells in the nasal tissues may displace γδ T cells from their niches, potentially enabling their long-term survival.

The mucosal surfaces of the trachea and primary bronchus are basically similar to that of the nasal mucosa except for the presence of hyaline cartilage and a poorly developed venous plexus (the latter presumably helps avoid accidental suffocation caused by tracheal hemorrhage). Tracheal epithelial cells are a major target for several viral infections, such as seasonal influenza virus, and a recent study has demonstrated that large numbers of antigen-specific effector $CD8^+$ T cells are recruited to the tracheal mucosa during the acute phase of the infection (137). By contrast, relatively few $CD4^+$ T cells are recruited to the tracheal mucosa (as compared to the LRT) during the acute phase of infection. This suggests that there are distinct sets of homing signals in the mucosa of the trachea and LRT (137). Although establishment of $CD8^+$ T_{RM} cells in the trachea was not determined in this study, $CD8^+$ T cells were still detectable in the trachea following the resolution of an influenza virus infection (day 14), suggesting that some of these cells may reside in the tracheal epithelium as T_{RM}.

The mucosa of the LRT is covered by pseudostratified ciliated epithelium (bronchiole) and columnar epithelium (terminal bronchiole to alveoli). A relatively thin interstitium underlies the epithelium and hosts both blood and lymphatic vessels. T cells in the LRT reside in at least two distinct compartments: the lung interstitium and the lung airways. T cells resident in the lung interstitium can be identified, and distinguished from circulating T cells, by intravenous labeling with an anti-T cell antibody (138). T cells in the lung airway are those that are collected by bronchoalevolar lavage taken *via* the trachea (139). Most of these cells are derived from the LRT (localized in the epithelial layer), although a few cells are also derived from the URT (trachea). $CD8^+$ T cells exhibiting memory phenotypes can be detected in the LRT of naïve animals or animals that had previously been infected or vaccinated at sites distant from the lung (6, 43, 50, 140–143). It is believed that there is a basal level of influx that enables continual surveillance of the lung by antigen-experienced $CD8^+$ T cells in the "lung-unconditioned" animals. For instance, some blood-borne cells are recruited to the airway under steady-state condition and CXCR3 expressed on antigen-experienced $CD8^+$ T cells is known to be involved in this process (140). Once recruited to the lung airways, T cells do not return to the interstitium or the circulation unless there is an infection or an inflammatory condition (144).

Upon pulmonary infection, epithelial cells, lung-resident populations of immune cells in the interstitium and airway epithelium (such as macrophages, DC, and ILC) cooperatively promote acute inflammation (145). Although the full array of adhesion molecules and chemokine receptors that specifically guide T cells to the lung has not yet been determined, it is known that CXCR3 is important for the recruitment of effector $CD8^+$ T cells to the epithelial layer of the interstitium as well as the airway (146). In addition, local inflammation-induced upregulation of CD69, and the activation of integrin α1β1 (very late antigen-1, CD49a) promotes transient localization and retention of $CD8^+$ T cells in the lung interstitium (134, 147). As with the other mucosal tissues, local TGF-β signaling is required for the expression of CD103 on $CD8^+$ T cells in the lung (135, 148), which then promotes localization of $CD8^+$ T cells along the walls of large airways (149). IL-15 [produced primarily by $CD11b^+$ macrophages in the interstitium during the early phases of a respiratory infection (150)] also facilitates the migration of effector $CD8^+$ T cells to the lung (151). However, IL-15 is dispensable for the differentiation and maintenance of $CD8^+$ T_{RM} cells in the lung (152).

Following the resolution of infection, substantial numbers of memory $CD8^+$ T cells are maintained in both the lung interstitium and the airways for several months (153). We have recently shown that memory $CD8^+$ T cells in both of these sites comprise a mixture of two distinct memory T cell populations: a major, stable population of T_{RM} cells, and a minor, dynamic population of T_{EM} cells that is continuously replenished by new cells from the circulation (134) (**Figure 4**). We also identified specific anatomical niches for $CD8^+$ T_{RM} cells around the bronchiole, which are temporarily created at sites of regeneration following tissue injury (134). We termed these sites repair-associated memory depots (RAMD). As with the epithelial layers in other mucosal surfaces, $CD8^+$ T_{RM} cells in the RAMD do not form clusters or lymphoid-like structures, but instead accumulate to relatively high densities in specific niches. By contrast, $CD8^+$ T_{EM} cells are widely, but sparsely, distributed throughout the unaffected lung interstitium. This rigid compartmentalization of memory $CD8^+$ T cell populations in the lung suggests that the two populations are maintained by separate signals. It is also important to note that residual antigen-driven reactivation in the mediastinal LN plays a role in driving the continual recruitment of $CD8^+$ T_{EM} cells to the lung for several months after infection (154–157). Local instructive signals induced by pulmonary infection, such as IL-33 and TNF, presumably also contribute to the transient retention of circulating $CD8^+$ T_{EM} cells in the lung interstitium (157). A more detailed analysis of the factors and mechanisms

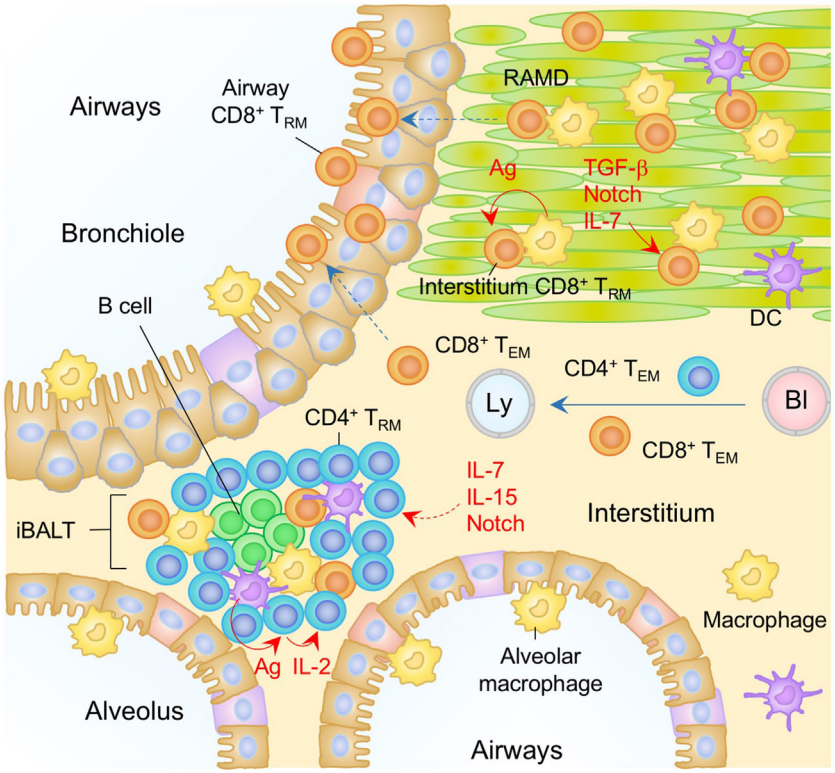

FIGURE 4 | T_{RM} niches in the in the lung. A majority of CD8$^+$ T_{RM} cells in the lung interstitium are maintained within the repair-associated memory depots (RAMD) that are temporarily created at the site of tissue injury, while CD8$^+$ T_{RM} cells are found sparsely in the unaffected areas. A complex of niche factors, including signals *via* cognate antigen, TGF-β, Notch, and IL-7, are known to be involved in the formation of CD8$^+$ T_{RM} cells in the lung interstitium. CD8$^+$ T_{RM} cells are also present in the lung airways, the number of which is presumably maintained by continual recruitment of cells from the pool of CD8$^+$ T_{RM} cells in the lung interstitium. CD4$^+$ T_{RM} cells in the lung interstitium are maintained predominantly within the inducible bronchus-associated lymphoid tissues (iBALT). Late antigen recognition triggers autocrine IL-2 signaling, which supports the proliferation and survival of CD4$^+$ T_{RM} cells. Homeostatic cytokines IL-7 and IL-15, and Notch signaling are also required for the maintenance of CD4$^+$ T_{RM} cells in the iBALT. T_{EM} cells are passing through the normal interstitium. Orange and blue cells indicate CD8$^+$ and CD4$^+$ T_{RM} cells, respectively, unless otherwise stated. Red lines indicate the representative niche factors that influence the maintenance of T_{RM} cells. A blue line indicates the migratory route. Dashed lines indicate potential impact of niche factors (red) or migratory routes (blue). Abbreviations: Ly, lymph vessel; Bl, blood vessel; Ag, antigen; T_{RM}, tissue-resident memory T cells; T_{EM}, effector memory T cells.

that regulate the continual recruitment of memory CD8$^+$ T cells to the lung has been presented in our previous review (5).

Interestingly, in our parabiosis experiments we also detected minimal, if any, conversion of CD8$^+$ T_{EM} cells into CD8$^+$ T_{RM} cells in the lung for several months post-infection, a time period when T_{RM} cells still comprise a large proportion of memory CD8$^+$ T cell pool in the lung (134). These studies further demonstrated that CD8$^+$ T cells recruited to the lung interstitium after the peak of the cellular immune response (around day 10 post-infection) are excluded from the RAMD, and fail to form T_{RM} cells (134). These data clearly demonstrated that T_{RM} niches in the lung interstitium are occupied at the peak of tissue damage, but are no longer available for latecomer CD8$^+$ T cells. In the skin and FRT sections, we noted that forced recruitment of CD8$^+$ T cells to the epithelial tissues by antigen-independent inflammation or topical administration of chemokines results in the establishment of T_{RM} cells (prime and pull) (37, 127). Importantly, however, we and others have demonstrated that this prime and pull strategy does not work for the establishment of CD8$^+$ T_{RM} cells in the lung, as CD8$^+$ T cells recruited to the lung by antigen-independent inflammation in the lung completely disappear after the inflammation in the lung has resolved (134, 158). The failure of the prime and pull strategy in the lung is likely due to the structural difference between the lung and other mucosal/surface tissues. For instance, skin CD8$^+$ T_{RM} cells can occupy DETC niches in the epidermis for their long-term survival, whereas normal lung mucosa does not exhibit such preformed niches. Administration of cognate antigen in combination with the prime and pull strategy results in the *de novo* creation of the RAMD, and significantly increases the numbers of antigen-specific, but not antigen-unrelated, CD8$^+$ T_{RM} cells in the lung interstitium and airways (134). This indicates that local antigen plays at least two distinct roles: the creation of damage-associated niches by generating antigen-bearing target cells in the lung in the presence of antigen-specific CD8$^+$ T cells in the circulation, and the antigen signaling necessary for the establishment, and/or survival, of T_{RM} (159). Following the establishment of T_{RM}, Notch signaling may be a potential niche factor that regulates the maintenance of T_{RM} cells in the lung, as the lack of Notch signaling results in the loss of CD103$^+$ CD8$^+$ T_{RM} cells from the

lung (160). Although cells that express Notch ligands are not yet identified in the RAMD, cell to cell contact seems important for sustaining T_{RM} cells in the lung. It is noteworthy that the size of the RAMD shrinks over time as tissue repair proceeds and tends to disappear several months post-infection (134). Such a transitional appearance of RAMD may account for the relatively shorter longevity of $CD8^+$ T_{RM} cells in the lung (149). Recently, Zhou et al. have reported that the addition of local 4-1BB signaling during recall (4-1BB is expressed mainly on memory but not naïve T cells) improves the generation of long-lived $CD8^+$ T_{RM} cells expressing IL-7 receptor (IL-7R)α (161), suggesting that IL-7 plays a key role in the maintenance of $CD8^+$ T_{RM} cells in the lung. It will be interesting to determine whether these cells can survive outside the RAMD.

In contrast to the lung interstitium, the histological nature of putative $CD8^+$ T_{RM} niches in the lung airways remains unclear. It has long been believed that the numbers of memory $CD8^+$ T cells in the lung airways are maintained by the continual recruitment from the circulation. Resident cells at this site are cleared by phagocytic cells or removed through mucociliary clearance, resulting in a relatively short half-life (~2 weeks) (144). Surprisingly, our parabiosis experiments have demonstrated no evidence for the continual replacement of host memory $CD8^+$ T cells in the lung airways by $CD8^+$ T_{EM} cells derived from the partner. Since it is unlikely that memory $CD8^+$ T cells can persist for long within the harsh airway environment, we assume that cells in the airways are continually replenished by $CD8^+$ T_{RM} cells from the RAMD (interstitium) but not by $CD8^+$ T_{EM} cells from the circulation. Thus, the major source of $CD8^+$ T cells in the lung airways may be RAMD located underneath the bronchoalveolar walls (**Figure 4**).

In contrast to $CD8^+$ T_{RM} cells, most $CD4^+$ T_{RM} cells in the lung are found in B cell follicles and are surrounded by T cell areas (134, 162–164) (**Figure 4**). Such lymphoid-like structures have been termed inducible bronchus-associated lymphoid tissues (iBALT) and are the primary niches for the maintenance of lung $CD4^+$ T_{RM} cells. The factors regulating the development of iBALT are reviewed elsewhere (165). Several other physiological niches for the generation and maintenance of lung $CD4^+$ T_{RM} cells have also been reported. As with the $CD8^+$ T_{RM} cells, local antigen also plays a role (163), as late antigen recognition at day 5–8 post-infection, which has been termed a "memory check point," is necessary for the formation of memory $CD4^+$ T cells in the lung and spleen (166). Antigen reactivation of the cells triggers autocrine IL-2 signaling, which prolongs the survival of $CD4^+$ T_{RM} cells by upregulating the IL-7Rα (166–168) and sustains the homeostasis of lung $CD4^+$ T_{RM} cells (162, 164). Interestingly, IL-15 signaling, as opposed to IL-2 signaling, can generate a separate but similar cohort of highly functional and protective $CD4^+$ T_{RM} cells in the lung (169). As with the $CD8^+$ T_{RM} cells, increased transcription levels of Notch signaling-associated molecules are observed in lung $CD4^+$ T_{RM} cells, suggesting the involvement of Notch signaling for the maintenance of lung $CD4^+$ T_{RM} cells (170).

Salivary Gland (SG)

The SGs are exocrine epithelial tissues that secrete saliva into the oral cavity. Humans and rodents have at least three pairs of major SGs (parotid, sublingual, and submandibular) and each gland has secretory units composed of an acinus, myoepithelial cells, and a duct (171). SGs also function as an effector site for IgA-mediated humoral immune responses that protect oral surfaces (172, 173).

It is well known that the SGs can be a target of a variety of bacterial as well as viral infections, such as mumps and cytomegalovirus (CMV). In the case of CMV, the virus is able to establish latent infection in the SGs and is able to evade $CD8^+$ T cell immunity by downregulating MHC class I molecules (174). Virus-specific $CD4^+$ T cells can control viral production, but are not able to eliminate latently infected cells (175, 176) such that persistent virus is selectively sequestered in the vacuoles of glandular acinar epithelial cells (177, 178). In latently infected individuals, resident populations of antigen-specific $CD8^+$ and $CD4^+$ T_{RM} cells are established in the SGs (179, 180) (**Figure 5**). However, their phenotypes, localization, and the local cues regulating their differentiation into T_{RM}, differ significantly (181). $CD4^+$ T_{RM} cells are located predominantly in the stroma of the SGs and their establishment depends on local antigen (179), presumably due to the upregulation of CD69 that antagonizes $S1P_1$-medaited tissue egress (181). By contrast, $CD8^+$ T_{RM} cells express CD103, and localize predominantly within the epithelium of the acini and ducts (179, 180) (**Figure 5**). Local TGF-β signaling in the SGs is required for upregulation of CD103 on

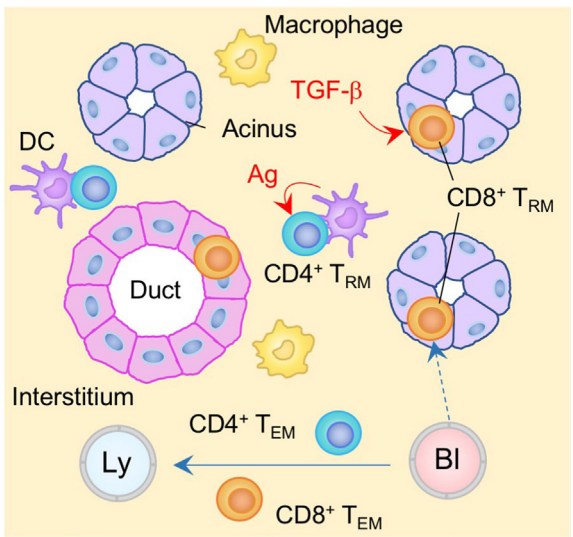

FIGURE 5 | T_{RM} niches in the in the salivary gland (SG). $CD8^+$ T_{RM} cells are localized predominantly within the epithelium of the acini and duct. Local TGF-β signaling is required for the formation of $CD8^+$ T_{RM} cells in the SG. By contrast, $CD4^+$ T_{RM} cells are localized predominantly within the stroma of the SG. In contrast to $CD8^+$ T_{RM} cells, cognate antigen plays a key role in the formation of $CD4^+$ T_{RM} cells. T_{EM} cells are passing through the normal interstitium. Red lines indicate the representative niche factors that influence the maintenance of T_{RM} cells. Orange and blue cells indicate $CD8^+$ and $CD4^+$ T_{RM} cells, respectively, unless otherwise stated. A blue line indicates the migratory route. A dashed line indicates potential migratory route. Abbreviations: Ly, lymph vessel; Bl, blood vessel; Ag, antigen; T_{RM}, tissue-resident memory T cells; T_{EM}, effector memory T cells.

$CD8^+$ T_{RM} cells and their localization into the epithelium (179, 180). Because CMV downregulates MHC class I molecules, particularly in infected acinar glandular epithelial cells in the SGs, local antigen does not appear to be required for the formation of $CD8^+$ T_{RM} cells in the SGs (179). Indeed, virus-specific $CD8^+$ T cells can be established in the SGs even in the absence of virus infection in this tissue (6, 182, 183). Furthermore, ongoing presentation of late antigens by non-hematopoietic cells in the LN or by virus-uninfected APC (*via* cross-presentation) during CMV infection results in substantial and sustained expansion of antigen-specific $CD8^+$ T cells in the circulation, a process known as memory inflation (184–187). Some of these memory $CD8^+$ T cells are also converted into T_{RM} cells in the SGs on a continual basis (180). Blockade of CXCR3, or the genetic deletion of either integrin α4β1 or E-cadherin on $CD8^+$ T cells reduces the accumulation of $CD8^+$ T_{RM} cells in the SGs (182, 183, 188), suggesting that these molecules promote the migration of circulating $CD8^+$ T cells to the glandular epithelium. In contrast to the inability of the primary $CD8^+$ T cell response to control the virus infection, $CD8^+$ T_{RM} cells resident in the SGs can confer protection upon recall by eliminating CMV infected non-epithelial cells, where CMV fails to achieve complete downregulation of MHC class I molecule (179).

Non-Barrier Tissues

Brain

Owing to the presence of the blood–brain barrier (BBB), the blood–cerebrospinal fluid (CSF) barrier (BCSFB), and the CSF–brain barrier, the central nervous system (CNS) is regarded as an immune privileged site with severely limited ingress of blood-borne T lymphocytes. Relatively few, if any, T cells are present in the healthy brain parenchyma under non-inflammatory conditions (189). Consequently, the aberrant accumulation of T cells in the brain parenchyma has generally been considered to be a pathogenic condition. However, it is now becoming clear that the few peripheral T cells present in the brain in the absence of inflammation play key a role in surveying the CNS and keeping the infectious pathogens in check (190), as the lack of these cells can result in opportunistic infections in the CNS (191).

The choroid plexus (CP) is recognized as a major gateway for peripheral T cell access to the CNS (192, 193). The CP is comprised of fenestrated blood capillaries lacking endothelial tight junctions (192). Thus, the barrier properties of the BCSFB at this site rely only on the monolayer of epithelial cells interconnected by tight junctions—a structure permissive for immune cell transit (192). Consequently, around 150,000–750,000 immune cells are present in the CSF of healthy individuals, and more than 90% of the T cells present are antigen-experienced (193). Recent studies have identified a lymphatic vessel network lining the dural sinuses that drain CSF and allow the transit of immune cells from the adjacent subarachnoid space and brain interstitial fluid to the cervical LN (194, 195). This implies that there is the continual trafficking of T_{EM} cells between CNS (e.g., meninges and FSC) and the circulation (196). Nevertheless, the brain parenchyma essentially lacks lymphatic vessels and is mostly devoid of T cells under steady-state conditions.

Upon infection with neurotropic pathogens, antigen-specific T cells infiltrate the subarachnoid spaces of the meninges as well as the perivascular spaces of the parenchymal post-capillary venule, where specialized APC reside (197, 198) (**Figure 6**). T cells are then activated to proliferate and produce cytokines and chemokines in the infected meninges (199–201). This results in local inflammation, which subsequently disrupts vascular tight junctions and the glia limitans, allowing infiltration of T cells into the parenchyma (190, 198) (**Figure 6**). During this process, the balance of local chemokine production regulates the transmigration of circulating T cells into the brain parenchyma (202). In brief, CXCL12 is constitutively expressed on the basolateral surface of endothelial cell layer in the CNS and is also upregulated during inflammation, which promotes $CXCR4^+$ T-cell recruitment to, and retention within, the perivascular space (203, 204). It is only after the local concentration of CXCL12 declines that effector T cells are able to migrate into the brain parenchyma in response to inflammatory chemokines, such as ligands for CXCR3 (205, 206) and CCR5 (207, 208). In the case of neuroinflammation associated with experimental autoimmune encephalomyelitis, the CXCL10–CXCR3 axis also functions to retain T cells within the perivascular space presumably due to differential inflammatory nature in the perivascular space (209).

After the clearance of a viral infection in the CNS, some of the antigen-specific $CD8^+$ T cells that had been recruited to the brain parenchyma differentiate into T_{RM} cells and become resident in the site (133). The numbers of $CD8^+$ T_{RM} cells that establish residency depends on the pathogen and is presumably linked to the tropism and pathogenesis of each virus (210). For example, following intranasal infection with vesicular stomatitis virus, which infects nerve endings, $CD8^+$ T_{RM} cells form clusters at the site of infection, and are widely distributed throughout the brain parenchyma (133). By contrast, intracerebral infection with LCMV, which infects non-neuronal cells in the brain (i.e., glial cells), $CD8^+$ T_{RM} cell populations are primarily established at brain surface structures, such as meninges and CP (around the ventricles or at anatomical borders between different brain regions) (211). In both cases, these $CD8^+$ T_{RM} cell populations are not pathogenic, but confer protection against reinfection even in the absence of circulating memory $CD8^+$ T cells (211).

Regardless of their location and the nature of the infecting pathogens, brain $CD8^+$ T_{RM} cells can be divided into at least two populations based on their expression of CD103 (133, 211–214). It has been proposed that the initial upregulation of CD103 is largely dependent on the local reactivation of $CD8^+$ T cells with cognate antigen in the brain (it remains elevated following antigen clearance) (133). However, it is clear that Treg-derived TGF-β (215, 216), inflammation, and other undefined local factors (213), can also upregulate CD103 on $CD8^+$ T_{RM} cells in the brain in an antigen-independent manner. These different types of instructive signals may account for the distinct gene expression profiles between $CD103^+$ and $CD103^-$ $CD8^+$ T_{RM} cells (212, 214) and the superior effector functions for the former (213, 214). It is noteworthy that retroviral knockdown of CD103 impairs the accumulation of $CD8^+$ T_{RM} cells in the brain, indicating the

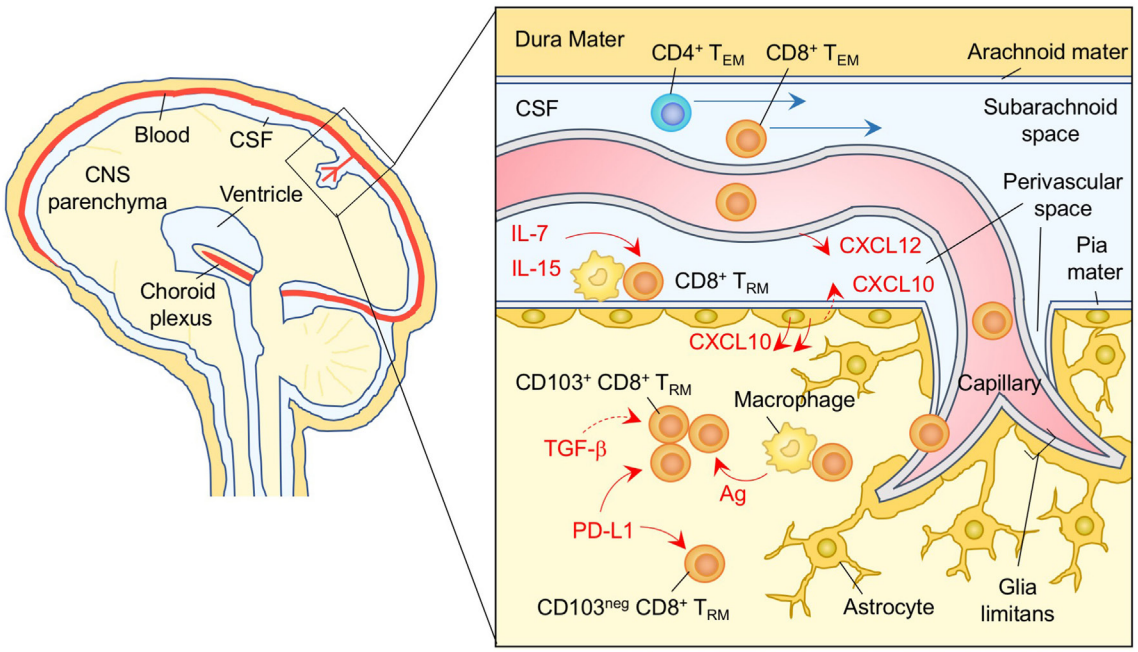

FIGURE 6 | T_{RM} niches in the in the brain. CXCL12 is constitutively expressed on the basolateral surface of endothelial cells, and recruits CXCR4$^+$ CD8$^+$ T cells to the perivascular spaces. Subsequent migration of cells in the brain parenchyma is promoted by chemokines such as ligands for CXCR3 and CCR5 secreted by astrocytes. CD8$^+$ T_{RM} cells are established in the parenchyma and perivascular spaces of the brain. Parenchymal CD8$^+$ T_{RM} cells are divided by the expression of CD103. Although upregulation of CD103 depends largely on cognate antigen, TGF-β may be involved in this process. PD-1 is highly expressed on both CD103$^+$ and CD103$^-$ CD8$^+$ T_{RM} cells in the brain parenchyma independent of either antigen or inflammatory signals. Signaling through PD-1 promotes the establishment of CD8$^+$ T_{RM} cells in the brain parenchyma. IL-7 and IL-15 are available in the perivascular spaces, and sustain the homeostatic proliferation of CD8$^+$ T_{RM} cells in this compartment. T_{EM} cells are passing through the cerebrospinal fluid (CSF). Orange and blue cells indicate CD8$^+$ and CD4$^+$ T_{RM} cells, respectively, unless otherwise stated. Red lines indicate the representative niche factors that influence the maintenance of T_{RM} cells. Blue lines indicate the migratory route. Dashed lines indicate potential impact of niche factors. Abbreviations: Ag, antigen; neg, negative; T_{RM}, tissue-resident memory T cells; T_{EM}, effector memory T cells.

importance of CD103 for the recruitment and/or retention of CD8$^+$ T cells early after infection, probably during transmigration through the BBB. Once recruited to the brain parenchyma, however, CD103 expression has no impact on the localization of CD8$^+$ T_{RM} cells (211), which may be attributed to the lack of E-cadherin expression in the adult brain (217). Taken together, it is possible that CD103 expressed on brain CD8$^+$ T_{RM} cells may reflect the prior acquisition of local education but is not functional as an adhesion molecule.

Programmed cell death protein 1 (PD-1) and CD69 are both expressed on CD8$^+$ T_{RM} cells in the brain (including both CD103$^+$ and CD103$^-$ T_{RM}) (213). Although the expression of both molecules on CD8$^+$ T_{RM} in non-CNS sites is generally dependent on repetitive antigen engagement (218), it has been demonstrated that both antigen and inflammation are dispensable for the sustained expression of PD-1 as well as CD69, and programmed cell death ligand 1 (PD-L1) in the brain (213). Furthermore, these cells remain functionally competent under these conditions (213). Interestingly, PD-1 expression on brain CD8$^+$ T_{RM} cells is found to be programmed, as environmental factors in the brain induce extensive demethylation of the *Pdcd1* promoter (which controls PD-1 expression) (213). In addition, genetic deletion of either PD-1 or PD-L1 diminishes the establishment of brain CD8$^+$ T_{RM} cells (219, 220). These findings suggest that signaling through PD-1 is a part of the T_{RM} differentiation program and may be attributed to the PD-1 signaling-induced upregulation of CPT1a, an enzyme necessary for fatty acid β-oxidation that promotes memory differentiation (221, 222). Since upregulation of PD-L1 expression is evident on parenchymal cells (e.g., microglia, astrocytes, and oligodendrocytes) following different types of viral infections in the CNS (223–227), it is reasonable to speculate that PD-1 expression by brain CD8$^+$ T_{RM} cells maintains a tolerable balance between immunopathology and immune control of the virus in the CNS (190).

Reports of Ki-67 expression on brain CD8$^+$ T_{RM} cells following resolution of virus infection suggests that these cells are maintained by homeostatic proliferation (211). CD8$^+$ T_{RM} cells located at the brain surface structures more frequently express Ki-67 and phosphorylated Stat5 than those in the brain parenchyma, suggesting that their anatomical location allows them access to the homeostatic cytokines, IL-7 and IL-15 (211). Furthermore, CD8$^+$ T_{RM} cells in the brain parenchyma are less responsive to homeostatic cytokines (212). Interestingly, CD8$^+$ T_{RM} cells in the brain parenchyma, especially the CD103$^+$ population, are not able to survive outside their tissue niche. The irreversible nature of tissue adaptation by CD8$^+$ T_{RM} cells in the brain parenchyma is very different to the situation in the lung airway where CD8$^+$

T_{RM} cells retain the plasticity to adapt to different environmental niches for their survival (133, 228).

Liver

The liver is a frontline immune tissue in which antigen-rich blood from the gastrointestinal tract enters *via* the portal vein and is passed through a network of sinusoids (the capillary bed of the liver). Antigens are effectively trapped by sinusoidal resident APC, such as Kupffer cells, liver sinusoidal endothelial cells (LSEC), and DC (229), and the relatively slow sinusoidal blood flow promotes effective interaction of circulating immune cells with these APC (230). Fenestrated sinusoidal endothelium also enables the direct surveillance of hepatocytes by circulating T cells (231).

Recent studies have demonstrated that liver-resident memory $CD8^+$ T cells are established in the sinusoid following systemic infection or vaccination (232) (**Figure 7**). Liver $CD8^+$ T_{RM} cells in mice are mostly $CD69^+$, $CXCR3^+$, and $CXCR6^+$, but lack the expression of CD103, presumably reflecting the lack of tight junctions in the sinusoidal endothelium. The situation in humans is slightly different since a subset of $CD8^+$ T_{RM} in the human liver are $CD103^+$ in both healthy and hepatitis B virus-infected individuals. In this case, the sequential exposure of the cells to IL-15 and TGF-β induces the development of liver-adapted $CD103^+$ $CD8^+$ T_{RM} cells (233). Interestingly, mouse liver $CD8^+$ T_{RM} cells exhibit an amoeboid shape and migrate with a crawling action along the sinusoids, whereas circulating $CD8^+$ T_{EM} cells exhibit a round shape and flow rapidly in the sinusoid (232). Lymphocyte function-associated antigen 1 (LFA-1) has been found to be crucial for the patrolling behavior of liver $CD8^+$ T_{RM} cells in the sinusoid (234). It is also known that Kupffer cells, macrophages, and LSEC in the sinusoid constitutively express CXCL16, a CXCR6 ligand (235–237), which attracts NK cells, another resident cell population in the sinusoid (238). This suggests that liver-resident $CD8^+$ T cells and NK cells share this chemokine niche (239), although competition between these populations for this niche has not been reported. Local antigen presentation is clearly important for the prolonged retention and establishment of $CD8^+$ T_{RM} cells in the sinusoid, as targeting antigen presentation to the hepatocytes in the presence of antigen-specific $CD8^+$ T cells in the circulation leads to the massive accumulation of $CD8^+$ T_{RM} cells in the sinusoid, a strategy termed as "prime and trap" (232). Since local antigen presentation in the liver can trigger the formation of tertiary immune structures known as intrahepatic myeloid cell aggregates for T cell population expansion (iMATE) (240), it is tempting to speculate that such follicle-like structures provide special T cell niches in the liver, especially for $CD4^+$ T_{RM} cells.

Kidneys

The kidney is a highly vascularized tissue that is crucial for filtering the blood and removing toxins from the body. Lymphocytes are relatively rare in healthy kidneys, although small numbers of resident immune cells such as DC, macrophages, and T cells can be found in the interstitium under steady-state condition (241, 242). $CD8^+$ T_{RM} cells can persist in extravascular renal compartments following direct (243) or regional infections with pathogens (6, 83, 99, 244), although their precise distribution is not clear (244). While the majority of renal $CD8^+$ T_{RM} cells express CD69, even in the absence of antigen, only small fraction of cells express CD103 (83, 243, 244). The tissue-derived factors that influence the formation of renal $CD8^+$ T_{RM} cells are poorly defined. However, it has been reported that a lack of TGF-β signaling leads to reduction in the formation of $CD8^+$ T_{RM} cells in the kidney (244). This has been attributed to the role of TGF-β signaling in promoting trans-endothelial migration of effector $CD8^+$ T cells by upregulating ligands for E- and P-selectin, including an activated form of CD43, and CXCR3 (244). IL-15 is also known to be essential for the upregulation of CD43 (245), which may explain the defective establishment of renal $CD8^+$ T_{RM} cells in the absence of IL-15 (99).

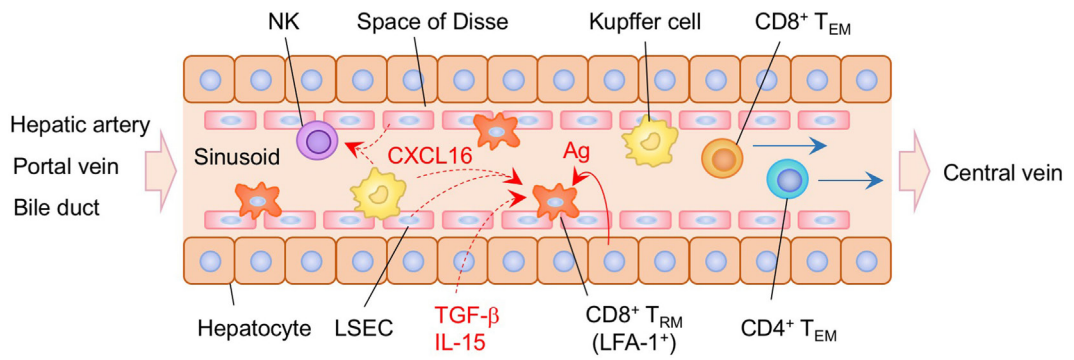

FIGURE 7 | T_{RM} niches in the in the liver. $CD8^+$ T_{RM} cells are localized within the sinusoid of the liver. These cells exhibit an amoeboid shape and crawling along the sinusoid dependent on the expression of lymphocyte function-associated antigen 1 (LFA-1). Antigen expressed on the hepatocytes play a key role in the establishment of $CD8^+$ T_{RM} cells in the liver sinusoid. Kupffer cells and NK cells are also resident populations in the sinusoid. CXCL16 secreted by Kupffer cells and liver sinusoidal endothelial cells (LSEC) may promote the retention of T_{RM} cells and NK cells within the sinusoid. T_{EM} cells are passing through the sinusoid without crawling. Orange and blue cells indicate $CD8^+$ and $CD4^+$ T_{RM} cells, respectively, unless otherwise stated. A red line indicates the representative niche factor that influences the maintenance of T_{RM} cells. Blue lines indicate the migratory route. Dashed lines indicate potential impact of niche factors. Abbreviations: Ag, antigen; T_{RM}, tissue-resident memory T cells; T_{EM}, effector memory T cells.

White Adipose Tissue (WAT)

While T_{RM} generally function locally to guard the vulnerable sites from reinfection, an interesting exception is the establishment of antigen-specific $CD8^+$ T_{RM} cells in the WAT (246). These cells exhibit a high turnover rate and active metabolism and can augment recall responses generated by non-lipid compartments, suggesting that the WAT functions as a reservoir of T_{RM} cells by improving their functional capacities and longevity. Notably, WAT T_{RM} cells also remodel the physiological function of the WAT, as reactivation of adipose T_{RM} cells lead to a sharp decrease in lipid synthesis. This elevates the antimicrobial responses within the adipose tissues, resulting in synergic immunological crosstalk between the tissue and the T_{RM} cells. Thus, it is of interesting to speculate that, beyond the role as the local sentinel, long-term maintenance of T_{RM} cells may influence the homeostasis and function of each tissue, leading to both beneficial and detrimental consequences.

Tumor

It has been reported that $CD8^+$ T cells with a T_{RM} phenotypes ($CD103^+$ and $CD49a^+$) are present in solid tumors (247, 248). Large-scale transcriptome analysis has revealed that $CD8^+$ tumor infiltrating lymphocytes (TIL) exhibit characteristics of T_{RM} cells and it has been observed that $CD103^+$ $CD8^+$ T_{RM} cells from neighboring peripheral tissues can infiltrate into solid tumors (249, 250). Runx3 expression appears to promote the infiltration of $CD8^+$ T_{RM} cells into tumors as *Runx3*-deficient $CD8^+$ T cells failed to accumulate in tumors (90). As with other tissues, local microenvironmental cues promote the acquisition of T_{RM} phenotypes of $CD8^+$ that infiltrate tumor tissues (251). It is important to note, however, that $CD8^+$ TIL with T_{RM} characteristics (termed as $CD8^+$ T_{RM} TIL hereafter) are no longer true "resting" T_{RM} cells as they are located in an effector site where cognate antigen is abundant and typically express checkpoint molecules to regulate their activity (249). This checkpoint molecule expression may be transient, or below suppressive levels, since $CD8^+$ T_{RM} TIL in tumors exhibit superior anti-tumor activities and a positive prognosis has been correlated with the quality and quantity of these cells (248–250, 252–256). It has also been found that $CD103^+$ $CD8^+$ T_{RM} TIL with the strongest CTL activity are located in the border area of the tumor. This contrasts with CD103 negative $CD8^+$ T_{RM} TIL that infiltrate the stroma of the tumor (a potentially highly immune suppressive environment), and mediate weak CTL activity (257). CD103-mediated efficient interaction of $CD8^+$ T_{RM} TIL with tumor cells of epithelial origin also promotes prolonged survival and enhanced CTL activity (251, 254, 258, 259). Based on these findings, the generation of $CD8^+$ T_{RM} cells in neighboring tissues to the tumor is a promising strategy to confer protection against tumor growth (250, 260–263). However, this protection is limited to primary tumors, and not metastases, since $CD8^+$ T_{RM} cells are segregated from the circulation (250).

LYMPHOID ORGANS

Secondary Lymphoid Organs

LNs, Spleen

The SLOs have generally been considered a transit site for T_{CM} and T_{EM} cells. In the case of the LN, these cells are transiting from the high endothelial venules and afferent lymphatics, respectively, into the circulation. However, recent studies have demonstrated that there are also small numbers of memory $CD4^+$ and $CD8^+$ T cells that are resident in the LN, spleen, PP, and tonsils without recirculation (264–268). The long-term residency of T_{RM} cells within the SLO has been demonstrated by parabiosis or photoconversion-based cell labeling studies (264, 265, 267, 268). Unlike circulating memory T cells, T_{RM} cells in the SLO share phenotypic characteristics and gene expression profiles with those in the NLT (110), including stable downregulation of $S1P_1$, a key molecule for regulating T-cell egress from the LN (55). Indeed, most T_{RM} cells in the SLO express CD69, which promotes the downregulation of $S1P_1$ (110, 264, 266, 268). Since surface expression of CD69 is generally transient, however, it is likely that repetitive antigen stimulation is required for the maintenance of CD69 expression and the retention of T_{RM} cells in the SLO (110). In this regard, there is considerable evidence that residual antigen persists in the draining LN for several months after vaccination or the resolution of an acute infection and presumably facilitates the accumulation of memory T cells (154–156, 269–272). In addition, a recent study by Beura et al. have demonstrated that some $CD8^+$ T_{RM} cells in the LN are derived from cells that exit the NLT (273), thereby enhancing the accumulation of antigen-specific $CD8^+$ T_{RM} cells in the draining LN.

The distribution of T_{RM} cells in the SLO depends on an antigen niche, as T_{RM} cells are preferentially localized at the common antigen entry sites: the marginal zone and red pulp of the spleen and the subcapsular sinuses of the LN (264) (**Figure 8**). Although the maintenance of murine T_{RM} cells in the SLO is relatively independent of IL-15, signaling *via* IL-15 and TGF-β are known to transcriptionally downregulate $S1P_1$ in human T cells. Indeed, T_{RM} cells in the tonsils are localized specifically near the epithelial barrier where IL-15 is constitutively expressed (266). This is indicative of cytokine niche-dependent compartmentalization of T_{RM} cells within the SLO. Since T_{CM} cells in the SLO are central to pathogen clearance by generating massively increased numbers of secondary effector T cells during a recall response, it will be important to determine the functional contribution of T_{RM} cells in the SLOs during the recall responses. It is possible that T_{RM} cells in the SLO do not actively contribute to the recall response to avoid unnecessary competition with T_{CM} cells, but are strategically positioned to protect the SLO from direct infection with pathogens.

Primary Lymphoid Organs

Thymus

Antigen-specific $CD8^+$ T_{RM} cells have also been found to persist in the thymus, a primary lymphoid organ (274). Thymic $CD8^+$ T_{RM} cells are established following infection with either thymus-tropic or non-tropic pathogens, with considerably higher numbers in the former. As with T_{RM} cells in the peripheral tissues, thymic $CD8^+$ T_{RM} cells exhibit a canonical T_{RM} phenotype ($CD69^+$ $CD103^+$). These cells localize predominantly in the medulla although a few cells lodge in the cortex (**Figure 9**). At least three mechanisms potentially explain the medullary localization of thymic $CD8^+$ T_{RM} cells. First, active TGF-β, which support the

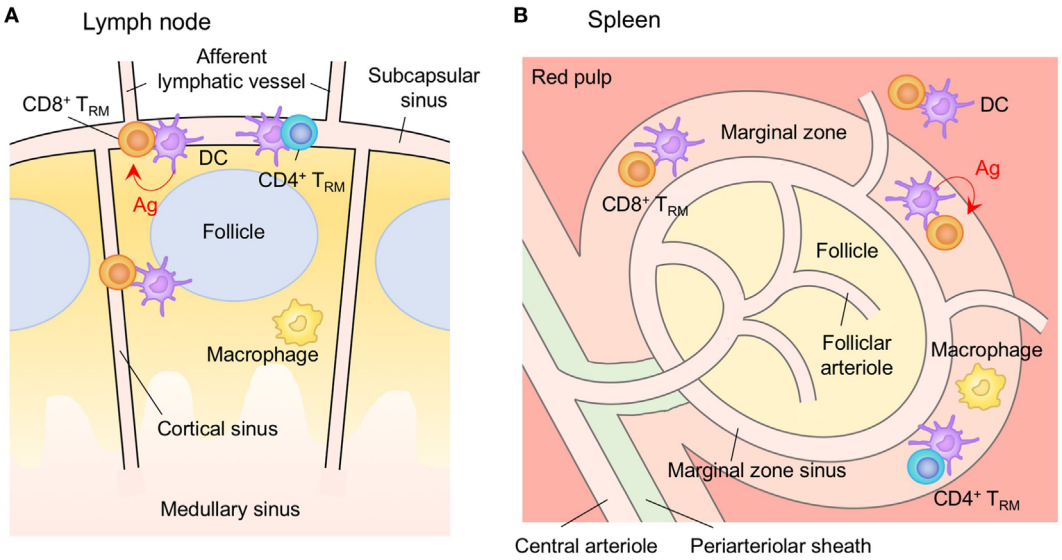

FIGURE 8 | T_{RM} niches in the in the secondary lymphoid organs (SLOs). Both CD4+ and CD8+ T_{RM} cells in the SLO are found at the common antigen entry sites, such as the subcapsular sinus in the lymph nodes **(A)**, and the marginal zone and red pulp in the spleen **(B)**. Retention of cells in these compartment is largely dependent on the expression of CD69 in response to antigen, although retention induced by CD69-independent mechanisms is also suspected. Orange cells indicate CD8+ T_{RM} cells unless otherwise stated. Red lines indicate the representative niche factors that influence the maintenance of T_{RM} cells. Abbreviations: Ag, antigen; T_{RM}, tissue-resident memory T cells.

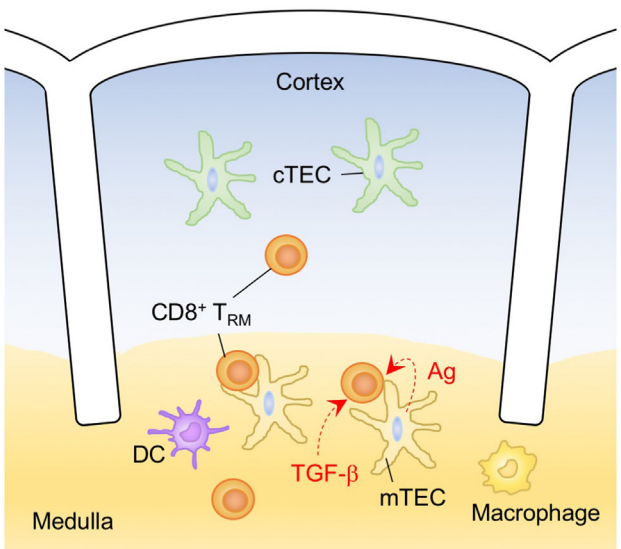

FIGURE 9 | T_{RM} niches in the in the thymus. CD8+ T_{RM} cells are localized predominantly within the thymic medulla, although some cells are also found in the cortex. A majority of CD8+ T_{RM} cells in the thymus express CD103 and CD69. TGF-β is rich in the medulla, and presumably influences the CD8+ T cell expression of CD103. Since persistent presentation of foreign antigen in the thymus is uncommon, self-antigen may drive the expression of CD69 on thymic CD8+ T_{RM} cells. Orange cells indicate CD8+ T_{RM} cells unless otherwise stated. Dashed lines indicate potential impact of niche factors. Abbreviations: Ag, antigen; cTEC, cortical thymic epithelial cell; mTEC, medullary thymic epithelial cell; T_{RM}, tissue-resident memory T cells.

generation of thymic Treg cells and potentially upregulates T cell expression of CD103, is predominantly localized in the thymic medulla (275). Second, E-cadherin is highly expressed in all thymic epithelial cells (TEC) of both the cortex and medulla (276) and promotes the interaction of TEC with CD103+ thymocytes (277). Third, mature thymocytes express CD69 which induces the downregulation of $S1P_1$ on CD8+ T_{RM} and blocks the departure of the cells *via* the medulla or cortico-medullary junction (278). The factors that induce the upregulation of CD69 on thymic CD8+ T_{RM} cells have not been determined (274). Since the immune activation process strongly inhibits the migration of peripheral DC populations to the thymus to avoid unfavorable induction of acquired tolerance to the invading pathogens (279, 280), it is reasonable to think that thymic CD8+ T_{RM} cells mainly function to protect the thymus, rather than contribute to the recall responses against systemic infections.

Bone Marrow (BM)

The BM is another primary lymphoid organ that facilitates the long-term maintenance of memory T cells by providing at least two distinct niches: a quiescence niche, that harbors a majority of quiescent memory T cells, and a self-renewal niche where memory T cells undergo homeostatic proliferation (281). Indeed, large numbers of memory CD8+ and CD4+ T cells accumulate in the BM (282, 283) and most of them express high levels of CD69, a hallmark of T_{RM} cells (164, 284, 285). TGF-β, secreted mainly by megakaryocytes in the BM, regulates the quiescence of memory T cells (286) and CXCL12 produced by reticular stromal cells promotes their co-localization with CXCR4+ memory

T cells (287). The reticular stromal cells, as well as myeloid cells, in the BM also provide niche factors for self-renewal such as IL-7 and IL-15 (283, 288, 289).

Recently, Di Rosa and Gebhardt have speculated that memory $CD8^+$ T cells in the BM are a circulating population that is transiting through the BM niches without establishing residence (290). This is largely based on the observation that memory $CD8^+$ T cells derived from the host and partner equilibrate in the BM in parabiosis experiments (65). By contrast, the deposition of memory $CD4^+$ T cells in the BM is relatively stable, as these cells persist in the BM for a long period even after most memory $CD4^+$ T cells disappear from the spleen and LN (283). Interestingly, BM memory $CD4^+$ T cells preferentially home back to the BM after adoptive transfer (283). A fraction of adoptively transferred splenic $CD8^+$ T cells, particularly those with a memory phenotype, also home to the BM (282, 284, 291). These data suggest that circulating memory T cells have high levels of access to BM niches. High levels of access of memory T cells to the BM niches could also explain the low detection of T_{RM} cells in the parabiosis experiments. More analyses are required for precise characterization of T_{RM} cells in the BM.

CONCLUDING REMARKS

The regulation, generation, and maintenance of T_{RM} cells depends on two primary cell-extrinsic factors: (i) local signals that enable microenvironmental adaptation of T cells in each tissue and (ii) the availability of tissue-specific anatomical niches. Non-immune cells as well as immune cell populations resident in each microenvironment provide these niche factors. Once established, T_{RM} cells function locally to guard the vulnerable sites from reinfection. Hence, a deep understanding the comprehensive picture of T_{RM} niches is required for the development of tissue-targeted vaccination strategies to effectively generate T_{RM} cells in each tissue. For example, "prime and pull" is a potential vaccination strategy for the skin and FRT, where T_{RM} cells can utilize niches that are originally occupied by other resident cells (37, 127). In sharp contrast, this strategy does not work for the lung due to the absence of preformed niches for T_{RM} cells to displace (134, 158). The creation of *de novo* niches in the lung by "prime and pull plus cognate antigen" partly resolves this problem (5, 134, 158). Antigen-niches also play a role in the establishment of T_{RM} cells in the vascularized tissues of the liver, a strategy referred to as "prime and trap" (232).

The description of T_{RM} niches in this review is based primarily on findings from mouse studies with occasional reference to work in humans. It is important to note, however, that the characteristics of T_{RM} cells in these species can vary. For example, the T_{RM} signature in humans is primarily defined by $CD69^+$ expression (292), while CD69 expression is insufficient to infer tissue residence in mice (6, 273). Furthermore, a key transcription factor Hobit that instructs tissue residency is highly expressed by murine T_{RM} cells (62), while its expression is relatively low in human T_{RM} cells (292–294). These, and other, species differences in T_{RM} indicate that many more studies in humans will be necessary for the development of effective vaccines in the clinic.

In summary, the factors regulating the formation of T_{RM} cells in each tissue and each species are far more complex than originally thought, and numerous hurdles exist in generating and maintaining T_{RM} cells in each tissue in terms of the efficacy, safety, and longevity. There is still much to learn.

AUTHOR CONTRIBUTIONS

ST participated in the concept, wrote the manuscript, and developed the figures.

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Memory Inflation Drives Tissue-Resident Memory CD8+ T Cell Maintenance in the Lung After Intranasal Vaccination With Murine Cytomegalovirus

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Tissue-resident memory T (T_{RM}) cells provide first-line defense against invading pathogens encountered at barrier sites. In the lungs, T_{RM} cells protect against respiratory infections, but wane more quickly than T_{RM} cells in other tissues. This lack of a sustained T_{RM} population in the lung parenchyma explains, at least in part, why infections with some pathogens, such as influenza virus and respiratory syncytial virus (RSV), recur throughout life. Intranasal (IN) vaccination with a murine cytomegalovirus (MCMV) vector expressing the M protein of RSV (MCMV-M) has been shown to elicit robust populations of $CD8^+$ T_{RM} cells that accumulate over time and mediate early viral clearance. To extend this finding, we compared the inflationary $CD8^+$ T cell population elicited by MCMV-M vaccination with a conventional $CD8^+$ T cell population elicited by an MCMV vector expressing the M2 protein of RSV (MCMV-M2). Vaccination with MCMV-M2 induced a population of M2-specific $CD8^+$ T_{RM} cells that waned rapidly, akin to the M2-specific $CD8^+$ T_{RM} cell population elicited by infection with RSV. In contrast to the natural immunodominance profile, however, coadministration of MCMV-M and MCMV-M2 did not suppress the M-specific $CD8^+$ T cell response, suggesting that progressive expansion was driven by continuous antigen presentation, irrespective of the competitive or regulatory effects of M2-specific $CD8^+$ T cells. Moreover, effective viral clearance mediated by M-specific $CD8^+$ T_{RM} cells was not affected by the coinduction of M2-specific $CD8^+$ T cells. These data show that memory inflation is required for the maintenance of $CD8^+$ T_{RM} cells in the lungs after IN vaccination with MCMV.

Keywords: $CD8^+$ T cells, cytomegalovirus, memory inflation, respiratory syncytial virus, tissue-resident memory, vaccine

INTRODUCTION

Tissue-resident memory T (T_{RM}) cells protect against invading pathogens in barrier tissues by direct killing of infected cells and by recruitment of other immune effector cell populations into the tissue. Much work has been done in recent years to characterize the migration pattern, function, and phenotype of T_{RM} cells in various anatomical locations (1–4). It has become clear that T_{RM} cells are heterogeneous, and that the requirements for localization and maintenance differ across tissues (4–9). In the lungs, T_{RM} cells have been shown to mediate immune protection against respiratory syncytial virus (RSV) (10–12) and heterosubtypic cross-protection against influenza virus (13–16). T_{RM} cells are also important for immune protection against cancer (17–23). In particular, T_{RM} cells have been shown to enhance the efficacy of intranasally administered cancer vaccines in mouse orthotopic head and neck tumor models (23). The abundance of T_{RM} cells in malignant lung tumors further correlated with survival in humans (23). However, lung-resident T_{RM} cells tend to wane over time, potentially reflecting a harsher and more dynamic environment compared with other barrier tissues (13, 14, 16, 24, 25). This progressive loss of T_{RM} cells likely explains why recurrent infections with RSV and influenza virus occur throughout life. Vaccination strategies aimed at maintaining high levels of T_{RM} cells in the lungs may therefore enhance immunity against respiratory pathogens and cancers.

Cytomegalovirus (CMV) has been shown to elicit robust populations of T_{RM} cells in some tissues (26, 27). The persistent nature of CMV leads to a unique phenomenon among memory $CD8^+$ T cells, which has been well characterized in mouse models using murine cytomegalovirus (MCMV). Specifically, MCMV infection generates two distinct populations of memory $CD8^+$ T cells, termed conventional and inflationary (28–32). Conventional $CD8^+$ T cell populations expand during acute infection and then contract, whereas inflationary $CD8^+$ T cell populations, which may not predominate in the early phase, continue to accumulate over time within the effector memory (EM) compartment. The ability to drive memory inflation may explain why CMV vectors have shown promise as vaccine candidates, protecting against various cancers and infectious agents and providing effective immunocontraception (33–41).

Several factors determine whether a particular epitope will elicit conventional or inflationary $CD8^+$ T cell populations. For inflationary memory responses, the source protein must be transcribed during latency, a feature that depends primarily on location within the genome (42). In addition, the derived epitope may require processing by constitutive proteasomes, because antigen presentation occurs predominantly on the surface of non-hematopoietic cells, which lack immunoproteasomes (43, 44). Interclonal competition may also play a role, given the observation that high-avidity clonotypes are preferentially selected for inflation during MCMV infection (45–47). Similar findings have been reported in the setting of human CMV infection (41, 48–50). Other potential contributors include epitope-dependent requirements for co-stimulation and $CD4^+$ T cell help (51–55). Memory inflation is therefore difficult to predict, even in well-defined mouse models, yet a detailed understanding of this phenomenon is critical for the design of effective vaccines that deliver protective antigens vectored by CMV.

Infection of CB6F1 mice with RSV elicits $CD8^+$ T cell responses that reproducibly target an immunodominant epitope from the M2 protein ($K^d/M2_{82–90}$) and a subdominant epitope from the M protein ($D^b/M_{187–195}$) (56). The M-specific $CD8^+$ T cell population typically incorporates high-avidity clonotypes expressing private T cell receptors with characteristic sequence motifs, leading to greater levels of cytokine production and more effective killing of virus-infected targets in side-by-side comparisons with the M2-specific $CD8^+$ T cell population (57–59). In addition, M-specific $CD8^+$ T cells regulate the magnitude of the otherwise numerically dominant M2-specific $CD8^+$ T cell population, an effect that mitigates the immunopathology associated with acute RSV infection (57).

Intranasal (IN) vaccination with an MCMV vector expressing the M protein of RSV (MCMV-M) has been shown to generate a robust population of M-specific $CD8^+$ T_{RM} cells with an effector/EM phenotype and augment early viral control relative to vaccination with MCMV alone or MCMV-M inoculated *via* the intraperitoneal (IP) route (60). In this study, we characterized the M2-specific $CD8^+$ T cell response to IN vaccination with an MCMV vector expressing the M2 protein of RSV (MCMV-M2). Vaccination with MCMV-M2 induced a population of M2-specific $CD8^+$ T_{RM} cells in the lungs that subsequently waned over time, whereas vaccination with MCMV-M induced a population of M-specific $CD8^+$ T_{RM} cells in the lungs that subsequently inflated over time. Coadministration of both vaccines diminished the M2-specific $CD8^+$ T cell response, but not the M-specific $CD8^+$ T cell response, during the acute phase of infection, but had no impact on the magnitude of the conventional M2-specific $CD8^+$ T cell population or the inflationary M-specific $CD8^+$ T cell population during the chronic phase of infection. Moreover, the inclusion of MCMV-M2 neither enhanced nor impaired the protective effects of vaccination with MCMV-M alone in challenge experiments with RSV.

MATERIALS AND METHODS

Mice

All experiments were conducted with age-matched (6–10 weeks) female CB6F1/J mice (Jackson Laboratories, Bar Harbor, ME, USA). Mice were maintained under specific-pathogen-free conditions on standard rodent chow and water supplied *ad libitum* in the Animal Care Facility at the National Institute of Allergy and Infectious Diseases. This study was carried out in accordance with the recommendations and guidelines of the NIH Guide to the Care and Use of Laboratory Animals. The protocol was approved by the Animal Care and Use Committee of the Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Mice were housed in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Animal procedures were conducted in strict accordance with all relevant federal and National Institutes of Health guidelines and regulations.

Cell Lines

CB6F1 mouse embryonic fibroblasts (MEFs) were isolated as described previously (60). MEFs were cultured in Advanced Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 10 U/ml penicillin G, 10 µg/ml streptomycin sulfate, and 0.1 M HEPES (DMEM-10). Human epithelial type 2 (HEp-2) cells were cultured in Eagle's Minimal Essential Medium (MEM; Invitrogen) containing 10% FBS, 2 mM glutamine, 10 U/ml penicillin G, 10 µg/ml streptomycin sulfate, and 0.1 M HEPES (MEM-10).

Viruses and Infection

Recombinant MCMVs were made using a bacterial artificial chromosome (BAC) system as described previously (35). Briefly, the M and M2 proteins from RSV were inserted into the IE2 gene of the K181Δm157 strain of MCMV using two-step allele replacement. BACs were extracted from *E. coli* using a NucleoBond Xtra Maxi Prep Kit (Clontech, Mountain View, CA, USA). MEFs were transfected with recombinant BACs by calcium phosphate precipitation (Clontech) as described previously (35). Single plaques were isolated by serial dilution after viral passage and selected based on excision of the BAC cassette determined by loss of GFP and confirmed by PCR. Viral stocks were made by sonication of infected MEFs, and plaque assays were performed in triplicate on CB6F1 MEFs. Mice were vaccinated IN with 3×10^5 PFU of recombinant MCMV-M and/or MCMV-M2 in 100 µl of DMEM-10 under isoflurane anesthesia (3%). For RSV challenge, stocks were generated from the A2 strain by sonication of infected HEp-2 monolayers as described previously (61). Mice were challenged IN with 2×10^6 PFU of RSV in 100 µl of MEM-10 under isoflurane anesthesia (3%). All mice were euthanized *via* the administration of pentobarbital (250 mg/kg).

Intravascular Staining and Flow Cytometry

Mice were injected intravenously (IV) with 3 µg of anti-CD45 (BD Biosciences, San Jose, CA, USA). Five minutes after intravascular staining, mice were euthanized with pentobarbital, and the lungs were harvested at various time points. Lymphocytes were isolated by physical disruption of tissue using a GentleMACs Machine (Miltenyi Biotec, San Diego, CA, USA) and separated using density gradient centrifugation with Fico-LITE (Thermo Fisher Scientific, Waltham, MA, USA). Isolated mononuclear cells were washed with phosphate-buffered saline (PBS) and resuspended in fluorescence-activated cell sorting buffer (PBS supplemented with 1% FBS and 0.05% sodium azide). Cells were stained with directly conjugated antibodies specific for the lineage markers CD3 (145-2C11) and CD8 (53-6.7) (BD Biosciences) and the memory markers CD44 (IM7), CD62L (MEL-14), CD127 (A7R34), KLRG1 (2F1/KLRG1), CD69 (H1.2F3), and CD103 (M290) (BD Biosciences or BioLegend, San Diego, CA, USA). Dead cells were excluded from the analysis using LIVE/DEAD Fixable Aqua (Invitrogen). Antigen-specific $CD8^+$ T cells were identified using $D^b/M_{187–195}$ (RSV M) or $K^d/M2_{82–90}$ (RSV M2) tetramers (MBL, Woburn, MA, USA). For validation of intravascular staining, cells were labeled with directly conjugated antibodies specific for CD3 (145-2C11), CD11c (N418), CD64 (X54-5/7.1), SiglecF (E50-2440), and CD11b (M1/70) (BD Biosciences or BioLegend). Data were acquired using an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software version 9.9.6 (TreeStar, Ashland, OR, USA). Memory phenotypes were further analyzed using Pestle version 1.6.2 and SPICE version 6.0 (http://exon.niaid.nih.gov/spice/).

ONX-0914 Inhibition Study

Mice were treated subcutaneously on days 0, 2, 4, and 6 with 2, 6, or 10 mg/kg of ONX-0914 (PR-957; Selleck Chemical, Houston, TX, USA) or vehicle control (10% captisol in 10 mM sodium citrate). On day 0, mice were infected IN as described above with 2×10^6 PFU of RSV. On day 7, mice were euthanized with pentobarbital, and the lungs were harvested and processed as described above.

Plaque Assay

Lungs were weighed and quick-frozen in 10% MEM-10, and plaque assays were performed as described previously (62). Briefly, thawed lung tissue was dissociated using a GentleMACs Machine (Miltenyi Biotec). Cell suspensions were pelleted to remove cellular debris, and clarified supernatants were serially diluted and inoculated in triplicate on 80% confluent HEp-2 cell monolayers. After rocking for 1 h at room temperature, monolayers were overlaid with 0.75% methyl cellulose in MEM-10 and incubated at 37°C. Cells were fixed with 10% buffered formalin and stained with hematoxylin and eosin on day 4. Plaques were counted and expressed as Log_{10} PFU/g of lung tissue. The limit of detection was 1.8 Log_{10} PFU/g.

Statistical Analysis

Statistical analyses were performed using a one-way or two-way ANOVA as appropriate for multiple comparisons (GraphPad Prism, San Diego, CA, USA). Memory phenotypes were compared using a permutation test (10,000 rounds) in SPICE version 6.0 (http://exon.niaid.nih.gov/spice/).

RESULTS

IN Vaccination With MCMV-M2 Elicits More Lung-Resident M2-Specific $CD8^+$ T Cells Than IP Vaccination

We and others have demonstrated that IN vaccination is necessary to elicit T_{RM} cells in the lungs (19, 23, 60). In particular, our earlier work showed that IN vaccination with MCMV-M elicited more M-specific $CD8^+$ T cells in the lung parenchyma than IP vaccination with MCMV-M (**Figure 1A**) (60). To extend this finding, we vaccinated mice with MCMV-M2 *via* the IN or IP route and used intravascular staining in conjunction with $K^d/M2_{82–90}$ tetramers to analyze M2-specific $CD8^+$ T cell responses in the blood and the lung parenchyma after 1 week. The intravascular staining protocol was validated in the context of IN vaccination to ensure that direct infection of the lungs did not lead to increased permeability due to inflammation (Figure S1 in Supplementary Material). Akin to the differences observed

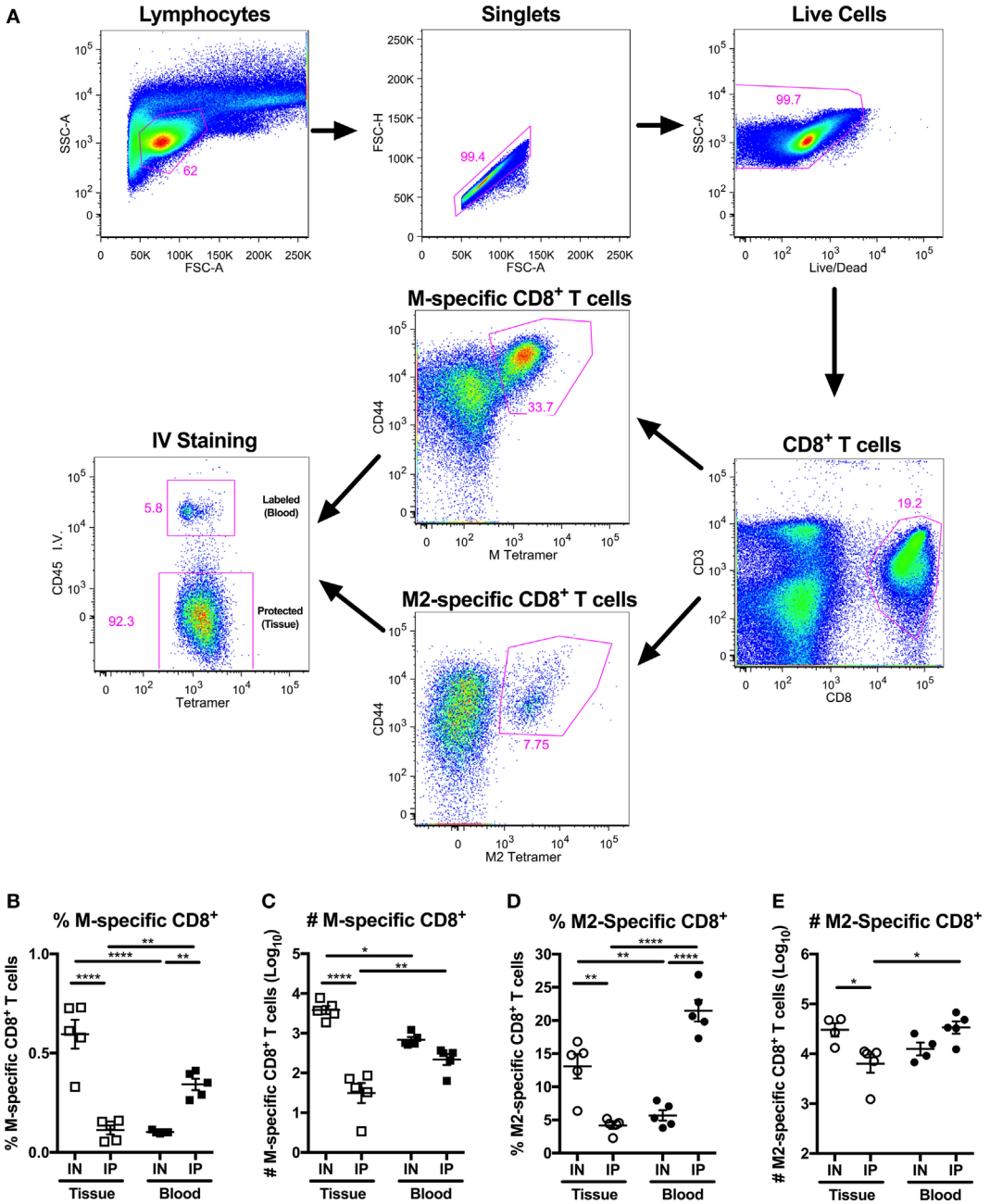
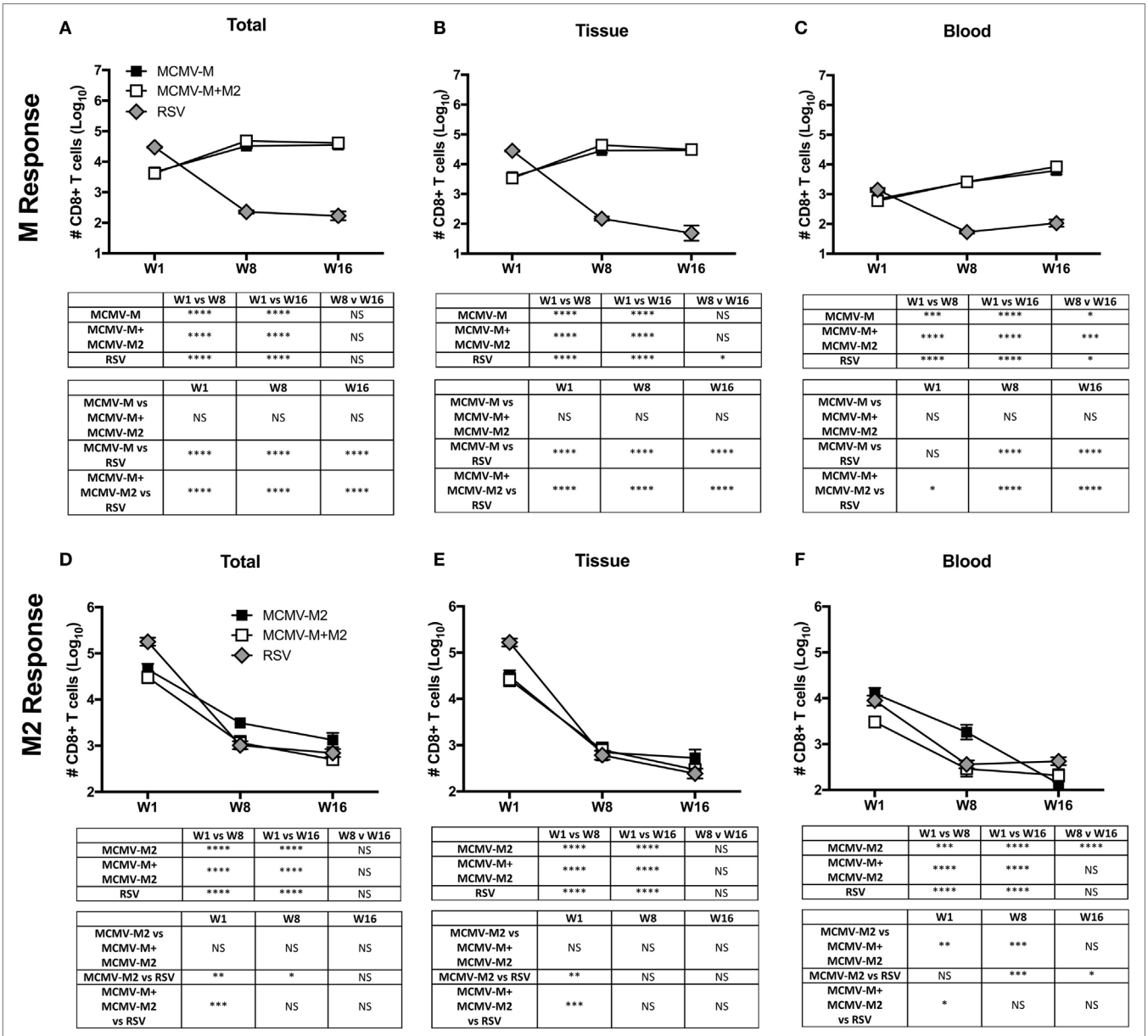


FIGURE 1 | Intranasal (IN) vaccination with murine cytomegalovirus (MCMV)-M2 elicits more lung-resident M2-specific CD8+ T cells than intraperitoneal (IP) vaccination. **(A–E)** Mice were vaccinated with MCMV-M or MCMV-M2 *via* the IN or IP route. Intravascular staining was used in conjunction with $D^b/M_{187–195}$ and $K^d/M2_{82–90}$ tetramers to quantify epitope-specific CD8+ T cells in the lung tissue and blood after 1 week. **(A)** Gating strategy used to identify M-specific and M2-specific CD8+ T cells in the tissue and blood of the lungs. **(B)** Frequency and **(C)** number of M-specific CD8+ T cells in the tissue and blood of lungs 1 week after MCMV-M vaccination. **(D)** Frequency and **(E)** number of M2-specific CD8+ T cells in the tissue and blood of the lungs 1 week after MCMV-M2 vaccination. Bars indicate mean ± SEM ($n = 5$ mice/group). $^{****}P < 0.0001$, $^{**}P < 0.01$, $^{*}P < 0.05$ by two-way ANOVA. Data are shown from one experiment and representative of two independent experiments.

after vaccination with MCMV-M (**Figures 1B,C**), we found that IN vaccination with MCMV-M2 induced significantly more lung-resident M2-specific CD8+ T cells than IP vaccination with MCMV-M2, both in terms of frequency ($P < 0.01$; **Figure 1D**) and number ($P < 0.05$; **Figure 1E**). By contrast, IP vaccination with MCMV-M2 elicited higher frequencies of M2-specific CD8+ T cells in the blood ($P < 0.0001$, **Figure 1D**) and in total ($P < 0.05$), but similar numbers of M2-specific CD8+ T cells in the blood and in total. We therefore focused on IN vaccination in our efforts to induce and maintain lung-resident CD8+ T cells.

The M-Specific CD8+ T Cell Population Inflates, Whereas the M2-Specific CD8+ T Cell Population Contracts, After Vaccination With MCMV

Next, we used a similar approach to evaluate CD8+ T cell responses at weeks 1, 8, and 16 after vaccination with MCMV-M or MCMV-M2 alone or a combination of MCMV-M and MCMV-M2. Intravascular staining was used as above in conjunction with $D^b/M_{187–195}$ and $K^d/M2_{82–90}$ tetramers to quantify epitope-specific CD8+ T cells in the blood and lung parenchyma. MCMV-M administered either alone or together with MCMV-M2 generated an M-specific CD8+ T cell population that inflated between weeks 1 and 8 ($P < 0.0001$) and remained stable through week 16 (**Figure 2A**). This trend was observed in the lung tissue and blood ($P < 0.0001$; **Figures 2B,C**). By contrast, M2-specific CD8+ T cells in the lung tissue and blood contracted over time ($P < 0.0001$; **Figures 2D–F**), irrespective of coadministration with MCMV-M. After RSV infection, which generates only conventional memory responses as a consequence of self-limited



	W1 vs W8	W1 vs W16	W8 v W16
MCMV-M	****	****	NS
MCMV-M+ MCMV-M2	****	****	NS
RSV	****	****	NS

	W1	W8	W16
MCMV-M vs MCMV-M+ MCMV-M2	NS	NS	NS
MCMV-M vs RSV	****	****	****
MCMV-M+ MCMV-M2 vs RSV	****	****	****

	W1 vs W8	W1 vs W16	W8 v W16
MCMV-M	****	****	NS
MCMV-M+ MCMV-M2	****	****	NS
RSV	****	****	*

	W1	W8	W16
MCMV-M vs MCMV-M+ MCMV-M2	NS	NS	NS
MCMV-M vs RSV	****	****	****
MCMV-M+ MCMV-M2 vs RSV	****	****	****

	W1 vs W8	W1 vs W16	W8 v W16
MCMV-M	***	****	*
MCMV-M+ MCMV-M2	****	****	***
RSV	****	****	*

	W1	W8	W16
MCMV-M vs MCMV-M+ MCMV-M2	NS	NS	NS
MCMV-M vs RSV	NS	****	****
MCMV-M+ MCMV-M2 vs RSV	*	****	****

	W1 vs W8	W1 vs W16	W8 v W16
MCMV-M2	****	****	NS
MCMV-M+ MCMV-M2	****	****	NS
RSV	****	****	NS

	W1	W8	W16
MCMV-M2 vs MCMV-M+ MCMV-M2	NS	NS	NS
MCMV-M2 vs RSV	**	*	NS
MCMV-M+ MCMV-M2 vs RSV	***	NS	NS

	W1 vs W8	W1 vs W16	W8 v W16
MCMV-M2	****	****	NS
MCMV-M+ MCMV-M2	****	****	NS
RSV	****	****	NS

	W1	W8	W16
MCMV-M2 vs MCMV-M+ MCMV-M2	NS	NS	NS
MCMV-M2 vs RSV	**	NS	NS
MCMV-M+ MCMV-M2 vs RSV	***	NS	NS

	W1 vs W8	W1 vs W16	W8 v W16
MCMV-M2	***	****	****
MCMV-M+ MCMV-M2	****	****	NS
RSV	****	****	NS

	W1	W8	W16
MCMV-M2 vs MCMV-M+ MCMV-M2	**	***	NS
MCMV-M2 vs RSV	NS	***	*
MCMV-M+ MCMV-M2 vs RSV	*	NS	NS

FIGURE 2 | The M-specific CD8+ T cell population inflates, whereas the M2-specific CD8+ T cell population contracts, after vaccination with murine cytomegalovirus (MCMV). **(A–F)** Mice were infected with respiratory syncytial virus (RSV) or vaccinated with MCMV-M or MCMV-M2 alone or a combination of MCMV-M and MCMV-M2 *via* the intranasal route. Intravascular staining was used in conjunction with $D^b/M_{187–195}$ and $K^d/M2_{82–90}$ tetramers to quantify M-specific **(A–C)** and M2-specific **(D–F)** CD8+ T cells in the lung tissue and blood at weeks 1 (W1), 8 (W8), and 16 (W16). Total **(A,D)** denotes all tetramer+ CD8+ T cells regardless of location. Bars indicate mean ± SEM ($n = 5$ mice/group). ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ by two-way ANOVA. Data are shown from one experiment and representative of two independent experiments.

antigen production, the M-specific and M2-specific CD8+ T cell populations both contracted dramatically between weeks 1 and 8 in the lung tissue and blood ($P < 0.001$; **Figures 2A–F**). Similar epitope-specific patterns were observed when assessing T cell frequency in the lung and spleen (Figure S2 in Supplementary Material). In addition, the MCMV-encoded M38-specific CD8+ T cell response was largely equivalent among experimental groups, suggesting that the observed loss of M2-specific CD8+ T cells over time was not attributable to clearance of MCMV-M2. Thus, the M-specific CD8+ T cell population is inflationary, whereas the M2-specific CD8+ T cell population is not inflationary, after vaccination with MCMV.

One week after vaccination, coadministration of MCMV-M and MCMV-M2 elicited an epitope-specific hierarchy equivalent to that observed after RSV infection, with a dominant CD8+ T cell response to $K^d/M2_{82–90}$ and a subdominant CD8+ T cell response to $D^b/M_{187–195}$ (**Figures 2A,D**). At weeks 8 and 16, this hierarchy was inverted as a consequence of M-specific CD8+ T cell inflation and M2-specific CD8+ T cell contraction (**Figures 2A,D**). Coadministration of MCMV-M and MCMV-M2 did not alter the number or frequency of M-specific CD8+ T cells in the blood or the tissue at any time point relative to vaccination with MCMV-M alone (**Figures 2B,C**; Figure S2B,C in Supplementary Material). By contrast, coadministration of MCMV-M and MCMV-M2 dampened the frequency, but not the overall magnitude, of the M2-specific CD8+ T cell response at week 1 ($P < 0.01$), but not at weeks 8 and 16 (**Figure 2D**; Figure S2D in Supplementary Material). This effect was anatomically discrepant. Specifically, coadministration of MCMV-M and MCMV-M2 did not significantly reduce the number or frequency of M2-specific CD8+ T cells in the lung tissue (**Figure 2E**; Figure S2E in Supplementary Material), but did significantly reduce the number and frequency of M2-specific CD8+ T cells in the blood at weeks 1 and 8 relative to vaccination with MCMV-M2 alone ($P < 0.01$; **Figure 2F**; Figure S2F in Supplementary Material). No significant differences in the frequency of M2-specific CD8+ T cells were observed after contraction of the response at week 16 (**Figures 2D–F**). The reduction of M2-specific CD8+ T cells at the acute time point after coadministration of MCMV-M and MCMV-M2 was not unexpected, because competition between the M-specific and M2-specific CD8+ T cell populations has been demonstrated after RSV infection of CB6F1 mice (57).

The M2 Epitope Is Preferentially Generated by the Immunoproteasome

Memory inflation likely requires epitope generation *via* the constitutive proteasome, because antigen processing and presentation are thought to occur predominantly by non-hematopoietic cells, which lack immunoproteasomes (43, 44). To determine if proteasomal processing impacted the M-specific or M2-specific CD8+ T cell responses, we infected mice with RSV and treated them with the immunoproteasome inhibitor ONX-0914 on days 0, 2, 4, and 6 at doses of 2, 6, or 10 mg/kg. On day 7, we evaluated M-specific and M2-specific CD8+ T cells in the lungs. Treatment with ONX-0914 significantly reduced the frequency and number of M2-specific CD8+ T cells, but not M-specific CD8+ T cells, in a dose-dependent manner (**Figures 3A,B**). These data suggest that the M2 peptide is preferentially generated by the immunoproteasome, whereas the M peptide is preferentially generated by the constitutive proteasome, which is unaffected by ONX-0914.

IN Vaccination With MCMV Elicits CD8+ T_{RM} Cells

A previous study demonstrated that IN vaccination with MCMV-M generated a robust population of T_{RM} cells, identified by expression of CD103 (60). However, it has also been shown that not all T_{RM} cells express CD103 (7). We therefore used intravascular staining to quantify M-specific and M2-specific T_{RM} cells in the lung parenchyma based on expression of CD69 and CD103 after infection with RSV or vaccination with MCMV-M or MCMV-M2 alone or a combination of MCMV-M and MCMV-M2. The administration of MCMV-M, either alone or together with MCMV-M2, generated a substantial population of CD69+ T_{RM} cells that was largely maintained between weeks 8 and 16, and significantly outnumbered the corresponding population

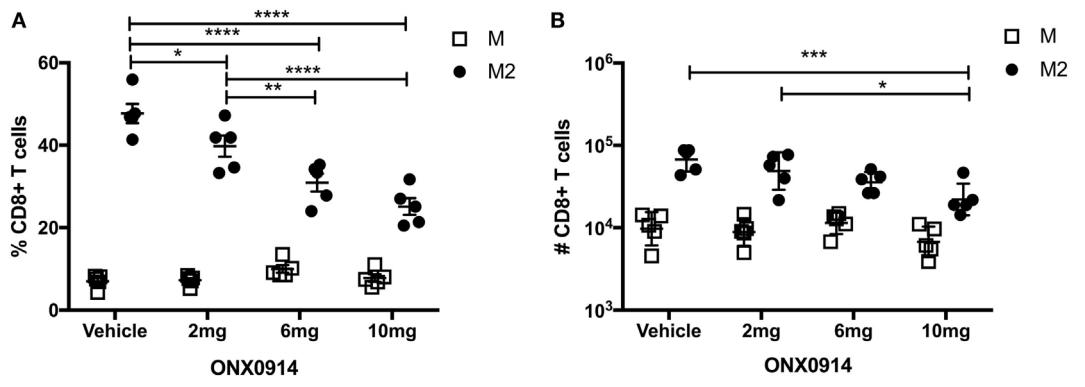


FIGURE 3 | The M2 epitope is preferentially generated by the immunoproteasome. **(A,B)** Mice were infected with respiratory syncytial virus (RSV) and treated with the immunoproteasome inhibitor ONX-0914 or vehicle control on days 0, 2, 4, and 6 at doses of 2, 6, or 10 mg/kg. $D^b/M_{187–195}$ and $K^d/M2_{82–90}$ tetramers were used to quantify the frequency **(A)** and number **(B)** of M-specific and M2-specific CD8+ T cells in the lungs on day 7. $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ by two-way ANOVA. Bars indicate mean ± SEM ($n = 5$ mice/group). Data are shown from one experiment and representative of two independent experiments.

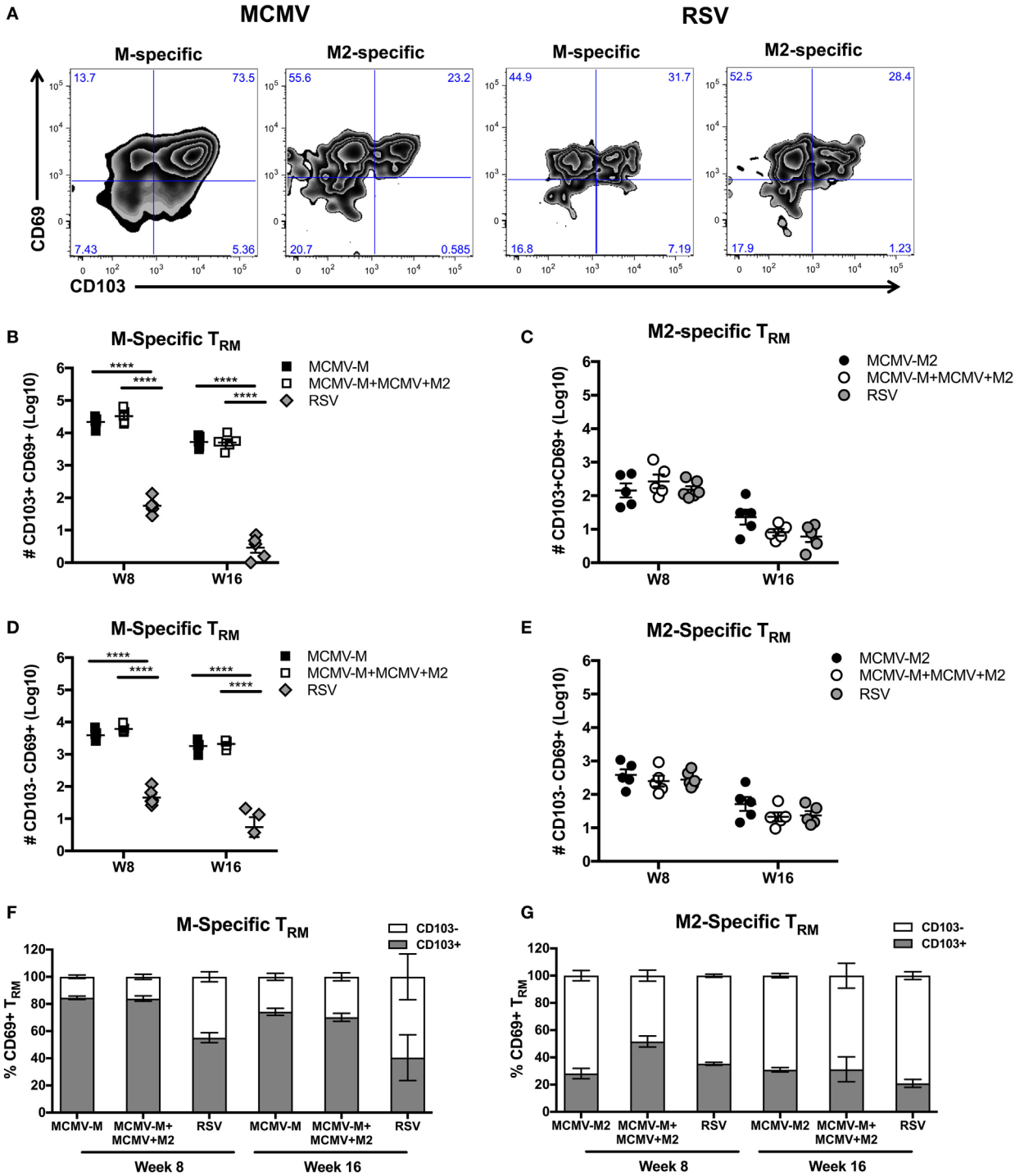


FIGURE 4 | Intranasal (IN) vaccination with murine cytomegalovirus (MCMV) elicits $CD8^+$ tissue-resident memory T (T_{RM}) cells. **(A–E)** Mice were infected with respiratory syncytial virus (RSV) or vaccinated with MCMV-M or MCMV-M2 alone or a combination of MCMV-M and MCMV-M2 *via* the IN route. Intravascular staining was used in conjunction with $D^b/M_{187–195}$ and $K^d/M2_{82–90}$ tetramers to quantify M-specific **(A,B,D,F)** and M2-specific **(A,C,E,G)** $CD8^+$ T cells in the lung tissue at weeks 8 (W8) and 16 (W16). **(A)** Representative flow cytometry plots showing expression of CD69 and CD103 on epitope-specific $CD8^+$ T cells in the lung parenchyma at week 8. **(B,D)** The number of M-specific $CD103^+CD69^+$ T_{RM} cells **(B)** and $CD103^-CD69^+$ T_{RM} cells **(D)** elicited by infection with RSV or vaccination with MCMV-M alone or together with MCMV-M2. **(C,E)** The number of M2-specific $CD103^+CD69^+$ T_{RM} cells **(C)** and $CD103^-CD69^+$ T_{RM} cells **(E)** elicited by infection with RSV or vaccination with MCMV-M2 alone or together with MCMV-M. **(F,G)** Percentage of $CD103^+$ and $CD103^-$ M-specific $CD69^+$ T_{RM} cells **(F)** and M2-specific $CD69^+$ T_{RM} cells **(G)**. $^{****}P < 0.0001$ by two-way ANOVA. Bars indicate mean ± SEM ($n = 5$ mice/group). Data are shown from one experiment and representative of two independent experiments.

of CD69$^+$ T_{RM} cells induced by RSV infection at both time points ($P < 0.0001$; **Figures 4A,B,D**). By contrast, the M2-specific CD69$^+$ T_{RM} population significantly decreased between weeks 8 and 16, irrespective of M2 protein expression *via* MCMV or RSV ($P < 0.01$; **Figures 4A,C, E**). There was no difference in the number of M2-specific T_{RM} cells elicited by vaccination with MCMV-M2 or infection with RSV. As the M-specific T_{RM} population induced by MCMV was maintained in the lungs and the M2-specific T_{RM} population induced by MCMV waned in the lungs, there were significantly more M-specific T_{RM} cells than M2-specific T_{RM} cells in the lung parenchyma at weeks 8 and 16 ($P < 0.0001$; **Figures 4B–E**). In the context of RSV infection, however, there were significantly more M2-specific T_{RM} cells than M-specific T_{RM} cells in the lung parenchyma at both time points ($P < 0.05$; **Figures 4B–E**). Coadministration of MCMV-M and MCMV-M2 did not affect the number of M-specific or M2-specific T_{RM} cells at either time point compared with the administration of MCMV-M or MCMV-M2 alone (**Figures 4B–E**).

Next, we assessed the expression of CD103 on CD69$^+$ T_{RM} cells. After vaccination with single MCMV vectors, a higher proportion of M-specific CD8$^+$ T cells coexpressed CD69 and CD103 compared with M2-specific cells at week 8 (84.6 vs. 28.1%; $P < 0.0001$) and week 16 (74.2 vs. 30.9%; $P < 0.05$) (**Figures 4F,G**). A similar trend was observed after coadministration of MCMV-M and MCMV-M2. At week 8, the vaccine-induced M-specific T_{RM} population also contained a significantly higher proportion of cells expressing CD103 than the M-specific T_{RM} population elicited by RSV infection (84.6% for MCMV-M and 83.9% for MCMV-M + MCMV-M2 vs. 55% for RSV; $P < 0.0001$).

These data show that inflation of the M-specific CD8$^+$ T cell population elicited by vaccination with MCMV enhances the frequency and number of T_{RM} cells relative to acute infection with RSV. By contrast, M2-specific CD8$^+$ T_{RM} cells were induced at similar levels irrespective of M2 protein expression *via* MCMV or RSV. It is also notable that a larger fraction of M-specific CD69$^+$ T_{RM} cells elicited by vaccination with MCMV coexpressed CD103 compared with either M2-specific CD69$^+$ T_{RM} cells elicited by vaccination with MCMV or T_{RM} cells of either specificity elicited by infection with RSV.

M-Specific and M2-Specific CD8$^+$ T Cells Are Phenotypically Distinct in the Lung Tissue and Blood After Vaccination With MCMV

Inflationary and conventional epitope-specific CD8$^+$ T cell populations have previously been shown to differ phenotypically after IP infection with MCMV (63). In this context, inflationary memory cells are predominantly CD127$^-$KLRG1$^+$ effectors, while conventional memory cells display a more CD127$^+$CD62L$^+$ central memory (CM)-like phenotype. This pattern is recapitulated after IP vaccination with MCMV-M. However IN vaccination with MCMV-M induces a CD8$^+$ T cell population with predominantly effector and EM phenotypes (60). We therefore analyzed the phenotype of antigen-specific CD8$^+$ T cells elicited by MCMV-M and/or MCMV-M2 vaccination at 8 weeks post-vaccination. We categorized the RSV-specific CD8$^+$ T cell populations as CM, EM, effector, or KLRG1$^+$ effectors (KLRG1$^+$) (**Figure 5**). Populations were defined as follows: all: tetramer$^+$ CD44$^+$; CM: CD127$^+$KLRG1$^-$CD62L$^+$; EM: CD127$^+$KLRG1$^-$CD62L$^-$; effector: CD127$^-$KLRG1$^-$CD62L$^-$; KLRG1$^+$ effector: CD62L$^-$KLRG1$^+$. Overall, there were no obvious phenotypic differences when the MCMV vectors were administered alone or in combination (**Figure 5B**). By contrast, distinct phenotypes were observed across anatomical compartments for both the M-specific and M2-specific CD8$^+$ T cell populations, with higher frequencies of KLRG1$^+$ effectors (yellow) and CM cells (blue) and lower frequencies of EM cells (green) in the blood compared with the tissue ($P < 0.05$). A comparison of M-specific and M2-specific CD8$^+$ T cells in the blood and tissue also showed that these antigen-specific populations were comprised of different proportions of memory subsets (**Figure 5**, $P < 0.05$). In the blood, the M2-specific CD8$^+$ T cell population incorporated larger fractions of CM (blue) and KLRG1$^+$ effectors (yellow) and smaller fractions of effector (orange) and EM (green) cells than the M-specific CD8$^+$ T cell population. Although statistically significant, the differences between the M-specific and M2-specific CD8$^+$ T cell population were more subtle in the tissue. Interestingly, we observed higher levels of CD44 expression on CD8$^+$ T cells in the lung tissue compared with CD8$^+$ T cells in the blood, irrespective of antigen specificity and vaccination modality (**Figure 5C**). When parsed out by location, expression of CD44 by M-specific and M2-specific CD8$^+$ T cells was relatively high compared with the corresponding bulk CD8$^+$ T cell populations in the blood and tissue of the lungs (Figure S3 in Supplementary Material).

MCMV-Elicited T_{RM} Cells Expedite Viral Clearance After Infection With RSV

To evaluate the biological relevance of these observations, we challenged mice with 2×10^6 PFU of RSV delivered *via* the IN route 16 weeks after vaccination with MCMV-M, MCMV-M2, or a combination of MCMV-M and MCMV-M2. Viral loads were measured on days 3 and 5 after infection with RSV. On day 3, mice vaccinated with MCMV-M or MCMV-M together with MCMV-M2 exhibited significantly lower viral loads in the lungs compared with mice vaccinated with the MCMV vector alone ($P < 0.01$ and $P < 0.05$, respectively; **Figure 6A**). By contrast, vaccination with MCMV-M2 did not lead to a significant reduction in viral load on day 3. All vaccination regimens significantly reduced viral loads on day 5 relative to the MCMV vector alone ($P < 0.0001$ for MCMV-M, $P < 0.01$ for MCMV-M2, $P < 0.0001$ for MCMV-M + MCMV-M2; **Figure 6B**). However, simultaneous vaccination with MCMV-M and MCMV-M2 did not enhance viral clearance relative to vaccination with MCMV-M alone (**Figure 6B**).

Inflation of the M-Specific CD8$^+$ T Cell Population Alters Immunodominance After Challenge With RSV

In further experiments, we assessed the frequency of antigen-specific CD8$^+$ T cells in the lung parenchyma on day 5 after challenge with RSV (**Figures 6C,D**). Mice vaccinated with the MCMV vector alone harbored relatively few M-specific or M2-specific

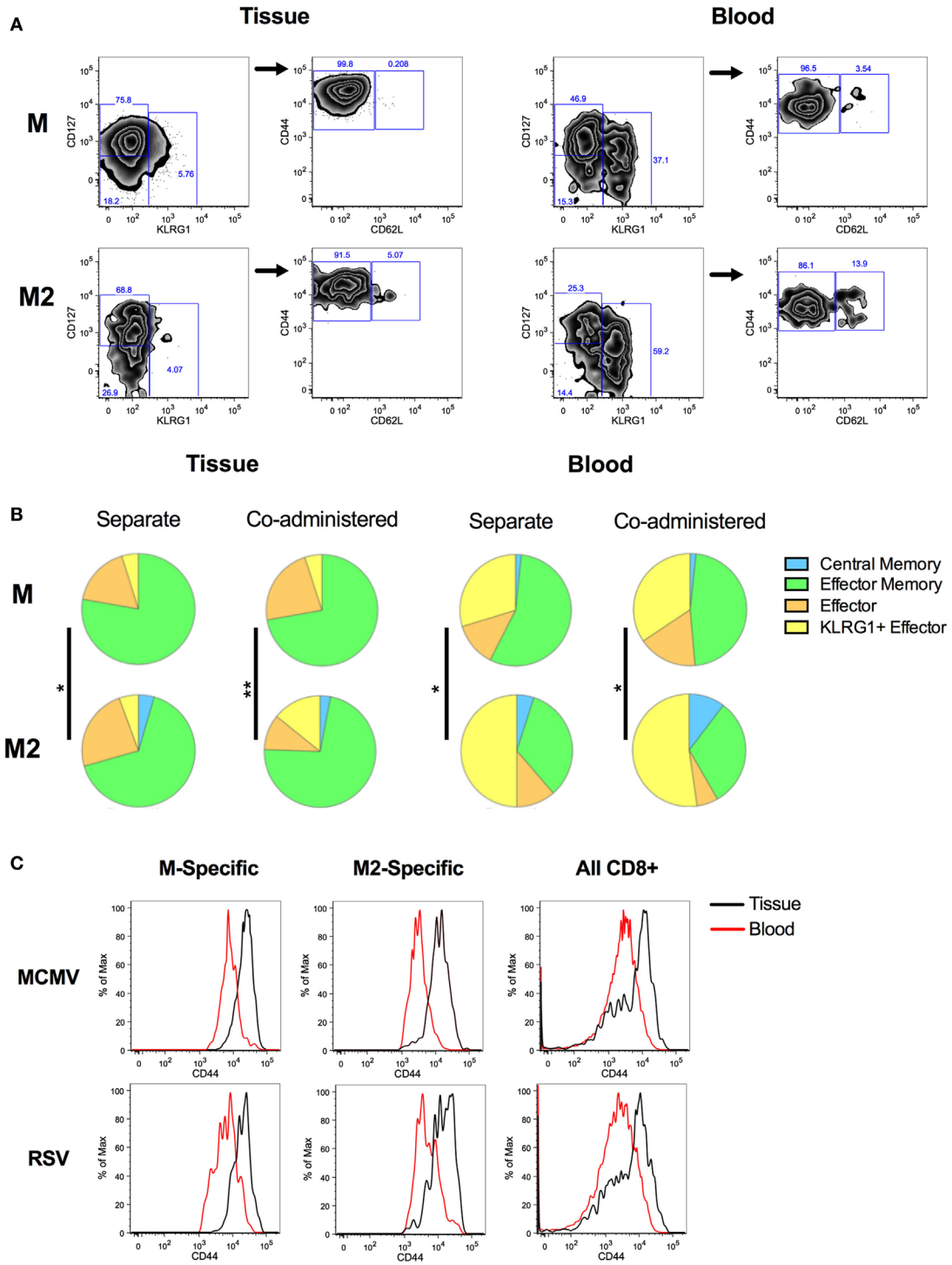


FIGURE 5 | Phenotype of M-specific and M2-specific CD8⁺ T cells elicited by murine cytomegalovirus (MCMV) vaccination. Mice were vaccinated with MCMV-M or MCMV-M2 alone or a combination of MCMV-M and MCMV-M2 *via* the IN route. Intravascular staining was used in conjunction with $D^b/M_{187–195}$ and $K^d/M2_{82–90}$ tetramers to identify M-specific and M2-specific CD8⁺ T cells in the blood and tissue of the lungs at week 8. **(A)** Gating strategy for phenotypic analysis. Populations were defined as follows: $CD127^+KLRG1^-CD62L^+$ [central memory (CM)]; $CD127^+KLRG1^-CD62L^-$ [effector memory (EM)]; $CD127^-KLRG1^-CD62L^-$ (effector); and $KLRG1^+CD62L^-$ ($KLRG1^+$ effector). **(B)** The proportions of CM cells (blue), EM cells (green), effectors (orange), and $KLRG1^+$ effectors (yellow) in the lungs are shown for each specificity. **(C)** CD44 expression on M-specific, M2-specific, and all CD8⁺ T cells in the tissue and blood of the lungs. $^*P \leq 0.05$, $^{**}P < 0.01$ by permutation test (SPICE). Data are shown from one experiment ($n = 5$/group) and representative of two independent experiments.

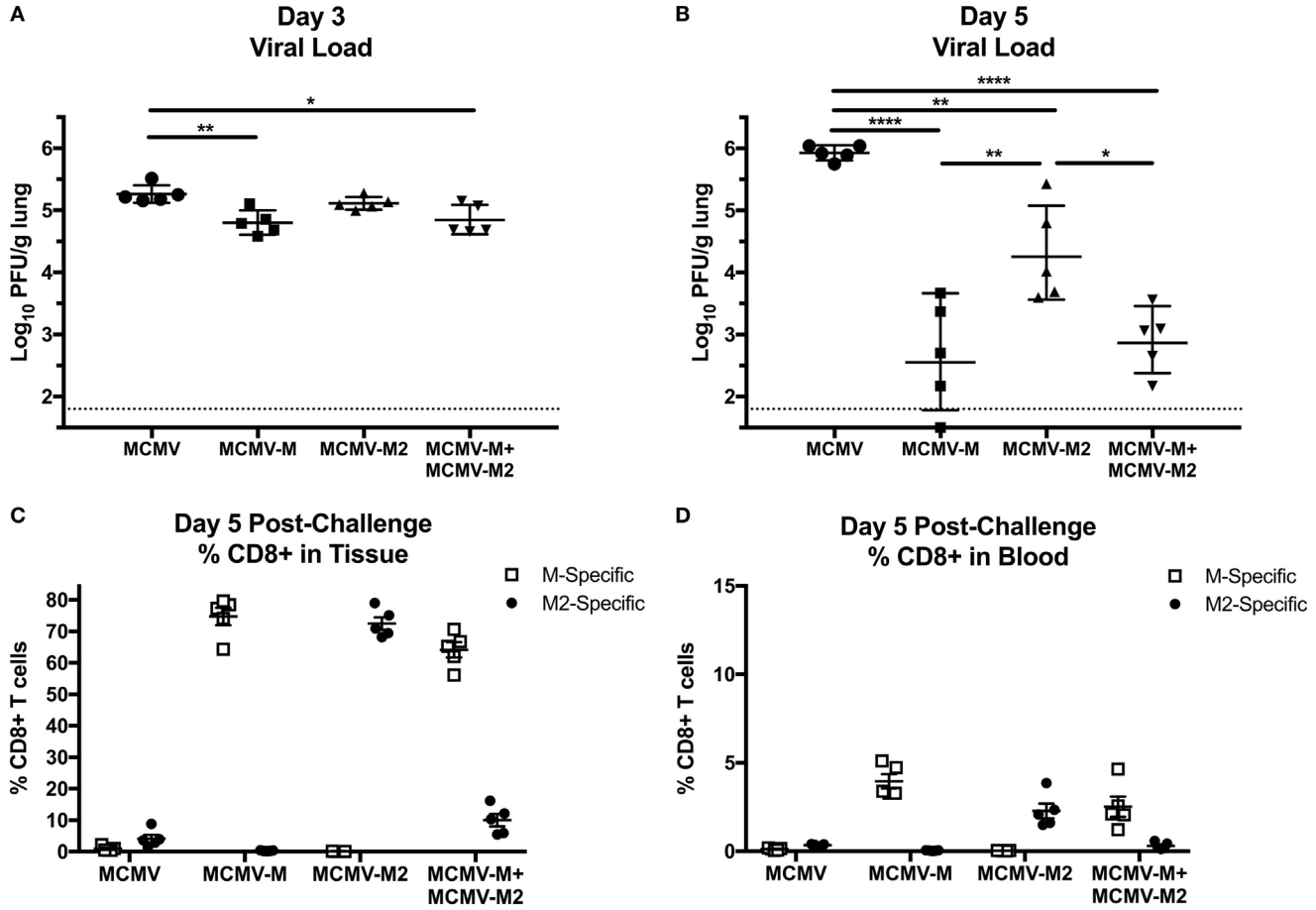


FIGURE 6 | Murine cytomegalovirus (MCMV)-elicited tissue-resident memory T cells expedite viral clearance after infection with respiratory syncytial virus (RSV). **(A–D)** Mice were vaccinated with MCMV vector, MCMV-M or MCMV-M2 alone, or a combination of MCMV-M and MCMV-M2 *via* the intranasal route and challenged with RSV at week 16. Viral titers in the lungs were measured by plaque assay on days 3 **(A)** and 5 **(B)**. $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ by one-way ANOVA. **(C,D)** Intravascular staining was used in conjunction with $D^b/M_{187–195}$ and $K^d/M2_{82–90}$ tetramers to quantify M-specific and M2-specific $CD8^+$ T cells in the lungs **(C)** and the blood **(D)**. $^{****}P < 0.0001$, $^{**}P < 0.01$, $^{*}P < 0.05$ by two-way ANOVA. Data shown from one experiment and representative of two independent experiments.

$CD8^+$ T cells in the lungs, but the M2-specific population was immunodominant, as typically observed in unvaccinated mice after infection with RSV. As expected, mice vaccinated with MCMV-M or MCMV-M2 alone mounted immunodominant $CD8^+$ T cell responses to the corresponding vaccine antigens, whereas mice vaccinated with both MCMV-M and MCMV-M2 displayed very high frequencies of M-specific $CD8^+$ T cells relative to M2-specific $CD8^+$ T cells, inverting the natural immunodominance hierarchy observed after infection with RSV. This finding may explain why the addition of MCMV-M2 did not enhance the protective effects of vaccination with MCMV-M alone in response to challenge with RSV.

DISCUSSION

Vaccination with MCMV-M *via* the IN route has been shown to generate a robust population of M-specific $CD8^+$ T_{RM} cells in the lungs that subsequently inflates over time (60). To extend this finding, we evaluated MCMV vaccine-induced $CD8^+$ T cell responses to the immunodominant M2 epitope. We found that IN vaccination with MCMV-M2 induced a conventional memory response, but failed to establish a stable population of lung-resident M2-specific $CD8^+$ T_{RM} cells. Moreover, coadministration of MCMV-M and MCMV-M2 inverted the natural immunodominance hierarchy, but did not significantly impact the generation of M-specific or M2-specific $CD8^+$ T_{RM} cells. As a consequence, the protective effects of vaccination with MCMV-M were neither impeded nor enhanced by the addition of MCMV-M2.

Memory inflation is essential for the maintenance of lung-resident $CD8^+$ T_{RM} cell populations. In the setting of self-limiting viral infections of the respiratory tract, conventional epitopes induce populations of $CD8^+$ T_{RM} cells in the lung parenchyma that wane over time (14). Our data further show that persistent antigen expression is insufficient to overcome this decline,

consistent with the findings of Smith et al., who demonstrated that T_{RM} cells are maintained in the salivary glands *via* continuous production rather than *via* long-term survival after infection with MCMV (26). In our previous work, we demonstrated that the robust population of M-specific $CD8^+$ T_{RM} cells induced by IN vaccination with MCMV-M contributed to early clearance of RSV (60). This effect was maintained after treatment with a sphingosine 1-phosphate receptor modulator, suggesting that protection was independent of recirculation *via* the lymph nodes. These data concur with the observation herein that IN vaccination with MCMV-M2 failed to mediate early immune control of RSV. Together, these studies highlight the importance of lung-tropic T_{RM} cells in protection against respiratory infection. Accordingly, immunization with a persistent vector offers no immediate advantages over traditional vaccine platforms for conventional epitopes like M2. By contrast, the induction and maintenance of inflationary epitope-specific $CD8^+$ T_{RM} cells in the lungs after vaccination with MCMV may enhance immune protection against respiratory pathogens, which typically induce only transient memory responses at the site of infection (14–16, 24).

Several factors determine the immunogenicity and memory characteristics of any given epitope. In this study, the M and M2 sequences were inserted into the IE2 gene, which naturally encodes inflationary epitopes, and the proteins were under the control of the constitutive promoter IE1 (30, 31, 45). Despite identical genomic locations, the M2 epitope failed to elicit inflationary $CD8^+$ T cell responses. This lack of inflation may reflect greater dependence on the immunoproteasome compared with the M-specific $CD8^+$ T cell response, consistent with previous studies that postulated a key role for antigen processing as a determinant of immunodominance patterns in the context of infection with MCMV (43, 44). In addition, M-specific $CD8^+$ T cells operate with higher composite avidities than M2-specific $CD8^+$ T cells after infection with RSV (57). However, this factor alone may not preclude M2-driven memory inflation, because recent work has demonstrated the existence of low-avidity inflationary $CD8^+$ T cell populations (41). It is also difficult to exclude other possible influences, such as competition between $CD8^+$ T cells with different antigen specificities and variable requirements for co-stimulation and $CD4^+$ T cell help, which are more difficult to assess directly. Any or all of these factors may contribute to the lack of inflation among M2-specific $CD8^+$ T cells. *In vivo* testing is therefore required to assess the true inflationary potential of any given epitope, a process that will become more difficult as vaccines advance from inbred animal models to human populations with diverse genetic backgrounds. A better understanding of the factors that govern memory inflation and how they can be manipulated will be important for the development of CMV vaccines.

As memory inflation is difficult to predict, it is important to study the effect of both inflationary and conventional epitopes in vaccine settings. Coadministration of MCMV-M and MCMV-M2 reduced the overall magnitude of the conventional M2-specific $CD8^+$ T cell response acutely after vaccination but did not impact the inflationary M-specific $CD8^+$ T cell response at any stage after vaccination. Moreover, dual immunization was equivalent to vaccination with MCMV-M alone in terms of protective efficacy after challenge with RSV. These data suggest that both conventional and inflationary epitopes can be included in a persistent vaccine without detrimental effects. However, it should be noted that competition for antigen can occur if inflationary epitopes are delivered by the same vector (45). Individual epitopes are therefore probably best expressed separately if polyvalency is required to prevent immune escape.

In summary, we have shown that memory inflation is required for the maintenance of $CD8^+$ T_{RM} cells in the lungs after IN vaccination with MCMV. These findings highlight an important consideration in the development of persistent vectors and suggest that epitope selection will be a central determinant of efficacy in the setting of vaccines that deliver antigens on a continuous basis.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations and guidelines of the NIH Guide to the Care and Use of Laboratory Animals. The protocol was approved by the Animal Care and Use Committee of the Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Mice were housed in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Animal procedures were conducted in strict accordance with all relevant federal and National Institutes of Health guidelines and regulations.

AUTHOR CONTRIBUTIONS

KM, TR, DP, and BG conceived and designed studies; KM, TR, EB-H, and DN performed animal studies and analyzed data; SM and AR designed and generated recombinant BACs; KM, TR, DP, and BG wrote the manuscript. All authors provided critical feedback and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01861/full#supplementary-material.

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SYSTEMATIC REVIEW
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A Systematic Review: The Role of Resident Memory T Cells in Infectious Diseases and Their Relevance for Vaccine Development

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Background: Resident memory T cells have emerged as key players in the immune response generated against a number of pathogens. Their ability to take residence in non-lymphoid peripheral tissues allows for the rapid deployment of secondary effector responses at the site of pathogen entry. This ability to provide enhanced regional immunity has gathered much attention, with the generation of resident memory T cells being the goal of many novel vaccines.

Objectives: This review aimed to systematically analyze published literature investigating the role of resident memory T cells in human infectious diseases. Known effector responses mounted by these cells are summarized and key strategies that are potentially influential in the rational design of resident memory T cell inducing vaccines have also been highlighted.

Methods: A Boolean search was applied to Medline, SCOPUS, and Web of Science. Studies that investigated the effector response generated by resident memory T cells and/or evaluated strategies for inducing these cells were included irrespective of published date. Studies must have utilized an established technique for identifying resident memory T cells such as T cell phenotyping.

Results: While over 600 publications were revealed by the search, 147 articles were eligible for inclusion. The reference lists of included articles were also screened for other eligible publications. This resulted in the inclusion of publications that studied resident memory T cells in the context of over 25 human pathogens. The vast majority of studies were conducted in mouse models and demonstrated that resident memory T cells mount protective immune responses.

Conclusion: Although the role resident memory T cells play in providing immunity varies depending on the pathogen and anatomical location they resided in, the evidence overall suggests that these cells are vital for the timely and optimal protection against a number of infectious diseases. The induction of resident memory T cells should be further investigated and seriously considered when designing new vaccines.

Keywords: resident memory T cells, infectious diseases, vaccine development, immunity, microbiology

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INTRODUCTION

Traditionally, memory T cells have been subdivided into two broad categories: effector memory and central memory T cells (T_{EM} and T_{CM}, respectively). After the realization that some memory T cells fail to egress out of peripheral tissues back into the blood stream, it became clear that this dichotomous distinction of memory T cells did not account for the complete diversity of the memory T cell population. This led to the discovery of a third subset of memory T cell. Appropriately, dubbed "Tissue-resident memory T cells" (here after referred to as T_{RM}), this newly defined population exhibits the unique feature of remaining localized in peripheral tissues (1). As such, these cells provide enhanced localized immunosurveillance and protection of peripheral tissues when compared to T_{EM} and T_{CM}. T_{RM} have been characterized in many peripheral tissues, including skin, lungs, brain, liver, the female reproductive tract, and the gastrointestinal mucosa. Given the huge variance in their location of residence, this subset of memory cell is highly heterogeneous, phenotypically varying depending on their anatomic location and the inflammatory cues produced by their respective microenvironment. Although experimental techniques such as parabiosis can definitively distinguish T_{RM} from circulating memory T cells, other less complex methods of identifying T_{RM} are more frequently used. The co-expression of CD69 and CD103 is commonly used as a marker of tissue residence, although it appears not all bona fide T_{RM} are defined by this particular phenotype. Regardless, T_{RM} have been implicated in a wide range of physiological functions, such as providing protection against pathogens and cancerous cells, as well as in many pathological states such as autoimmune and other inflammatory diseases. The exploration of T_{RM} biology and the role they play in maintaining homeostasis has broad implications for human health. Currently, our understanding of T_{RM} function is largely constrained within the context of infectious diseases. As of now, it appears that T_{RM} are better adapted to providing rapid protection against pathogen invasion when compared to their circulating counter parts (2–4). Thus, vaccines of the future would ideally establish a population of protective T_{RM} at the portals of entry most at risk of pathogen invasion to provide immediate and effective immunity, rather than relying on the delayed recruitment of effector cells from the circulating pool of memory cells. Since parenterally administered vaccines induce minimal tissue-specific protection, current routes of administering vaccines may need to be revised (5, 6). The present review will primarily focus on the role of T_{RM} in the immune response generated to a range of human pathogens and discuss future avenues for the development of T_{RM}-based vaccines.

METHODOLOGY

A systematic search of published literature was conducted. Literature was critically evaluated for evidence of the role T_{RM} play during infections and in vaccinology. A flowchart summarizing our methodology has been included (**Figure 1**). The preparation of this review was guided by the *PRISMA-P 2015 guideline* (7).

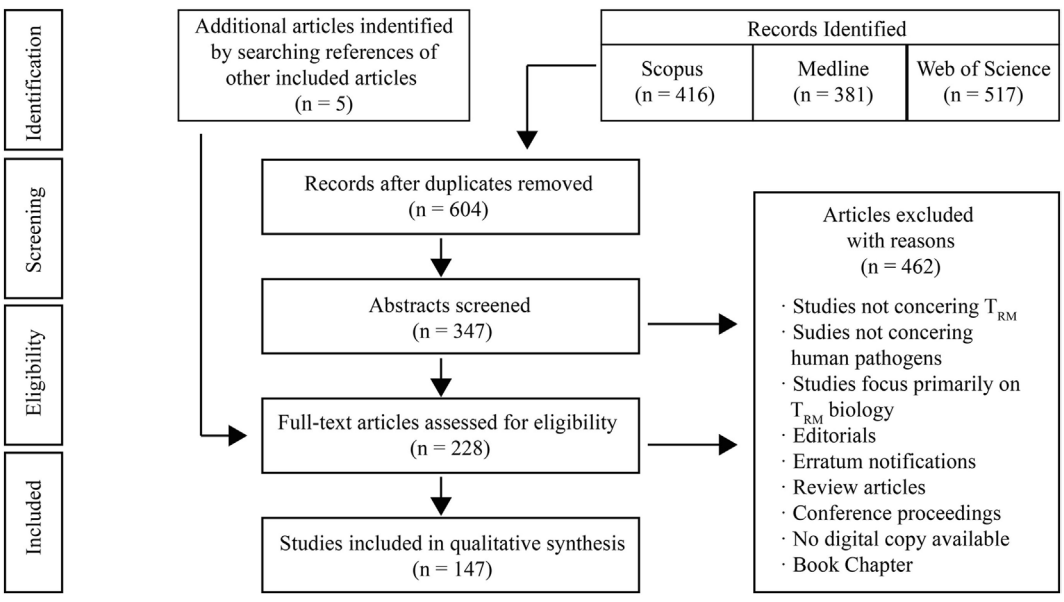

FIGURE 1 | Literature search strategy. The search strategy used revealed 381 records in Medline (Ovid), 416 in SCOPUS, and 517 in Web of Science. This resulted in a total number of 1,314 records. After removing duplicates, there were 604 records. Screening of titles resulted in the exclusion of 257 records, as they did not address resident memory T cells, human infectious diseases, or neither. Others records were excluded as they were reviews, editorials, meeting abstracts, book chapters, poster presentations, or erratum notifications. The abstracts of the remaining 347 records were analyzed and a further 124 publications were excluded due to their focus on T_{RM} biology. The full texts of the remaining studies were reviewed. 81 of these texts were excluded for aforementioned reasons. Co-authors were consulted when there was ambiguity regarding the relevance of a study. In total, 142 publications from the search were included. 5 additional studies were included by screening the references of studies from the search results and following external review.

Final searches of literature were performed on March 23, 2018 in Medline, SCOPUS and Web of Science by the first author. The Boolean search strategy used was as following ("resident memory t cell*" OR "t resident memory cell*" OR "tissue resident memory cell*" OR "resident memory" OR "tissue memory"). The references of included studies were also screened for other relevant publications.

Both human and animal studies that use surface markers of residence or other established techniques such as intravascular staining and parabiosis to illustrate localization of T cells to peripheral tissues, as well as T cell phenotyping were included. Studies were also screened for their relevance to human pathogens, and thus animal infection models that are analogous to human infectious diseases were included. Studies were included irrespective of published date. Only published and accepted manuscripts of original research were included. Publications that primarily focused on T_{RM} biology (ontogeny, cellular metabolism, etc.) or non-infectious diseases were not included. Certain non-communicable diseases such as hepatocellular carcinoma and cervical cancer that can be caused by pathogens are briefly mentioned within the broader discussion of T_{RM}-mediated immunity.

RESULTS OF SEARCH

The results of the search strategy are summarized in **Figure 1**.

DATA SYNTHESIS AND ANALYSIS

The first author conducted extraction of data from relevant studies. This review has been divided into sections based on pathogen type: viruses, bacteria, parasites/helminths, and fungi (**Figure 2**). The studies included in this review contain the most relevant findings related to immune responses generated by T_{RM} against human pathogens, or make use of novel strategies for T_{RM} generation. We apologize to authors whose work could not be included in this review.

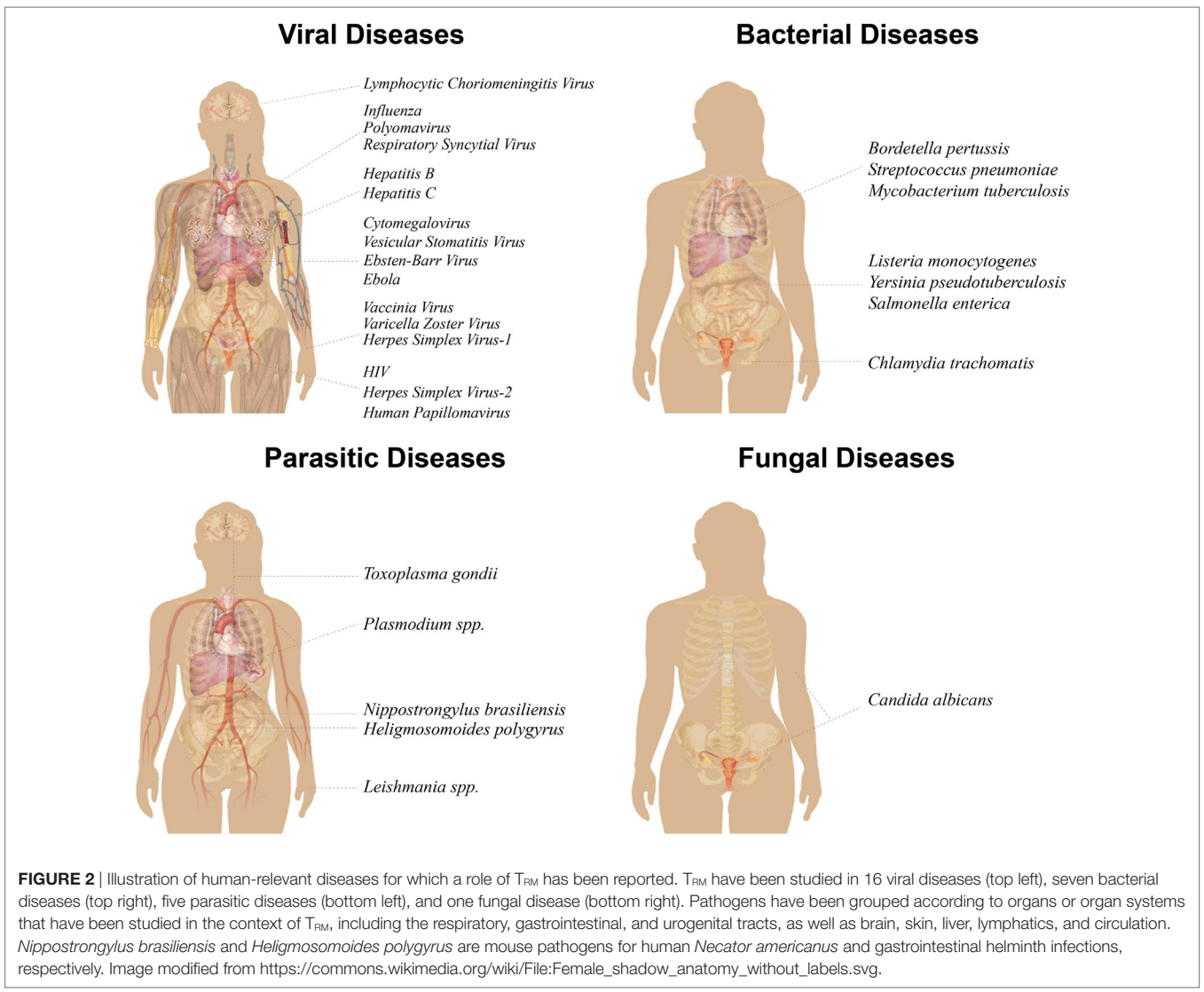

FIGURE 2 | Illustration of human-relevant diseases for which a role of T_{RM} has been reported. T_{RM} have been studied in 16 viral diseases (top left), seven bacterial diseases (top right), five parasitic diseases (bottom left), and one fungal disease (bottom right). Pathogens have been grouped according to organs or organ systems that have been studied in the context of T_{RM}, including the respiratory, gastrointestinal, and urogenital tracts, as well as brain, skin, liver, lymphatics, and circulation. *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* are mouse pathogens for human *Necator americanus* and gastrointestinal helminth infections, respectively. Image modified from https://commons.wikimedia.org/wiki/File:Female_shadow_anatomy_without_labels.svg.

THE ROLE OF T_{RM} IN VIRAL INFECTIONS

As of present, T_{RM} immune responses are by far mostly studied in the context of viral pathogens. The following section will present findings by specific viral pathogen/viral disease.

HERPES SIMPLEX VIRUS (HSV)

Herpes simplex virus causes infections that present with a varying range of symptoms. The primary clinical manifestations of HSV infection are intraepithelial vesicles. There are two antigenically distinct HSV subtypes: HSV-1 and HSV-2, causing cold sores and genital warts, respectively (8). However, both sub-types can be the etiology of either clinical disease as sexual transmission allows for spread between the two sites (9). Both viruses establish a life-long latent infection within the surrounding nervous tissue, and control of HSV infection requires effective cell-mediated immune responses to prevent reactivation. However, co-morbid illnesses, immunosuppressive drugs, UV exposure, and psychological stress can hinder immune control. A number of studies suggest that T_{RM} are implicated in controlling HSV-1 latency in the trigeminal ganglia (10). HSV-1 infection models primarily focus on infections of the skin and nervous tissue such as the trigeminal ganglia and the eyes (11–22). Following acute infection with HSV-1, $CD8^+$ T_{RM} remain localized to the skin initially infected and are also found surrounding latently infected sensory ganglia (11). However, evidence suggests that multiple exposures to cognate antigen can substantially increase the T_{RM} population in not only the site of HSV infection, but also in distant skin (12). $CD8^+$ skin T_{RM} appear to resemble the antigen-presenting Langerhans cells of the skin, extending dendritic projections into the surrounding tissue, probably in an attempt to survey the local area for antigen (13). This is supported by evidence from confocal microscopy and intravital imaging (21) that suggests these T_{RM} can travel between keratinocytes (13). However, unlike Langerhans cells, these T_{RM} do not extend into the stratum corneum (13, 17). It also appears that skin T_{RM} are not specifically attracted to virally infected cells, and thus migrate throughout the epidermis in a random manner (17, 21). By extension, it can be inferred that skin T_{RM} may take a considerable period of time before identifying virally affected cells. As such, it may be safe to assume that a critical mass of skin T_{RM} is needed in order to afford timely protection. This notion is supported by the observation that protection appears to be dependent upon the local density of T_{RM} (17). Upon antigen recognition, skin T_{RM} undergo a change in their morphological and motility pattern, decelerating their migratory rate and losing their dendricity (13, 17). This is probably indicative of a shift in role from immunosurveillance to effector function. Furthermore, the maintenance of HSV-1-specific T_{RM} populations appears to be independent of circulating T cells in both skin and trigeminal ganglia (14, 17). Skin T_{RM} appear to be able to sustain their numbers through local proliferation after secondary infection (17). However, after a combined corticosterone and stress-induced reduction of trigeminal ganglia $CD8^+$ T cells (presumably T_{RM}), there appeared to be no increased proliferation of remaining T cells when compared to the homeostatic proliferation rates as indicated by BrdU incorporation (14). Analysis of HSV-1-specific skin and dorsal root ganglia T_{RM} during acute immunity and later time points revealed that transcription of cytolytic molecules decreases with time. As such, T_{RM}-based immunity in the long term may not be reliant on enhanced cytolytic effector functions, but rather on the localization of these cells at sites susceptible to reinfection (15). The chronic inflammatory response induced by persistent viral gene expression during latency leads to T_{RM} exhaustion in the brain ependymal region, rendering them unable to control HSV-1 infection (16). Perhaps the reason why T_{RM} downregulate their cytolytic genes during times of homeostasis is because continuous expression may lead to exhaustion. Nevertheless, it appears that the generation of $CD8^+$ T_{RM} in skin and ganglia may be a viable option for protection against HSV-1 infection or reactivation. Local inflammation of the skin and mucosa alone can encourage the recruitment of T_{EM} to these peripheral sites where differentiation into the T_{RM} phenotype occurs. This was demonstrated using 2,4-dinitrofluorobenzene, a contact-sensitizing agent. Furthermore, the application of nonoxynol-9 (a spermicide agent) to the female genital tract enhanced protection against HSV challenge, correlating with higher numbers of $CD103^+$ T cells localizing to the epithelium (19). Hence, agents that can be applied to specific tissue and that cause a localized, general inflammatory response may be a strategy worth exploring for the generation of T_{RM}. More specifically, however, the CXCL10/CXCR3 chemokine pathway appears to be vital in generating T_{RM}, as mice deficient in either CXCL10 or CXCR3 were unprotected against HSV-1 UV-B light-induced reactivation challenge. Furthermore, the administration of CXCL10 into deficient mice through the use of a neurotropic virus vector amplified T_{RM} in the trigeminal ganglia, conferring better protections against reactivation challenge (20). CXCL10 administration through the use of a rAAV8-CamKIIa-GFP-CamKIIa-CXCL10 vector showed similar results (18). Samples from human patients that were asymptomatic but seropositive for HSV-1 infection were used to determine which 467 HLA-A*0201-restircted $CD8^+$ T cell epitopes were immunodominant in the HSV-1-specific immune response. These asymptomatic individuals generated a high number of polyfunctional $CD8^+$ T_{EM} against three epitopes. HLA-A*0201 transgenic mice were primed with these epitopes and subsequently treated with an ocular topical preparation containing rAAV8-CamKIIa-GFP-CamKIIa-CXCL10 to deliver exogenous CXCL10 chemokine. Results from UV-B reactivation challenge demonstrated that this strategy was able to reduce viral shedding in tears and recurrent herpetic ocular disease (18). This strategy may be beneficial in rationally developing novel vaccines against other diseases.

Human studies have also been conducted in HSV-2 infection. Samples from the genital tract of HSV-2-infected women contained populations of HSV-2-specific T cells with a T_{RM} phenotype (23). More interestingly, a population of $CD8\alpha\alpha^+$ T_{RM} that reside at the dermal–epidermal junction have also been described in biopsies of HSV-2-infected humans. This unique positioning suggests that these cells may be able to survey the neural tissue from

which virus travels to the skin during reactivation (24). Thus, T_{RM} play a role in the natural immune response against HSV-2 infection. Although it was already demonstrated that T cells could be recruited to peripheral tissues using inflammatory agents (19), the "prime and pull" vaccine strategy was first described in a HSV-2 infection model (25). In this study, the investigators explored the novel idea of parenterally immunizing mice and subsequently topically administering CXCL10 into the vagina before challenging with HSV-2. Mice that underwent the prime and pull protocol showed minimal signs of clinical disease and had a survival rate of 100%. Naive and parenterally immunized mice that did not receive the pull treatment developed clinical disease and exhibited high mortality rates. This strategy also demonstrated the capacity to prevent infection of sensory neurons (25). Further investigation of this protocol revealed that immunity was largely dependent on INF-γ produced by $CD8^+$ T_{RM} (26). Re-stimulation of this $CD8^+$ T_{RM} was dependent on a population of $CD301b^+$ dendritic cells that resided in the lamina propria. In fact, depletion of $CD301b^+$ dendritic cells using a diphtheria toxin model rendered the prime and pull strategy ineffective and mice suffered high morbidity and mortality rates (26). Although a non-specific inflammatory stimulus such as nonoxynol-9 may be sufficient to pull $CD8^+$ T cells in to the female reproductive tract and subsequently convert them to T_{RM}, it appears that antigen presentation by $CD301b^+$ dendritic cells is needed for $CD8^+$ T_{RM}-mediated immunity at this site. A different study that made use of a topical vaccine containing a human papillomavirus (HPV) vector expressing gB and gD ectodomains of HSV-2 has shown the capacity to generate T_{RM} in the reproductive tract, and reduce viral shedding and clinical disease (27). This study highlights the capacity of HPV vectors to induce T_{RM} in the genital tract, a vaccine strategy that may be applicable to other sexually transmitted infections. HSV-2-specific $CD8^+$ T_{RM} can also be generated using a "chemical-free and biological-free" laser adjuvant, a protocol that could be explored in other infectious models (28). While the vast majority of studies have assessed the protective capabilities of $CD8^+$ T_{RM} during HSV infection, very few studies have analyzed the role of $CD4^+$ T_{RM} in HSV infections (21, 29). Intravaginal vaccination of mice with thymidine kinase negative HSV-2 (an attenuated form of the virus) provided full protection against challenge with wild-type HSV-2, independent of $CD8^+$ T cells and B cells. Instead, parabiosis studies demonstrated that $CD4^+$ T_{RM} are required within the genital tract mucosa for immunity in this model. These $CD4^+$ T_{RM} are polyfunctional, secreting IFN-γ, TNF-α, and IL-2, and resided in organized, non-tertiary immune structures called memory lymphocyte clusters (MLCs). These MLCs appear to assemble under the influence of macrophage-secreted CCL5. Upon antigen stimulation, the $CD4^+$ T_{RM} within the MLCs expand and secrete high levels of IFN-γ. The investigators of this study also report that circulating memory T cells were "barely recruited" when MLCs were present in the mucosa. This suggests that $CD4^+$ T_{RM} may be capable of clearing or controlling infection even before recruiting signals are generated in a magnitude large enough to attract circulating T cells to the site of infection (29). It still remains necessary to explore whether a critical mass of $CD4^+$ T_{RM}-containing MLCs are needed within the genital tract to provide protection. It is likely that this profound role of $CD4^+$ T_{RM} in mediating immunity during HSV-2 infection is due to the location of the infection (genital tract) rather than the viral factors alone. This is supported by the fact that MLCs have also been found in the genital tract of both human and mice secondary to infections caused by a bacterial pathogen [refer to *Chlamydia trachomatis* (*Ct*) section of this review]. Despite the difficulties in generating $CD4^+$ TRM following prime and pull vaccination, an ideal vaccine against HSV-2 should generate $CD8^+$ and $CD4^+$ T_{RM} (25).

INFLUENZA

Influenza viruses are a major cause of respiratory infections. Although influenza vaccines have been in use for many years, antigenic drift of surface hemagglutinin and neuraminidase proteins require annual immunizations. Antigenic shift can result in highly virulent strains of influenza that cause devastating pandemics (30). The ideal influenza vaccine would provide heterotypic immunity that prevents the escape of newly mutated viruses. Current influenza vaccines rely on generating high neutralizing antibody titers to protect against infection. Although this strategy has demonstrated efficacy in mediating protection, the inability of antibodies to neutralize new variants of the virus has sparked research into alternate strategies (31). Growing evidence suggests that efforts should be focused on developing vaccines that generate T_{RM}-mediated immunity (32). Analysis of human samples has revealed that influenza-specific T_{RM} can be found in substantial numbers in lung tissue, highlighting their role in natural infection (33, 34). Despite expressing low levels of granzyme B and CD107a, these $CD8^+$ T_{RM} had a diverse T cell receptor (TCR) repertoire, high proliferative capacities, and were polyfunctional (34). Influenza infection history suggests a greater level of protection against re-infections likely due to the accumulation of $CD8^+$ T_{RM} in the lungs (35). Furthermore, the natural immune response to influenza A virus infection in a rhesus monkey model demonstrated that a large portion of influenza-specific $CD8^+$ T cells generated in the lungs were phenotypically confirmed as $CD69^+CD103^+$ T_{RM} (36). Unlike lung parenchymal T_{RM}, airway $CD8^+$ T_{RM} are poorly cytolytic and participate in early viral replication control by producing a rapid and robust IFN-γ response (37, 38). Bystander $CD8^+$ T_{RM} may also take part in the early immune response to infection through antigen non-specific, NKG2D-mediated immunity (39). The generation of functional T_{RM} that protect against heterosubtypic influenza infection appear to be dependent on signals from $CD4^+$ T cells (40). A role for $CD4^+$ T_{RM} has also been reported (41). Much like their $CD8^+$ counterparts, $CD4^+$ T_{RM} also produce a significant IFN-γ response during early infection (42, 43). Aside from the $CD8^+$ and $CD4^+$ subsets of T_{RM}, a subset of $NK1.1^+$ double negative T memory cells which reside in the lungs also play a role in influenza infection (44). Taken together, these studies and others (45–47) demonstrate that T_{RM} are required for optimal protection. However, unlike T_{RM} in other locations, such as the skin, lung T_{RM} are not maintained for extended periods of time. This gradual loss of lung T_{RM} appears to be the reason for the loss in heterotypic immunity against influenza infection (45, 46, 48). Lung T_{RM} exhibit a transcriptional profile that renders them

susceptible to apoptosis (48). Despite conflicting evidence (49), it appears that maintenance of the lung $CD8^+$ T_{RM} populations relies on the continual seeding from circulating $CD8^+$ T cells. However, with time, circulating $CD8^+$ T cells adopt a transcriptional profile that reduces their capacity to differentiate into T_{RM}. Expanding the $CD8^+$ T_{EM} compartment through booster vaccination may circumvent the problem of these time-sensitive transcriptional changes (48). There is also conflicting evidence regarding the requirement of local antigen for the generation of T_{RM} within the lung (48, 50). Continuing to find ways to generate and maintain lung T_{RM} is of great importance for vaccines against pulmonary infections. Intranasal administration of vaccines seems to encourage the development of a strong mucosal immune response (51, 52). Intranasal administration of Live Attenuated Influenza Vaccine (FluMist) in a mouse model induced both $CD4^+$ and $CD8^+$ T_{RM} that provided a degree of cross-strain protection independent of T_{CM} and antibodies (53). The intranasal administration of a PamCys2 or Adjuplex has demonstrated capacity for producing protective influenza-specific lung $CD8^+$ T_{RM} in similar numbers and IFN-γ secreting potential when compared to the natural response to influenza infection (54, 55). Furthermore, a vaccine containing virus-like particles with tandem repeat M2e epitopes generated heterotypic immunity through the induction of antibodies, and protection correlated with IFN-γ-secreting $CD8^+$ T_{RM} (56). A Modified Vaccinia Ankara-vectored virus expressing conserved influenza nucleoprotein and matrix protein 1 elicited an IFN-γ secreting $CD4^+$ T cell and $CD8^+$ T_{RM} response (57). Co-administration of 4-1BBL (CD137 signal) along with an influenza nucleoprotein expressing replication defective adenovirus vector *via* the intranasal route stimulated and boosted a lung $CD8^+$ T_{RM} response through the recruitment of circulating T cells (58). Intranasal administration of 4-1BBL may serve as a promising "pull" strategy in systemically primed individuals. Another potential "pull" strategy is the intranasal administration of Fc-fused IL-7. This protocol was used as a pre-treatment before influenza A infection, and demonstrated protective capacities in mice against lethal challenge. It appears that Fc-fused IL-7 recruits polyclonal circulating T cells into the lungs, which subsequently reside in the lung tissue as "T_{RM}-like cells" (59). Intranasal administration of Fc-fused IL-7 after systemic priming may be able to recruit influenza-specific T cells into the lungs, and may be a strategy for inducing lung T_{RM}. An antibody targeted vaccination strategy in which antigens are coupled to monoclonal antibodies against $CD103^+$ or $DNGR\text{-}1^+$ dendritic cells has also been shown to elicit a protective $CD8^+$ T_{RM} response (47, 60).

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Human immunodeficiency virus is a retrovirus that is transmitted *via* contact with infected blood and other fluids such as semen and vaginal secretions. The virus specifically targets the surface proteins CD4, CXCR4, and CCR5, with the natural progression of disease resulting in the depletion of $CD4^+$ T cells. As a consequence, infected individuals are left in an immunocompromised state referred to as aquired immunodeficieny syndrome (AIDS), which is characterized by fatal opportunistic infections and malignancies. Although the development of therapeutics such as anti-retroviral therapy has reduced the incidence of AIDS, HIV/AIDS continues to contribute significantly to global morbidity and mortality. Evidence shows that $CD8^+$ T cells are vital in controlling early infection (61). Studies of human tissue samples have revealed that T_{RM} are generated in response to HIV infection in multiple locations including the gastrointestinal tract and the female reproductive tract (62–65). Furthermore, individuals who appeared to naturally control infection had T_{RM} that were capable of producing the highest polyfunctional immune responses when compared to individuals who did not. However, the T_{RM} population within the HIV-specific $CD8^+$ T cell compartment in individuals who controlled infection was under-represented when compared to individuals who were viremic (62). Although not confirmed, this may be due to the higher ability of polyfunctional T_{RM} in these individuals to recruit circulating T cells, thereby only altering the T_{RM} proportion. Similar to other infections in various sites, $CD8^+$ T_{RM} in the context of HIV can be sub-divided into two subsets based on the expression of CD103 (62, 63). Analysis of the ectocervial epithelium and menstrual blood revealed that HIV-infected women were more likely to have $CD103^-$ T_{RM} when compared to healthy individuals (63, 64). This reduced expression of CD103 may be explained by the HIV-induced depletion of $CD4^+$ T cells which appear to be vital in providing help to $CD8^+$ T cells for up-regulating CD103 (64). The $CD103^-$ populations of the ectocervix resided closer to the basement membrane of the epithelium when compared to their $CD103^+$ counterparts. Interestingly, the $CD103^+$ population from infected individuals appears to express higher levels of PD-1 (63). In a separate study, adipose $PD\text{-}1^+$ $CD4^+$ T_{RM}, appeared to remain relatively inactive during HIV infection and may serve as a reservoir for HIV (65). As such chronically activated T_{RM} and T_{RM} exposed to immunomodulated environments (such as the adipose tissue) may be unable to elicit a full effector response, favoring the progression of HIV infection. It also appears that HIV has the ability to disrupt CCR5-mediated $CD8^+$ T cell migration into the cervical mucosa, thereby impairing the development of T_{RM} populations (66). Regardless, human studies suggest that T_{RM}, especially $CD8^+$ T_{RM}, play an important role in combating HIV infection and thus may be valuable targets for vaccine development. Since the most common mode of transmission of HIV is through sexual intercourse, it may be desirable to explore strategies that induce anti-HIV $CD8^+$ T_{RM} in the female and male reproductive tract and rectosigmoid epithelium. In a Simian Immunodeficiency Virus model of rhesus macaques, intravenous administration of SIVmac239Δ*nef* generated a population of $CD8^+$ T_{RM} in the vaginal tissue and the gut that participated in protection (67). In a murine model, a mucosal vaccination strategy in which intranasal administration of an influenza-vector expressing the HIV-1 Gag protein p24 followed by an intravaginal booster induced $CD8^+$ T_{RM} in the vagina. Antigen stimulation of these $CD8^+$ T_{RM} resulted in the recruitment of B cells, natural killer cells, and $CD4^+$ T cells (68). While the recruitment of innate

and adaptive immune cells may be beneficial in early viral clearance, the recruitment of $CD4^+$ T cells may be detrimental in the context of HIV as they are the target for HIV. Hence, incidental recruitment of $CD4^+$ T cells to sites of HIV entry (female reproductive tract and rectum) by prime and pull vaccination strategies may unintentionally increase susceptibility to infection. A micro-needle array delivery system that utilizes a recombinant adenovirus vector containing the HIV-1 protein Gag, has also produced promising results in generating T_{RM}. These HIV-specific T_{RM} were found in the female reproductive tract and respiratory tract of immunized mice and responded to local antigenic stimulation through expansion and production of IFN-γ and granzyme B (69). Using this micro-needle array delivery system as a priming strategy followed by intravaginal delivery of a booster concoction serving as a pull strategy may be an interesting protocol worth exploring.

VACCINIA

Vaccinia is a poxvirus that usually causes a very mild or asymptomatic infection in immunocompetent individuals. Immunity to vaccinia virus also provides sufficient protection against smallpox, which allowed for its eradication following administration of the live vaccinia virus (70). Despite elimination, smallpox remains a priority on the global agenda given the potential for the virus to be used as a biological weapon (71). For this reason and its ability to serve as a vector, vaccinia virus continues to be used in research. Murine models demonstrate that T_{RM} are generated in response to vaccinia and play a significant role in mediating protection against infection (72–76). Dermal-resident γδ T cells have also been implicated in the immune response against cutaneous vaccinia infection (77). Following skin infection $CD8^+$ T cells are recruited independently of CD4 T^+ cells and IFN-γ (72), many of which subsequently assume the T_{RM} phenotype (72, 73, 75, 78, 79) and are capable of initiating potent inflammatory responses upon re-stimulation (79). Of particular interest is the capacity of local vaccinia skin inoculation to globally seed skin tissue even at remote sites with long lasting T_{RM} (72) as well as generating T_{RM} responses in non-related non-lymphoid organs such as the lungs and liver (76). Multiple exposures to cognate viral antigens have also shown to selectively expand T_{RM} (72, 73, 78, 79). In a lung infection model of vaccinia, higher numbers of lung T_{RM} correlated with better protection against subsequent infection as indicated by a rapid reduction in viral loads. T_{RM} seem to expand more rapidly and localize to the infection site as indicated by a 5-ethynyl-2′-deoxyuridine proliferation assay when compared to their circulating counter parts. Depletion of lung $CD8^+$ T cells by intranasal administration of αCD8 antibody, resulted in previously protected mice becoming susceptible to infection, indicating that $CD8^+$ T_{RM} play a vital role in mediating immunity (74). In another study, parabiosis experiments demonstrated that T_{RM} were exceedingly better at clearing vaccinia virus skin infection than T_{CM} within a shorter timeframe. In fact, it appears that skin T_{RM} can clear vaccinia skin infection even in the absence of neutralizing antibodies and T_{CM} (72). However, vaccinia-specific $CD8^+$ skin T_{RM} appear to have an impaired ability to recruit circulating effector cells during polymicrobial sepsis infection (80). Whether there are other physiologically challenging conditions that impair skin T_{RM} functionality remains largely unexplored. Surprisingly, vaccinia lung infection revealed that not all T_{RM} are equally capable of conferring protection. T_{RM} that resided in the lung interstitium were better positioned to rapidly kill infected lung cells in a contact-dependent manner when compared to T_{RM} situated in association with the tissue vasculature. Furthermore, T_{RM} found within the interstitium, unlike vascular-associated T_{RM}, were able to up-regulate CD69 expression, potentially indicating an enhanced ability to respond during early infection (74). Investigations of vaccinia infection has also reinforced that epithelial immunization routes, such as skin scarification and intranasal exposure, demonstrate significant efficacy for generating protective T_{RM} responses (72–76, 78). In fact, vaccination *via* skin scarification is capable of protecting against clinical disease (pock lesions of the skin) whereas not all mice vaccinated *via* systemic routes such as intramuscular and intraperitoneal were protected from pock lesions. More astonishingly, mice immunized *via* skin scarification demonstrated greater resistance to disease when challenged *via* a heterologous route (intranasal), compared to mice immunized subcutaneously or intraperitoneally, in spite of generating reduced antibody titers (75). These observations may be attributed to T_{RM}-mediated immunity given the evidence that T_{RM} can be generated in distant tissues after skin scarification (76). Overall, studies that use vaccinia infection models have shed light on the ability of skin scarification to elicit a robust and somewhat unique immune response.

RESPIRATORY SYNCYTIAL VIRUS (RSV)

Respiratory syncytial virus is a common cause of lower respiratory tract infections in children and the elderly. Common reinfection with RSV suggests absence of protective immunity (81). A number of studies have shown the importance of T_{RM} in providing protection against RSV (82–86). An experimental human infection study showed that adults with higher frequencies of RSV-specific $CD8^+$ T cells, many of which displayed a T_{RM} phenotype, developed less severe lower respiratory tract symptoms and reduced viral loads. This increase in protection was not correlated with higher numbers of circulating $CD8^+$ T cells, suggesting the localization of T_{RM} was vital for mediating immediate protection (82). T_{RM} induction in lung tissue and airway fluid was also demonstrated following intranasal RSV infection in mice. Adoptive transfer of airway lymphocytes from RSV-infected mice into naïve recipients reduced disease burden upon infection challenge, compared to adoptive transfer of airway lymphocytes from sham-infected mice. It was concluded that both airway $CD8^+$ and $CD4^+$ T cells play a role in protecting against RSV infection and reducing disease severity, respectively (83). However, given that only bulk $CD4^+$ or $CD8^+$ T cells were transferred, it remains to be investigated if the protective capacity is mediated by airway T_{EM} or T_{RM} cells. In support of the latter, an African green monkey model of RSV infection illustrated that antibody and $CD4^+$ T cell responses are unlikely to protect against reinfection. On the contrary, it appears that lung $CD8^+$ T cells, of

which up to half displayed a T_{RM} phenotype, were more capable of protecting against secondary infection (84). From the available evidence (82–86), it appears that an ideal RSV vaccine should elicit a $CD8^+$ T_{RM} response in the lung. Of note, some experimental RSV vaccines have already shown promising results with regards to T_{RM} generation: intranasal administration of an RSV antigen-expressing murine cytomegalovirus generated an IFNγ- and MIP-1β-secreting population of T_{RM} (85); co-administration of the TLR9 agonist CpG and an inhibitor of notch signaling (L-685,458) with formalin-inactivated RSV elicited a strong protective T_{RM} response (86); intranasal administration of virus-like particles containing RSV M and M2 proteins as antigen delivery systems has also shown propensity to induce the production of T_{RM} (87); and a dendritic cell-*Listeria monocytogenes* immunization strategy, when administered locally, was able to avoid circulating T cell-induced immunopathology and protect against RSV infection challenge through the generation of T_{RM} (88).

CYTOMEGALOVIRUS (CMV)

Cytomegalovirus establishes life-long latency in many organs including mucosal tissues. It has long been known that CMV infection induces a sustained clonal expansion of specific $CD8^+$ T cells, a phenomenon referred to as memory inflation (89). However, only recently has it been explicated that CMV infection promotes the formation of T_{RM} in various mucosal tissues, especially the salivary glands (90–92). Although the $CD8^+$ T cell response is vital for the control of CMV infection, the virus-induced downregulation of MHC I on acinar glandular cells of the salivary glands (long-term target tissue of CMV) resulting in the reliance on $CD4^+$ T cells for control of lytic replication at this site (93). Surprisingly, salivary gland $CD8^+$ T_{RM} were capable of controlling viral replication. It appears that murine CMV is unable to completely inhibit the expression of MHC I on $CD8^+$ T_{RM} of the salivary glands, thereby providing an opportunity for these T cells to mediate localized immunity (90). Although it remains unclear whether these T_{RM} inhibit viral replication through effector cytokines or direct cytotoxicity, it certainly appears that salivary gland T_{RM} may inhibit the shedding of CMV, hence reducing the chances of transmission. These mucosal T_{RM} typically form early after infection. However, mucosal seeding continuously occurs through the recruitment and differentiation of circulating populations. As such, the immunodominance of mucosal T_{RM} against CMV changes with time, favoring the TCR repertoire that remains high in circulation (91). T_{RM} have also been found in brain tissue after murine CMV infection (94–96). In the brain, CMV-specific T_{RM} formation seems to be dependent on regulatory T cell (T_{reg}) activity. Furthermore, T_{reg} cells seem to have a suppressive effect on brain T_{RM}'s capacity to produce granzyme B, potentially a precautionary measure to prevent detrimental neuroinflammation (94). From the studies that have dissected the role of T_{RM} in protecting against CMV infection and inhibiting reactivation there seems to be a clear role for these tissue tropic T cells in limiting CMV replication. A number of studies also demonstrate the capacity of CMV to be used as a viral vector in novel vaccines that generate T_{RM}-mediated immunity (85, 91, 97). Manipulating CMV's capacity to induce a robust $CD8^+$ T cell response within mucosal tissues may be a promising avenue for the generation of new vaccines.

LYMPHOCYTIC CHORIOMENINGITIS VIRUS (LCMV)

Lymphocytic choriomeningitis virus, a rodent-borne disease can cause meningoencephalitis in humans (98). While LCMV infection models have been used to study T_{RM} in multiple tissues (99), the protective role of T_{RM} has only been clearly investigated in the brain, thymus, and female reproductive tract. Depletion of circulating T cells or NK cells demonstrated that T_{RM} have the capacity to protect against infection independently of NK cells, T_{CM}, and T_{EM} populations (100, 101). Upon MHC-I-antigen stimulation, LCMV brain T_{RM} displayed effector functions and mediated virus control through IFN-γ release and perforin-mediated cytotoxicity (100). Thymic T_{RM}, when stimulated with gp33, released both IFN-γ and TNF-α, suggesting that T_{RM} at this location may be polyfunctional. It also appears that T cells that took residence in the thymus were more likely to respond to antigen stimulation when compared to their splenic counterparts, further exemplifying the protective nature of these cells (101). Since infection of the thymus can significantly reduce T cell generation due to increased thymocyte deletion and reduced proliferation, it is vital to have protective mechanisms in place that act rapidly to minimize pathogen-induced damage in the thymus. From the available evidence, thymic T_{RM} seem to be capable of adequately fulfilling this task. LCMV infection also induces the production of T_{RM} in various peripheral tissues, such as the lungs, intestines, and female reproductive tract (102–104). While the role of T_{RM} in the lung and intestines following LMCV infection is not well established, it was found that re-activation of $CD8^+$ T_{RM} in the female reproductive tract was able to produce a general anti-viral immune response that is almost able to confer sterilizing immunity when challenged with an non-cognate virus (105). This T_{RM} induced antiviral state may be of great interest in the aim to generate vaccines that create heterotypic protection.

VARICELLA ZOSTER VIRUS

Varicella zoster virus, the cause of chicken pox, is an alpha-herpes virus that can establish latency within the dorsal root ganglia. Reactivation of the virus results in a painful disease called shingles. Although vaccines are available against both chicken pox and shingles (106), recent evidence suggests that T_{RM} may be key players in controlling latent infection, a phenomenon that could be exploited to improve current vaccines. One study analyzed skin samples from human donors of varying ages who were serologically confirmed VZV positive. 80–90% of T cells from the sampled tissue expressed CD69, suggesting that the majority of T cells in skin were T_{RM}. IL-2 responses from stimulated VZV-specific T cells demonstrated that host age did not influence the numbers of responsive cells. However, it was found that skin from older donors demonstrated a lesser capacity to mount a clinical response and

decreased $CD4^+$ T cell infiltration when challenged with VZV antigen. This correlated with higher proportions of $Foxp3^+$ cells. Furthermore, T_{RM} of older skin expressed PD-1 in higher amounts (107). Together, this data suggest that VZV-specific T_{RM} may be suppressed with age. This may be a reason for the high incidence of reactivation of VZV in older individuals. Results from a different study that utilized samples of human trigeminal ganglia suggests that T_{RM} do not seem to play a role in controlling latent infection in the trigeminal ganglia (10). Regardless, further investigation into the role of T_{RM} in controlling latent VZV infection may help to develop therapeutics or vaccines that prevent shingles.

HUMAN PAPILLOMAVIRUS

Human papillomavirus is a sexually transmitted pathogen that generally causes an asymptomatic, self-limiting infection. However, certain subtypes of HPV can cause cancer of the cervix, anus, and oropharynx (108). The routine administration of HPV preventative vaccines has led to a significant reduction in the incidence of infection in many parts of the world. However, immunization of individuals with an established HPV infection has not shown to protect against the progression of HPV-induced lesions into carcinoma. As such, a therapeutic vaccine that is administered by post infection may subvert this problem. Current HPV vaccines rely on the induction of antibodies to neutralize viral particles (109). The potential for generating anti-HPV T_{RM} as a strategy for eliminating previously established HPV infection is yet to be fully explored. One study evaluated the capacity of two adenoviruses (Ad26 and Ad35) that express a fusion of the HPV16 oncoproteins E6 and E7 to elicit a protective response in the cervicovaginal mucosa. Intra-vaginal administration of either vector was able to elicit the generation of $CD8^+$ T_{RM} within the cervicovaginal mucosa. Furthermore, systemic priming with Ad35 followed by an intra-vaginal booster immunization of Ad26 induced polyfunctional, E6/E7-specific, cytokine-secreting $CD8^+$ T cells within the cervicovaginal mucosa (110, 111). Although it remains to be resolved if protection against established HPV infection causally relies on T_{RM}, this and other studies (111) provide impetus to further explore the intra-vaginal route of administration and the use of viral vectors as strategies for the induction of cervicovaginal T_{RM}.

VIRAL HEPATITIS

Viral hepatitis is an inflammatory disease of the liver that is caused by a range of viruses (112). Two studies, both of which utilized human donor liver tissue and paired blood samples, analyzed the role of T_{RM} in the context of viral hepatitis. One study focused on patients with hepatitis B viral infections (HBV), while the other study included patients with HBV or hepatitis C viral infections. A higher proportion of liver T cells from patients who demonstrated partial control of HBV infection had a T_{RM} phenotype, when compared to healthy controls. Given that the overall numbers of T cells in the liver of healthy and HBV-infected individuals were similar, this threefold increase in T_{RM} numbers appear to be due to an increased predisposition of T cells to adopt the T_{RM} phenotype in virally infected liver tissue, rather than expansion of pre-existing T_{RM} (113). The numbers of T cells co-expressing CD69 and CD103 increased by fourfold in chronic hepatitis C patients (114). Furthermore, the reciprocal relationship between viral loads and liver T_{RM} numbers indicates that T_{RM} play a vital role in infection control (113). *Ex vivo* stimulation of T_{RM} showed heterogeneous antigen specificity, with a number of HBV antigens being able to initiate effector responses. However, viral envelope peptides seemed to generate the greatest capacity to induce production of IFNγ, TNFα, and IL-2. Analysis of T_{RM} from healthy liver tissue revealed a noticeably reduced expression of granzyme B, when compared to non-resident counter parts. This suggests that hepatic T_{RM} have less cytolytic capacity than circulating T cells (113, 114). However, liver T_{RM} of patients with chronic hepatitis B expressed markedly higher amounts of granzyme B when compared to healthy controls (114). Liver T_{RM} also showed increased expression of the inhibitory molecule PD-1 compared to non-resident T memory cells (113, 114). The downregulation of granzyme B and upregulation of PD-1 in healthy liver tissue may be a precautionary measure intended to prevent immunopathology, given the liver's role in filtering high amounts of antigen draining from the mesenteric circulation. This is of great importance in viral hepatitis infections as immunopathology is largely involved in the progression of viral hepatitis that leads to cirrhosis and hepatocellular cancer. The increased production of granzyme by T_{RM} in CHB patients may be part of the pathogenesis of fulminant hepatitis. Further exploring the role of T_{RM} in protection against viral hepatitis (including hepatitis A, D, and E) and the immunopathology implicated in the progression of the disease may aid in the development of immunomodulatory therapeutics to prevent viral cirrhosis and hepatocellular cancer.

EPSTEIN–BARR VIRUS (EBV)

Epstein–Barr virus is one of the most prominent causes of infectious mononucleosis. After exposure to infected saliva, the virus infects and replicates in B cells and epithelial cells of the new host. Although the clinical disease of glandular fever is usually self-limiting, EBV remains latent in circulating B cells and episodes of reactivation are known to occur. It appears that reactivation of EBV occurs in the lymphoid tissue of the oropharynx, where the virus switches from a latent form into a lytic cycle. Control of infection is mediated by a T cell response against infected B cells (115). EBV-specific $CD8^+$ memory T cells localize to the epithelium of the oropharynx (116), where they up-regulate CD69 and CD103 in an IL-15- and TGF-β-dependent fashion (117). $CD103^+$ EBV-specific T memory cells found in tonsillar tissue are more sensitive to antigen stimulation and produce a greater effector response when compared to circulating EBV-specific T cells (116). Furthermore, a substantial $CD103^+$ T cell population only seems to appear as viral replication and disease tapers (118). Taken together, it appears as though T_{RM} play a crucial role in rapidly controlling viral replication of EBV within the oropharyngeal tissue upon reactivation to prevent full clinical relapse.

VESICULAR STOMATITIS INFECTION (VSV)

Vesicular stomatitis infection is a zoonotic disease that can cause a mild febrile illness in humans (119). Intranasal infection of mice with VSV has shown to produce $CD103^+$ CD8 T_{RM} population in the brain (120, 121), as the virus travels along the olfactory bulb to the brain where it causes infection. These brain T_{RM} were found to be functional *in situ*, responding to cognate antigen (120, 121). Staining for effector molecules revealed that many of these T_{RM} cells were positive for granzyme B, suggesting cytolytic abilities. Once removed from the brain parenchyma, these cells appear dysfunctional, suggesting they are highly adapted to the brain microenvironment. Maintenance of this population of T_{RM} appears to be independent of circulating T cells, and BrdU incorporation indicates a slow homeostatic rate of proliferation to sustain the population (120). Interestingly, brain T_{RM} appear to form clusters within specific sites of the brain parenchyma that contain $CD4^+$ T cells, perhaps indicating a role for $CD4^+$ T cells in the generation and/or maintenance of brain $CD8^+$ T_{RM}. These clusters may have formed around sites of previous VSV replication sites, where persisting antigen may be drawing the T_{RM} to these locations. Although, viral RNA could not be detected at these sites (120), this does not exclude the possibility that undetectable levels of antigen may be present at these sites. T_{RM} may also form clusters around local dendritic cells that are still presenting antigen from a previous infection. This hypothesis is supported by the observation that antigen presentation by bone marrow-derived-APCs was able to support CD103 expression by T_{RM} (120).

OTHER VIRUSES

Polyomaviruses are opportunistic pathogens that usually remain latent following infection. However, in immunocompromised individuals, infection can cause multifocal leukoencephalopathy (122). T_{RM} are generated in the context of polyomarvirus infection (123–126), and polyomavirus-specific brain $CD8^+$ T_{RM} in mice maintain a high TCR affinity for pathogen epitopes. In fact, T_{RM} TCR affinity appears to be higher than the TCR affinity of T cells from the spleen. This observation supports a role of T_{RM} in mediating rapid control of viral replication during reactivation, as high TCR affinity allows for the early detection of low amounts of virus (123). In contradiction to this finding, evidence from another study suggests that lower TCR stimulation increases the generation of brain T_{RM} (125). One way of interpreting these seemingly contradicting observations is that brain T_{RM} initially differentiate from circulating effector T cells with low TCR stimulation capacity, but after taking residence in the brain, undergo functional avidity maturation (127) increasing their ability to respond to antigen. A renal transplant clinical study suggests that renal BK Polyomavirus-specific T_{RM} were rendered incapable of protecting against infection leading to interstitial nephritis, likening these T_{RM} to dysfunctional tumor-infiltrating lymphocytes (126).

Ebola virus causes a form of hemorrhagic fever characterized by intravascular coagulation and maculopapular rash. Although the natural reservoirs for the virus are thought to be fruit bats, human-to-human transmission can occur when contaminated body fluids breach mucosal barriers or skin. Absence of specific treatment and epidemic potential of the virus highlights the need for a vaccine (128). Aerosol administration of a human parainfluenza virus type 3-vectored vaccine expressing an Ebola envelope glycoprotein was capable of not only eliciting neutralizing antibodies but also a $CD103^+$ T cell response in the lungs of macaques. A large proportion of these T_{RM} were polyfunctional, demonstrating positivity for two or more activation markers. Furthermore, a single dose of this vaccine conferred 100% protection against infection challenge (129). Since a large proportion of transmission in the recent Ebola epidemic was through skin contact, vaccination *via* scarification is worth exploring.

Norovirus is a highly infectious virus, and is a common cause of gastroenteritis. Although infection is generally self-limiting, chronic forms have been reported in immunocompromised patients. A clinical study has implicated $CD8^+$ T cells resembling T_{RM} in the immune response against norovirus (130). However, a genetically manipulated strain of murine norovirus causing chronic infection revealed that despite a robust and functional T_{RM} response being generated, clearance of the virus was not achieved, likely due to inadequate antigen sensing (131).

THE ROLE OF T_{RM} IN BACTERIAL INFECTIONS

Although there is significantly less literature about T_{RM} in the context of bacterial infections, the evidence largely implies that T_{RM} have a noteworthy role in protecting against pathogenic bacteria. The following section groups bacterial pathogens together depending on their location of primary infection.

BACTERIAL INFECTIONS OF THE LUNGS AND AIRWAYS

Pertussis, also known as whooping cough, is caused by *Bordetella pertussis*, a Gram-negative coccobacillus. Despite high vaccination coverage, whooping cough remains a serious public health concern. T cell responses are critical for immunity against *B. pertussis* (132). While the existing whole-cell pertussis (wP) vaccine is generally associated with a strong Th1 response, immunization with the widely used acellular pertussis (aP) vaccine induces a Th2-dominated humoral response (133). Immunity to the aP vaccine wanes over time compared to wP vaccines (134). This diminished immunity allows for the transmission of *B. pertussis* to susceptible individuals. A recent study reported that following *B. pertussis* infection, IL-17- and IFN-γ-secreting $CD4^+$ T_{RM} congregate in the lungs of infected mice where they persisted for 120 days, and expanded up to sixfold upon reinfection. Egress inhibitor FTY720 did not affect the control of bacterial burden during secondary infection, suggesting that T_{RM} were capable of providing immunity irrespective of peripheral T cell recruitment. Bacterial clearance in reinfected mice also correlated with $CD4^+$ T_{RM} expansion, with a large portion of cells displaying a Th17 phenotype (135).

Adoptive transfer of lung $CD4^+$ T_{RM} from infected mice into naïve hosts conferred protection against *B. pertussis* challenge (135), suggesting that Th17-like $CD4^+$ T_{RM} seemed to play a crucial role in long-term immunity. Interestingly, γδ T cells that express CD69 and CD103, classically known to provide innate-like protection during primary infection, also provided a significant early-release IL-17 response during secondary infection in convalescent mice. However, γδ T_{RM}, especially $V\gamma4^+$ γδ T cells persisted in the lungs of convalescent mice and produced a greater IL-17 response on re-exposure to *B. pertussis* in an antigen-specific manner (136). Therefore, a long-lasting *B. pertussis* vaccine should not only promote the generation of *B. pertussis*-specific $CD4^+$ T_{RM} but also γδ T_{RM}.

Pneumonia is one of the largest infectious causes of mortality in children worldwide (137). The most common cause of community-acquired pneumonia is *Streptococcus pneumoniae*, a Gram-positive polysaccharide-encapsulated bacterium (138). Modern pneumococcal vaccines are polysaccharide based and are thus poorly immunogenic, providing serotype-specific immunity that wanes over time. Although $CD4^+$ Th17 responses are considered vital in providing protection against pneumococcal infections, the role of T_{RM} is yet to be fully characterized. Experimental *S. pneumoniae* infection was found to promote the production of heterotypic $CD4^+$ T_{RM} of both Th17 and Th1 phenotypes in niches located within pneumonia-affected lobes of the lung. It was also observed that immunity was restricted to pathogen-experienced tissue, suggesting that T_{RM} reside in primary infection sites, rather than providing immunosurveillance throughout the entire respiratory mucosa. Despite spatial restriction, T_{RM} provided superior protection to the local tissue when compared to systemic immune responses elicited by antigen-specific $CD4^+$ T_{CM}. Neither adoptive transfer of splenic $CD4^+$ T cells from infected mice into naïve recipients, nor inhibiting lung translocation of circulating $CD4^+$ T cells with FTY720 in pathogen-experienced mice, had a significant effect on protection against pneumococcal infection challenge. Therefore, protective immunity against bacterial pneumonia is likely due to the aggregation of $CD4^+$ T_{RM} in susceptible tissues (139). Interestingly, combining whole virion influenza and whole cell pneumococcal vaccine also promoted the generation of lung $CD4^+$ T_{RM}. It is likely that these T_{RM}, in combination with the accompanying high antibody titers elicited by the combined vaccine, played a role in providing protection against pneumococcal-influenza co-infection (140). Overall $CD4^+$ T_{RM} may play a role in the generation of naturally acquired immunity against pneumococcal infections, and should be considered in the development of heterotypic pneumococcal vaccines.

Mycobacterium tuberculosis (*Mtb*), an acid-fast staining intracellular bacterium, is the causative agent of tuberculosis (TB). The deadly infection can present as pulmonary, as well as extra pulmonary disease (141). Currently, *Bacillus Calmette–Guérin* (BCG) is the only licensed vaccine against TB, and prevents dissemination in children. However, BCG does not provide strong enough immunity against pulmonary TB in adults, therefore, allowing transmission (142). Immune control of *Mtb* infection largely relies on the production of IFNγ by $CD4^+$ T cells, which enhances macrophage killing of persisting intracellular *Mtb* and leads to the formation of granulomas around sites of bacterial replication (141). A clinical study revealed that individuals previously exposed to tuberculosis were likely to have a population of lung-resident Th1 effector memory cells that released IFN-γ in response to *Mtb* antigen re-exposure (143). However, the delay in activation and recruitment of TB-specific T cells to the lungs during primary infection allowed for *Mtb* to proliferate, resulting in a high bacterial burden. The importance of airway-residing memory T cells (then called airway luminal cells) in mediating protection against TB has been described well before the dawn of T_{RM} (144–146). However, these cells most likely represent the same cell type. Lung T_{RM} induced by mucosal vaccination have shown to be effective in limiting the early control of bacterial replication (147). Despite the defined role of $CD4^+$ T cells in controlling TB, recent evidence from vaccine studies suggest that $CD8^+$ lung T_{RM} also play an important role in protection against *Mtb* (148, 149). Only mucosal administration of BCG led to the generation of airway T_{RM} that produce higher levels of pro-inflammatory cytokines, including IFN-γ than $CD8^+$ T_{EM}. Furthermore, adoptive transfer of sorted airway $CD8^+$ T_{RM} from BCG-vaccinated mice demonstrated enhanced protection against *Mtb* challenge in recipient mice. Transfer of $CD8^+$ T_{RM} decreased the numbers of alveolar macrophages, while increasing the number of $CD4^+$ T cells and B cells in the infected lung tissues (148). It was hypothesized that $CD8^+$ T_{RM} kill *Mtb*-infected alveolar macrophages, thereby depleting intracellular reservoirs of the bacteria and limiting the entry into the lung parenchyma (148). Likewise, the viral-vectored vaccines SeV85AB and AdAg85A, administered *via* the intranasal route have also shown to elicit an immune response that favors the production of $CD8^+$ rather than $CD4^+$ T_{RM} (149, 150). In a rhesus monkey model, a cytomegalovirus vector delivering a range of *Mtb* antigens (RhCMV/TB) provided significant protection against tuberculosis, presumably through it its ability to generate and maintain pathogen-specific $CD4^+$ and $CD8^+$ circulating and more importantly resident memory T cells that selectively express VLA-1 (151). Finally, aerosol vaccination with an attenuated *Mtb* strain lacking *sigH* not only led to an enormous influx of T cells expressing CD69 into the lung airways (likely to include T_{RM}), but also to a significant long-term protection against virulent *Mtb* challenge (152). Collectively, these studies indicate that rationally designed TB vaccines should generate immune responses that prevent the establishment of infection and/or provide sterilizing immunity by inducing both lung $CD4^+$ T_{RM} and $CD8^+$ T_{RM} in the lungs.

BACTERIAL INFECTIONS OF THE UROGENITAL TRACT

A number of bacteria cause disease of the reproductive tract and urinary system. One such example is *Ct*, an obligate intracellular bacterium that causes infections of the genitals and eyes. It is the leading cause of infectious blindness worldwide, and can cause infertility when sexually transmitted (153). According to clinical evidence, it appears that spontaneous clearance of clinical infection correlates with at least partial protection against *C. trachomatis* through the production of INF-γ-secreting cells

such as $CD4^+$ Th1 cells. However, IFN-γ responses alone do not seem to provide complete protection. It has also been documented that B cell-antibody responses are involved in immunity, especially against secondary infection (154–156). Importantly, intraepithelial $CD8^+$ lymphocytes and MLCs composed of B cells and $CD4^+$ T cells border the vaginal and uterine tract, respectively in pathogen-experienced tissue. These immunocyte structures hinder *C. trachomatis* from replicating and establishing a clinical infection (157). Optimal protection against *Chlamydia* requires both the recruitment of T_{CM} and the presence of T_{RM} within the urogenital tract (158, 159). It is likely that protective immunity occurs in response to chronic or repeated infection, which leads to the seeding of T_{RM} throughout the epithelial surface (160). A vaccine composed of *Chlamydia* major outer membrane protein and ISOCMATRIX adjuvant was able to provide enough protection to prevent the sexual transmission of *C. trachomatis*, however, was not capable of providing complete immunity. This may be attributed to the inability of the vaccine to generate a large enough T_{RM} population, underscoring the essential role of T_{RM} in *Chlamydia* infection (159). In a separate study, mice were either inoculated with infectious *C. trachomatis* or UV-inactivated *C. trachomatis* (UV-*Ct*). Mice infected with the infectious form demonstrated capacity to control future infections better than naïve controls, which may be attributable to the production of both *Chlamydia*-specific T_{CM} and T_{RM} populations. However, the group of mice inoculated with UV-*Ct* suffered higher bacterial burdens when compared to naïve controls. This data in conjunction with the generation of *de novo Ct*-specific T_{reg} suggest that a tolerogenic immune response occurred in these mice. On the contrary, intra-uterine administration of UV-*Ct* conjugated with charge-switching synthetic adjuvant peptides (UV-*Ct*-cSAP) conferred a superior protection to *Ct* in both conventional and humanized mice. The rapid clearance of *Ct* in UV-*Ct*-cSAP-vaccinated mice has been attributed to the immediate release of IFN-γ by mucosal T_{RM} (158). Taken together, the ideal vaccine against *Chlamydia* should promote the generation of local MLC, T_{CM}, and T_{RM} in the epithelium, even though partial protection appears to be sufficient to prevent disease transmission.

BACTERIAL INFECTIONS OF THE GASTROINTESTINAL TRACT

Gastrointestinal infections are generally acquired through ingestion of contaminated food or water. These bacteria may remain in the gut, or may disseminate to other parts of the body causing systemic disease (161). Targeted induction of T_{RM} along the gastrointestinal epithelium could enhance protection against these pathogens. *Listeria monocytogenes*, a food-borne Gram-positive coccobacillus, is of particular concern in immunocompromised and pregnant individuals, and can cause meningitis and stillbirth (162). Its capacity to replicate within host cells facilitates immune evasion. Thus, protection against *L. monocytogenes* is largely dependent on cell-mediated immunity (163). First observed in 1981 as a distinct population of long-lived T memory cells "positioned" in tissue following listeriosis (164), a more recent study highlighted the role of intestinal $CD8^+$ T_{RM} in mediating immunity against *L. monocytogenes* following oral infection. In fact, blockage of integrin $\alpha_4\beta_7$ prevented the formation of these intestinal TGF-β-dependent T_{RM} resulting in diminished protection upon re-challenge (165). Revealed by multi-photon dynamic microscopy, a population of $V\gamma4^+$ γδ T_{RM} was found within the mesenteric lymph nodes in response to *L. monocytogenes* infection, which remained largely stationary under homeostatic conditions. However, upon re-challenge, activation of these cells resulted in organized clusters around bacterial replication foci where they released IL-17 and subsequently, the recruitment of neutrophils to facilitate bacterial elimination. Similarly to γδ T_{RM} function seen in *B. pertussis* infection (136), neutralization of IL-17 hindered bacterial clearance, highlighting the importance of early IL-17 release by γδ T_{RM} (166).

Yersinia pseudotuberculosis (*Yptb*), a food-borne pathogen causing of Far East scarlet-like fever, is a Gram-negative bacterium responsible for gastroenteritis, mesenteric lymphadenitis, and can clinically mimic acute appendicitis (167). A *Yptb* oral infection mouse model showed a robust $CD8^+$ T cell response in the intestines including a population of *Yptb*-specific $CD103^+$ $CD8^+$ T_{RM} uniformly distributed throughout the intestine, while $CD103^-$ T_{RM} formed around sites of primary infection where they carried out effector functions (168). Although found in antigen-rich areas, their development is independent of local antigen stimulation (168). The development of T_{RM} populations in the intestine seems to rely on inflammatory signals from the site of infection rather than antigens (169). Production of IFN-β and IL-12 from intestinal macrophages effectively suppresses TGF-β-mediated CD103 expression thereby leading to the development of $CD69^+$ $CD103^-$ T_{RM} population in mice during *Yptb* infection. This data suggest a central role for inflammatory monocytes in the differentiation and maintenance of different $CD8^+$ T_{RM} populations to achieve optimal protection against intestinal infections. Additionally, this study raises the question as to whether CD103 is necessary for residence within the intestinal tissue, or whether it negatively regulates T_{RM} capacity to migrate within their residential tissues.

Salmonella spp. is a group of Gram-negative bacilli that is a common cause of gastrointestinal infections responsible for "food poisoning." Transmitted orally, this heterogeneous group of bacteria contains typhoid or enteric-fever causing serovars that can be potentially fatal to humans (170). Current vaccines against *Salmonella* are poorly immunogenic and risk disease in immunocompromised individuals (171). An effective vaccine that prevents gastrointestinal infection is much needed to prevent outbreaks of salmonellosis. Subcutaneous co-administration of *Salmonella* SseB and flagellin has shown to provide protection against systemic disease in mice. However, parabiosis studies suggest that this protection can be transferred *via* the circulation, diminishing the role of T_{RM} in the observed immunity (172). Nevertheless, it may be beneficial to assess the capacity of this vaccine and others to protect against gastrointestinal infection by oral administration. However, the barriers of oral tolerance and destruction of vaccine components by digestive enzymes and chemicals must be overcome in order to develop oral vaccines.

In summary, T_{RM} responses in bacterial infections appear to be more diverse compared to viral infections, an observation that

may be attributed to the varying locations of bacterial replication (intracellular versus extracellular), the more complex lifestyles and the presence or more sophisticated immune evasion mechanisms. Future studies should assess the role of T_{RM} in the natural immune response to other bacterial infections.

THE ROLE OF T_{RM} IN PARASITIC (PROTOZOA AND HELMINTHS) INFECTIONS

Protozoa are unicellular organisms that are of great importance to human health. Most prevalent in tropical regions of the world, protozoan infections are difficult to treat due to their complex life cycles and their ability to evade host immune responses through antigenic variation, residence within various intracellular compartments, and their capacity to assume protective forms such as cysts (173, 174). At this juncture, our collective understanding of T_{RM} responses to protozoan infections remains relatively deficient. However, studies have shown that T_{RM} play a significant role in protecting against a few protozoan species.

Malaria, the most prevalent protozoan infection of humans, is caused by five species of *Plasmodium*. Transmitted by the bite of an infected female *Anopheles* mosquito, *Plasmodium* parasites enter the circulation and take residence inside erythrocytes during part of their complex life cycle. Natural immunity to *Plasmodium* infection involves a mixture of humoral, $CD4^+$, and $CD8^+$ T cell responses (175). However, liver T_{RM} have emerged as a promising target for protecting against malaria (176). Unlike T_{RM} of the epithelium, such as the lungs, intestines, and skin, liver-T_{RM} appear to reside in sinusoids (the blood vessels of the liver), rather than the parenchymal tissue (177–179). The heavily fenestrated architecture of these blood vessels and the distinct slow flow rate of blood allows for T_{RM} to traverse through the organ without being dispatched into circulation. Furthermore, liver sinusoids provide a prime niche for close interaction of T_{RM} and antigen presenting cells, such as Kupffer cells and dendritic cells. This allows for the rapid detection of antigen. Each hepatocyte is also in close association with a sinusoid, thereby providing easy access to liver T_{RM} for assessment of surface antigen presentation (177). Intravital imaging revealed that these T_{RM} traversed around 10 μm per minute and, as reported in HSV-1 infection of skin, T_{RM} assume an amoeboid form, extending dendrites to survey the liver for antigens (2). Rather than relying on CD103-αE integrin interactions for maintaining tissue residence, liver T_{RM} appear to utilize the adhesion molecule LFA-1 (3). A rhesus monkey *P. knowlesi* infection model that assessed sporozoite immunization demonstrated capacity for generating liver T_{RM}. These T_{RM} appear to be protective as their depletion resulted in the loss of immunity (1). Experiments with radiation-attenuated sporozoites also support the notion that inducing high numbers of liver T_{RM} can afford protection against malaria. In this study, a "prime-and-trap" strategy was used in which primed T cells from the spleen were drawn to the liver using a recombinant adeno-associated virus that infected hepatocytes and subsequently caused them to express *Plasmodium* antigen. The immunity generated by this strategy was attributed to the increased numbers of $CD8^+$ liver T_{RM} (2). The emergence of liver T_{RM} as a potential target for pre-erythrocytic malaria vaccines warrants further research.

Leishmaniasis is a heterogeneous vector-borne disease that is caused by an intracellular protozoa parasite. There are over 20 known *leishmania* species, all of which are transmitted by bites from infected female *Phlebotomine* sandflies. Clinical disease presents in three main forms: cutaneous, mucocutanous, and visceral. Subjugation of *Leishmania* parasites relies on the establishment of IFN-γ-producing $CD4^+$ Th1 cells (180). While it is widely known that clearance of primary infection can lead to protective immunity, the effector response that leads to protection remains unclear. It has, however, become apparent that T_{RM} play a crucial role in providing such protection (181–183). *L. major* infection models illustrated that following infection, long-lived T_{RM} are rapidly fabricated and seeded universally throughout the skin, as they can be detected in tissue far away from the primary site of infection (181). It was previously believed that these T_{RM} provided immunity by rapidly recruiting circulating effector T cells. However, more recent studies suggest that the recruitment of circulating T cells may not be as important as previously thought, as FTY720 and αCXCR3-treated mice re-challenged with *L. major* showed minimal difference in early parasite control (182, 183). Furthermore, parabiosis studies demonstrated that *Leishmania*-specific circulating T cells alone provide little or no protection during early infection. Data exemplifies that $CD4^+$ T_{RM} are rather likely to provide immunity by eliciting a delayed-type hypersensitivity response. Early immunity is attributable to the capacity of T_{RM} to rapidly recruit reactive oxygen species/nitric oxide producing inflammatory monocytes to control parasite burden (182). Liver T_{RM} have also been implicated in the immune response against *Leishmania*. The recombinant proteins LirCyp1 and LirSOD of *L. infantum* appear to be good candidates for promoting the expansion of liver memory T cells (184). No current vaccine exists for this potentially fatal disease. Together, these data suggest that Leishmaniasis vaccines should be tailored to generate T_{RM} to provide heterotypic protection against the many species that cause disease.

Toxoplasma gondii, the causative agent of toxoplasmosis, is an intracellular protozoan that is generally acquired through contaminated food. In humans, *T. gondii* can form persistent cysts in multiple tissues (185). In both acute and chronic infection, cell-mediated immunity and effector cytokines play vital roles in limiting the progression of disease (186). Mice deficient in TNF-α suffer from increased pathology, and IFN-γ production in the brain stimulates microglia and astrocytes to inhibit protozoan proliferation. In a chronic infection model of *T. gondii*, $CD103^+$ $CD8^+$ T_{RM} established in the brain produced a more robust IFN-γ and TNF-α response when compared to $CD103^-$ T cell subsets (187). It appears that brain T_{RM} provide superior protection against *T. gondii* infection of the central nervous system when compared to $CD8^+$ T_{EM} and T_{CM}. During *T. gondii* and *Y. pseudotuberculosis* infection T_{RM} also seem to

accumulate in white adipose tissue in what appears to be a depot of protective memory cells (188).

Helminthic infections are highly prevalent around the world. Although most infections are not fatal, they account for a large proportion of disease burden, causing secondary conditions, such as anemia and malnutrition. Immunity against helminthic infection is largely mediated by the Th2 effector arm of the adaptive immune system (189). The role of T_{RM} in protecting against helminthic infections, however, has only been explored recently in two species: *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* (190, 191). While neither of these species are human pathogens, they provide analogous models to gastrointestinal helminthic infections and *Necator americanus* infection in humans, respectively (192, 193). Adoptive transfer of peritoneal-cavity $CD4^+$ T_{RM} from convalescent mice into naïve mice prior to *H. polygrus* infection challenge, has demonstrated that peritoneal-cavity derived $CD4^+$ T_{RM} are capable of hindering the reproductive capacity of female worms without reducing worm burden (190). This phenomenon provides new insight into what appears to be a unique interaction between T_{RM} and pathogen. A different study that used a *N. brasiliensis* model demonstrated that even a small number of lung-interstitial T_{RM} were capable of providing protective immunity. This was confirmed as cognate mice treated with FTY720 and lymphotoxin beta-receptor fusion protein (which causes lymphopenia) were able to clear secondary infection, suggesting that circulating T cells are not necessary to mount a protective secondary response (191). In spite of the lack of knowledge surrounding the interaction between T_{RM} and helminths, there is a clear role for this subset of T cells in worm infections that needs to be explored further.

THE ROLE OF T_{RM} IN FUNGAL INFECTIONS

Typically, fungal infections are less frequent compared to viral and bacterial diseases. However, due to the increasing use of immunomodulatory drugs for cancer and organ transplant patients, the increasing incidence of mycosis is of clinical importance (194). T_{RM} responses are least studied in the context of fungal infections. In fact, only one fungus appears to have been used in T_{RM} studies.

Candida albicans is a dimorphic yeast and opportunistic pathogen. Although it forms part of the normal commensal biome of humans, it can cause infections known as candidiasis especially in immunocompromised individuals (195). Skin and tongue-resident $CD4^+$ IL-17-producing T_{RM} can provide effective protection against *C. albicans* (196, 197). Murine skin and oral infection models demonstrated that during early infection, γδ T cells release IL-17 in response to *C. albicans* invasion (196, 197). In skin, by day 7 post-infection, the vast majority of IL-17-producing cells are of the Th17 phenotype. Eventually, the T cells at the site of initial infection upregulate CD103 and CD69 suggesting they assume the $CD4^+$ T_{RM} phenotype between 30 and 90 days post infection. During this time, the cells first become less motile, eventually "sessile" and localize to the papillary dermis. However, upon reinfection, these T_{RM} were capable of rapidly clearing infection and appear to be superior at doing so than circulating T_{EM}. It was also reported that *C. albicans*-specific Th17 cells were found in high numbers in normal human skin (196), and that low doses of *C. albicans* antigen exposure stimulates the production of regulatory skin T_{RM} that substantially suppress the activity of skin T_{EM} (198). This may be attributed to the widespread presence of *C. albicans* in human tissue. Resident memory Treg cells may play a protective role in preventing a hyper-inflammatory response to benign *C. albicans* antigen exposure. Although the development of a vaccine against *C. albicans* is not of vital importance, these studies provide an initial insight into understanding T_{RM} responses to fungal infections. Other fungal infections that would be of interest include tinea, cryptococcosis, and aspergillosis.

DISCUSSION

The discovery of T_{RM} has enhanced the possibility to develop new and improved vaccines. From the available literature, it appears that the most important factor for generating T_{RM} is to match the route of vaccination to the route of pathogen entry. In general, these are the mucosal and epithelial barriers that provide the first line of defense against pathogens: the respiratory, gastrointestinal, urogenital mucosa, and integumentary epithelium (**Figure 3**). Thus, the long-standing method of administering vaccines parenterally may be less effective at conferring optimal protection when compared to the novel mucosal and epithelial vaccine strategies highlighted throughout this review. The "prime and pull" method in which a parenteral vaccine is administered (prime) and an inflammatory agent is applied at a later time point to the desired peripheral tissue (pull) has also proven itself to be an effective vaccine strategy for generating T_{RM}. The combination of mucosal vaccination and "pull" strategies may be an avenue worth exploring in future experiments. Another promising vaccine strategy is the use of viral-vectored vaccines. Regardless, it is also imperative to keep in mind the balance that these tissues must constantly strike in inducing a tolerogenic versus effector immune response to antigens, given their dual function in both their physiological roles (respiration, digestion, reproduction, etc.) and in serving as barriers against infection. Additionally, it will be important to consider the local cytokine milieu that influences T_{RM} generation (199). Generating memory T cells in peripheral tissues with cytolytic effector functions and the capacity to recruit inflammatory cells may pose the risk of inducing a hyper-inflammatory state leading to immunopathology. However, the natural persistence of T_{RM} in most peripheral tissues for extended periods of time under homeostatic conditions implies that mechanisms are in place to regulate T_{RM} responses. Developing a greater understanding of these mechanisms is vital in creating safe T_{RM}-based vaccines.

A very appealing aspect of T_{RM}-based vaccines is that it may be possible to generate heterotypic protection, as shown by studies using influenza infection models. This is vital for protecting against a range of rapidly mutating pathogens, such as HIV, as well as infectious diseases such as leishmaniasis that are caused

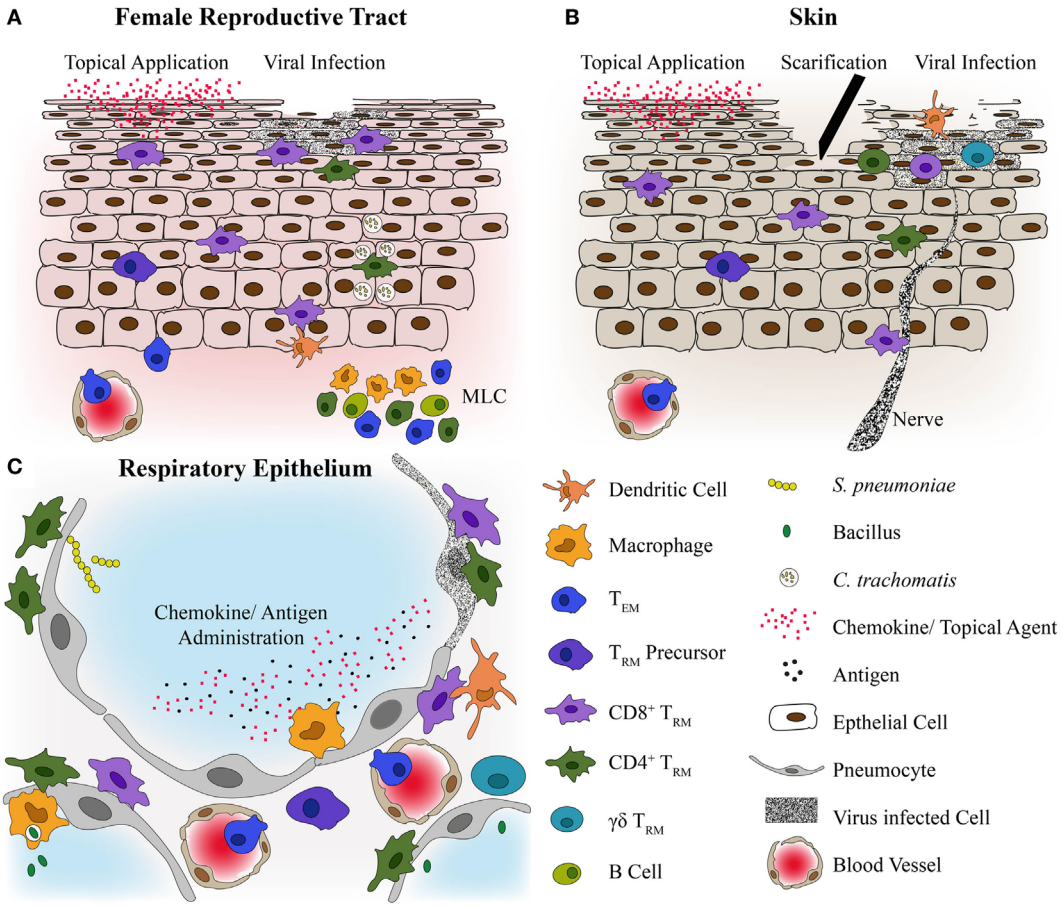

FIGURE 3 | Visual summary of key T_{RM} effector responses and vaccine strategies at epithelial surfaces. **(A)** Represents the female reproductive tract. Topical application of both specific chemokines and general inflammatory agents such as nonoxynol-9 can be used to "pull" systemically primed T_{EM} into the mucosal tissue. $CD103^+$ T_{RM} reside closer toward the apical surface of the mucosa. Both $CD4^+$ and $CD8^+$ T_{RM} play a role in controlling viral infections. Memory lymphocyte clusters have been shown to be important in controlling infections at this site. $CD8^+$ T_{RM} re-stimulation appears to be dependent on $CD301b^+$ dendritic cells that reside in the lamina propria. **(B)** Represent the integumentary epithelium. Topical application of both specific chemokines and general inflammatory agents such as 2,4-dinitroflourobenzene can be used to "pull" systemically primed T_{EM} into the epidermal tissue. Skin scarification as a route of vaccination encourages the development of skin T_{RM}. Upon antigen recognition, skin T_{RM} lose their dendricity and become less motile. γδ T_{RM} can mediate early immune responses. $CD8^+$ $\alpha\alpha^+$ T_{RM} have been found in the dermal–epidermal junction where they may be able to survey local neural tissue for reactivation of latent viral infections. **(C)** Represents the respiratory epithelium. While different chemokines have shown the ability to "pull" T_{RM} into the respiratory epithelium and airways, the presence of antigen appears to be important at this site. T_{RM}-mediated control of *Streptococcus pneumoniae* is largely dependent on the $CD4^+$ subset. Control of viral and *Mycobacterium tuberculosis* infection requires both $CD4^+$ and $CD8^+$ T_{RM}. γδ T_{RM}, in conjunction with $CD4^+$ T_{RM} have been shown to mediate immunity against *Bordetella pertussis*.

by heterogeneous pathogens. Furthermore, the promptitude with which T_{RM} mediate immune responses is also of great interest when generating protection against infections such as tuberculosis that are capable of establishing latent infections. In order to achieve immediate T_{RM} protection, it appears that there must be a minimal density of T_{RM} within peripheral tissues to ensure pathogens are identified and eliminated in a timely manner. This may pose a challenge in developing T_{RM}-based vaccines as sufficient numbers of T_{RM} need to be generated and maintained evenly throughout infection-susceptible tissues. The spatial capacity in non-lymphoid tissue to accommodate for T_{RM} may be limited, and thus establishing the capacity of different tissues has as well as determining the minimum threshold of T_{RM} needed to provide protection is much needed.

CONCLUSION

In summary, the findings of this review largely accentuate the importance of T_{RM} in protecting against a range of pathogens. Their localization to sites prone to infection appears to give T_{RM} an enhanced capacity to mount swifter immune responses when compared to circulating memory T cells. Previous vaccine development has been largely centered on the generation of systemic memory response, which at times has shown to be ineffective. The capacity to form tissue-specific immunity through T_{RM} may shape vaccines of the future. Continuing to foster the growing pool of knowledge about T_{RM} will help to guide the field of T_{RM} vaccinology and may lead to the generation of new and more effective vaccines which may help to reduce the incidence of many infectious diseases.

AUTHOR CONTRIBUTIONS

VM performed the literature search and conducted extraction of data from relevant studies. AK, HS, and SN critically reviewed the literature search. VM and AK wrote the manuscript. All coauthors read and approved the final version of the manuscript.

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CD4 T_{RM} Cells Following Infection and Immunization: Implications for More Effective Vaccine Design

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The induction of immunological memory, which is mediated by memory T and B cells, is central to adaptive protective immunity to pathogens induced by previous infection and is the cornerstone of effective vaccine design. Recent studies in mice have suggested that memory T cells that accumulate in tissues, termed tissue-resident memory T (T_{RM}) cells, play a crucial role in maintaining long-term protective immunity to mucosal pathogens. CD4 and CD8 T_{RM} cells can be induced following infection at mucosal sites or the skin, where they are maintained and poised to respond rapidly to reinfection with the same pathogen. T_{RM} cells can also be generated by vaccination, but their induction is influenced by a number of factors, including the type of vaccine, the adjuvant, and the route of immunization. Live attenuated vaccines appear to be more effective than killed or subunit vaccines at inducing T_{RM} cells and mucosal immunization, especially by intranasal route, is more effective than parenteral delivery. However, evidence is emerging that formulation of killed or subunit vaccines with novel adjuvants, especially those that generate Th1 and Th17 responses, can promote the induction of T_{RM} cells. While T_{RM} cells are also present at high number in mucosal tissues in humans, one of the challenge will be to develop methodologies for routine quantification of these cells in humans. Nevertheless, the identification of approaches for optimum induction of T_{RM} cells in mice should assist in the design of more effective vaccines that sustain protective immunity against a range of human pathogens.

Keywords: memory CD4 T cell, tissue-resident memory T cell, infection, immunization, vaccine, protective immunity, Th1 cell, Th17 cell

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INTRODUCTION

The induction of immunological memory is central to antipathogen adaptive immunity induced by previous infection or vaccination. While circulating antibodies can confer protection against infection with certain pathogens, antibodies in the circulation and at mucosal sites usually wane over time and long-term protection is dependent on the induction of memory T and B cells. There is growing recognition that memory T cells that reside in tissues, called tissue-resident memory T (T_{RM}) cells, play a crucial role in maintain long-term immunity, especially against pathogens that infect mucosal surfaces (1). T_{RM} cells were identified as cells that retained in the non-lymphoid organs with limited ability to recirculate. Tissue-resident lymphocytes constitutively express adhesion molecules and integrins that help them to remain in the tissue. These include CD44, a receptor for hyaluronic acid that can also bind to collagens or matrix metalloproteinases, and CD69, a transmembrane C-type

lectin that is critical for regulating the T cell egress from lymphoid organs and retention in peripheral tissues (2, 3). CD103, αE integrin, is often expressed on intraepithelial and airway CD8 T_{RM} cells. As a receptor for E-cadherin, CD103 helps T_{RM} cells to adhere to the epithelium and be positioned on the first line of defense (4). However, the expression of CD103 by CD4 T_{RM} cells is more controversial. A study by Collins et al. demonstrated that CD103 can be expressed on CD4 memory T cells egressing from the skin and suggested that this marker may be modulated as CD4 T cells enter and leave the skin (5). In contrast, CD4 effector T cells that infiltrated and resided in the skin after primary infection with *Candida albicans* acquired expression of CD69 and CD103 (6). We have recently reported that infection with *Bordetella pertussis* induces $CD69^+$ CD4 T_{RM} cells and a significant proportion of these cells stably express CD103 through the course of infection and after clearance of the bacteria (7). Following reinfection with *B. pertussis*, CD103 was rapidly upregulated on these cells, and this was not affected by treatment with FTY720, which inhibits lymphocyte egress from the draining lymph node and tissues (7). Retention of T_{RM} cells in tissues is facilitated by downregulation of CD62L and CCR7, "homing receptors" that allow T cells to enter secondary lymphoid organs, and sphingosine-1-phosphate receptor 1, which enables cells to egress from lymphoid tissues (8–10). The expression of other molecules like chemokine receptors on T_{RM} cells are often shaped by the specific tissue environmental cues.

Newborns and infants are in a greater risk from infections than adult humans. For example, high levels of morbidity and mortality have been reported in infants following respiratory infections with pathogens like influenza virus or *B. pertussis*, suggesting impaired protective immunity in infants compared with adults (11, 12). A possible explanation is that in pediatric tissues, the dominant population of T cells are naïve T cell emigrants from the thymus, whereas adult tissues contain predominantly memory T cells (13). Moreover, results from a mouse model of influenza infection have indicated that impaired protective immunity induced by previous infection or vaccination during infancy may reflect reduced generation of T_{RM} cells (14). Collectively, the emerging data on T_{RM} cells suggest that they play a critical role in long-term protective immunity induced by previous infection or vaccination.

INDUCTION, PERSISTENCE, AND FUNCTION OF CD4 T_{RM} CELLS IN INFECTION

The key function of T_{RM} cells is to rapidly respond to infection or reinfection with a pathogen and to orchestrate local immune responses in the tissue that mediate clearance of the pathogen. T_{RM} cells that are generated by infection are sustained in the local tissue after clearance of the pathogen (15). During a life time, T_{RM} cells accumulate in many tissues and provide long-term local protection against subsequent infection by reactivation with specific antigen (15, 16). The persistence of T_{RM} cells in tissue after pathogen clearance and the mechanism of maintenance in the tissues is unclear. Although memory T cells classically require antigen-specific stimulation through the T cell receptor (TCR) and costimulation for proliferation, there is evidence that T_{RM} cells may respond in an innate-like manner to cytokines, including IL-18, IL-12, IL-15, and tumor necrosis factor (TNF)-like cytokine 1A (TL1a), without TCR activation (17). Signaling from IL-15 and TGF-β has been shown to be critical for persistence of mature CD8 T_{RM} cells (**Figure 1**) (18). Furthermore, while much of the focus has been on pathogen-induced T_{RM} cells, these cells can also be generated by non-infectious agents, including allergens or autoantigens, and can mediate pathology in asthma or autoimmune disorders (19, 20). Nonetheless, there is growing evidence from mouse studies of a beneficial role for CD4 and CD8 T_{RM} cells in protection against a variety of infectious pathogens.

Viral Infections

CD8 cytotoxic T lymphocytes play a critical role in the control of viral infections by killing virally infected cells. However, CD4 T cells also play a vital role in protective immunity to viruses by helping antibody production and facilitate the induction and expansion of virus-specific memory CD8 T cells, including CD8 T_{RM} cells (21–23). Recent studies demonstrated that respiratory infection with a number of different viruses can also induce CD4 T_{RM} cells. It has been reported that influenza virus infection induces polyclonal, virus-specific memory CD4 T cells in the lungs and the spleens of infected mice (24). Adoptive transfer experiments with CD4 T cells specific for influenza hemagglutinin (HA) showed that lung-derived memory CD4 T cells were almost exclusively found in the lungs 7 days after transfer to naïve mice. In contrast, transferred splenic memory CD4 T cells were distributed in multiple tissues and were not retained in these tissues. Furthermore, only HA-specific CD4 T_{RM} cells from the lungs conferred protection against lethal influenza infection (24). These findings suggested that lung-resident CD4 T_{RM} cells, but not spleen memory CD4 T cells, play a crucial role in local protective immunity against influenza virus infection in the lungs.

Antigen-specific T_{RM} cells induced during influenza virus infection are localized near the airways and bronchovascular bundles and are maintained long after viral clearance independently of replenishment from lymphoid stores (25). IL-2-dependent and -independent mechanisms have been described for generation of influenza-specific CD4 T_{RM} cells, contributing to heterogeneity of protective T_{RM} cells. The formation of IL-2-independent subset of T_{RM} cells required a direct IL-15 signal to CD4 T-cell effectors (26, 27). Similarly, intranasal infection of mice with lymphocytic choriomeningitis virus (LCMV) required IL-2 signaling for the generation of virus-specific CD4 T_{RM} cells. CD4 T cells that lacked CD25, the IL-2 receptor α chain, failed to develop into lung T_{RM} cells in LCMV-infected mice (28). These studies suggest a broad mechanism involving IL-2 signaling pathway for the formation of CD4 T_{RM} cells.

Similar to respiratory tissue, the female reproductive track is vulnerable for repeated infections. In a model of herpes simplex virus-2 (HSV-2) infection, where thymidine-kinase defective (TK^-) HSV-2 was used to avoid neurovirulence (29), CD4 T cells infiltrated the female genital mucosa during infection and provided help for mobilizing cytotoxic effector CD8 T cells that cleared the infection (30). In addition, infection with TK^- HSV-2

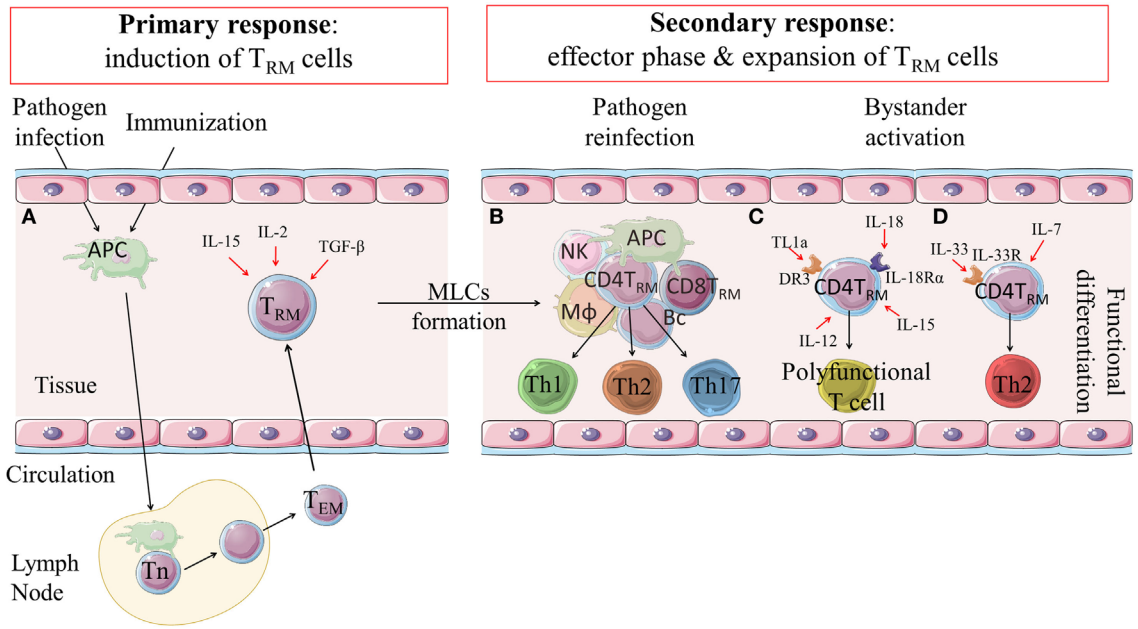

FIGURE 1 | Induction, expansion and function of CD4 T_{RM} cells. **(A)** Following infection or immunization effector and memory T cell responses are induced in lymph nodes. Effector-memory T cells can migrate to the tissue where they are retained as CD4 T_{RM} cells through expression of integrins and adhesion molecules, such as CD44, CD69 and/or CD103. Their induction is influenced by IL-2, IL-15 and TGF-β. **(B)** CD4 T_{RM} cells are retained in the tissues and can be reactivated locally in MLCs during secondary responses following reinfection or after infection in an immunized host. CD4 T_{RM} cells are expanded and can become effector Th1, Th17 or Th2-type cells that mediate rapid clearance of the infection. **(C)** There is emerging evidence that CD4 T_{RM} cells can be non-specifically activated in the tissues by cytokines, such as IL-18, IL-12, IL-15 and TL1a, and develop into polyfunctional T cells that have potent anti-pathogen activity. **(D)** It has also been suggested that IL-7 and IL-33 may non-specifically activate CD4 T_{RM} cells to become effector Th2 cells. DR3: death receptor 3; TL1a: tumor necrosis factor (TNF)-like cytokine 1a; MLCs: memory lymphocyte clusters; APC: antigen presenting cell; Mϕ: macrophage; NK: natural killer cell; Bc: B cells; Th: T helper (cell); T_n: naïve T cell; T_{EM}: effector memory T cells; T_{RM}: tissue-resident memory T cells.

provided local, long-term protection against a secondary infection with wild-type HSV-2 based on the formation of CD4 T_{RM} cells, which were retained mainly among memory lymphocyte clusters (MLCs) (31). Therefore, an effective vaccine against HSV-2 infection may be possible by targeting the induction of T_{RM} cells.

Bacterial Infections

A number of recent studies have indicated that CD4 T_{RM} cells established in non-lymphoid tissues after primary infection provide protection against reinfection with the same pathogen. In a mouse model of *B. pertussis* infection, it was demonstrated that transfer of Th1-like cells resulted in pathogen clearance in the absence of specific antibodies (32). We have recently reported that infection of mice with *B. pertussis* induce the development of $CD69^{+}CD103^{+/-}$ CD4 T_{RM} cells in the lungs (7). Treatment of convalescent mice with FTY720 did not affect clearance of a secondary infection with *B. pertussis*, suggesting that an established population of T_{RM} cells mediates local protective immunity against reinfection. Moreover, adoptive transfer of CD4 T_{RM} cells from the lungs of convalescent mice conferred protection against *B. pertussis* infection in naïve mice (7). It has also been demonstrated that pulmonary infection with *Mycobacterium tuberculosis* is controlled by a subset of lung parenchymal-homing CD4 T cells. Adoptive transfer of parenchymal T_{RM} cells into susceptible T cell-deficient hosts showed preferential migration back to the lung and superior control of infection compared with the intravascular CD4 T cells (33).

In a mouse model of pneumonia, repeated respiratory infections with *Streptococcus pneumoniae* (pneumococcus) seeded the lungs with antibacterial CD4 T_{RM} cells that mediated heterotypic protection (34). Furthermore, oral infection of mice with *Listeria monocytogenes* induced robust pathogen-specific CD4 T cell response, the majority of which migrated to the intestine and were transitioned to long-lived T_{RM} cells with a polyfunctional Th1 profile, secreting predominantly IFN-γ, TNF, and IL-2, and detectable level of IL-17 (35).

There is also emerging data to suggest that CD4 T_{RM} cells play a central role in protection against *Chlamydia trachomatis* infection (36). It has been shown that lymphoid aggregates, which contained CD4 T cells, are formed in the genital tract of mice during infection with *C. trachomatis*. These aggregates, which resembled MLCs described by Iijima and Iwasaki (31), persisted long after the infection had resolved (37). The formation of lymphoid aggregates with T_{RM} cells during primary infection provided a robust response to secondary infectious challenge and was dependent on B cell antigen presentation in established MLCs (38). These findings demonstrate that bacterial infection at various mucosal site (lungs, gut, and gential tract) induce CD4 T_{RM} cells that mediate protective immunity against reinfection of the mucosa with the relevant pathogen.

Parasite Infection

The development of Th2-type immune responses are required for protective immunity against infection with helminths, such as *Nippostrongylus brasiliensis* (39). Recent studies on lung infection with *N. brasiliensis* revealed that a Th2-type polarized pulmonary CD4 T cell population established during infection and can drive effective local adaptive immunity to reinfection with the same parasite (40). In a mouse model of intestinal infection with *Heligmosomoides polygyrus*, functional memory Th2 cells persisted in the lamina propria and the peritoneal cavity after resolution of infection. Interestingly, cells at both locations produced Th2 cytokines after restimulation; however, only peritoneal CD4 T_{RM} cells mediated protective immunity against the helminth infection. The Th2-type CD4 T_{RM} cells expressed high levels of the IL-33 receptor and produced effector cytokines in response to IL-33 and IL-7 independently to TCR activation (41). CD4 T_{RM} cells have also been identified in the skin after infection with *Leishmania major* where they persisted long after the pathogen was cleared (42, 43). Interestingly, CD4 T_{RM} cells were also found in the flank skin far from the primary infection site in the ear. Pathogen-specific CD4 T_{RM} cells produced IFN-γ in response to secondary infection and rapidly recruited other memory cells from the circulation; however, recruitment and activation of inflammatory monocytes was required for optimal protection (42, 43). These findings suggest that Th1- and Th2-type T_{RM} cells are induced by infection with different parasites and these cells mediate host protective immunity against the relevant parasite.

Distinct Subtypes of Infection-Induced T_{RM} Cells

A key research question that is beginning to be addressed is whether there are distinct Th1, Th2, and Th17 subtypes of T_{RM} cells and whether effector Th1, Th2, and Th17 arise from T_{RM} cells in the tissues after reinfection with a pathogen. It has been reported that skin infection with *C. albicans* in humans or mice leads to formation of IL-17-producing CD4 T_{RM} cells that reside in papillary dermis and rapidly clear the infection after re-exposure to the pathogen (6). It was also shown that protection against oropharyngeal candidiasis is mediated by oral-resident natural Th17 cells (44). Th1 cells have an established protective role in immunity to viruses and intracelluar bacteria and evidence is emerging that IFN-γ-seceting T_{RM} cells are critical for long-term protection against these pathogens. The findings from the parasite field also suggest that Th2 or Th1-type T_{RM} cells may play key roles in protective immunity against extracellular and intracellular parasites, respectively. However, the factors that control the development or specific activation of effector Th1, Th2, and Th17 from T_{RM} cells in the tissues after reinfection with a pathogen are still unclear (**Figure 1**).

VACCINE-INDUCED T_{RM} CD4 T CELLS

While most successful vaccines in use today mediate protective immunity through the induction of antibodies, optimum protection against many pathogens requires the generation of appropriate cellular immune responses, including CD4 T cells. Indeed, there are increasing number of studies showing that the formation of CD4 T_{RM} cells after natural infection mediates protective immunity against secondary exposure to the same pathogen. Although there is less evidence of a role for CD4 T_{RM} cells in protective immunity generated with vaccines in use today, the recent studies in mice have suggested that the induction of CD4 T_{RM} cells may be central to persistent vaccine-induced protection against a range of mucosal pathogens. Immunization approaches that induce systemic and tissue-retained memory CD4 T cells may be critical to persistent protection, because they are long-lived in the tissues and are more polyclonal than CD8 T cells (45, 46). It has also been suggested that CD4 T cell are less prone than CD8 T cell to immune escape from antigenic variation in T cell epitopes (47). Therefore, the induction of CD4 T_{RM} cells may be a promising approach for the design of new or improved vaccines. In the light of recent findings on the development of T_{RM} cells in different mucosal tissues, several important factors have to be considered in the development optimal immunization approaches for the induction of these cells.

LOCATION, COMPARTMENTALIZATION, AND ROUTE OF IMMUNIZATION

The efficacy of certain vaccines is influenced by the route of immunization. A comparison of two different licensed influenza vaccines given by intranasal or parenteral routes demonstrated that the route of administration, as well the type of vaccine (live versus killed), influenced the induction of CD4 T_{RM} cells. Intranasal administration of attenuated influenza virus vaccine (FluMist) generated CD4 T_{RM} cells in the lungs, which mediated long-term protection against non-vaccine strains of influenza virus. In contrast, an inactivated influenza virus vaccine (Fluzone) induced strain-specific neutralizing antibodies, but failed to induce T_{RM} cells, even when delivered intranasally (48). Studies with coronaviruses (CoVs), which cause a severe respiratory disease in humans, showed that intranasal, but not subcutaneous, immunization with SARS-CoV nucleocapsid (N) protein induced airway and lung-parenchymal antigen-specific memory CD4 T_{RM} cells (49). However, protection was lost following depletion of airway, but not parenchymal, memory T_{RM} cells. These results provide evidence of compartmentalization of the immune response induced by vaccination and suggest that T_{RM} cells may preferentially populate the site of induction/immunization.

Vaccine-induced T_{RM} cells can be localized not only near the site of immunization but can be spread to other parts of the same tissue. Mucosal vaccination can induce broad mucosal-tropic memory lymphocytes. Intranasal immunization with attenuated TK^- HSV-2 resulted in long-lasting protection mediated by HSV-2-specific CD4 T_{RM} in distant tissues, the vaginal mucosa (50, 51). Similarly, transmucosal protection against *Chlamydia muridarum* infection was established after oral vaccination. Colonization of the gastrointestinal tract with non-pathogenic bacterium induced protective immunity in the genital tract (52). Furthermore, it was shown that intranasal, but not subcutaneous, vaccination with ultraviolet light (UV)-inactivated *C. trachomatis* complexed with charge-switching synthetic adjuvant particles

induced protective CD4 T cells that rapidly populated uterine mucosa with T_{RM} cells (53).

Conserved vaccine antigens have the potential to induce broadly cross-protective immunity against many strains of the same pathogen. This is particularly important for pathogens like influenza virus, where the HA molecule, the target antigen for neutralizing antibodies, undergoes significant antigen variation allowing escape from protective immunity against seasonal strains of influenza virus. It was reported that intranasal immunization with influenza virus matrix protein ectodomain (M2e) adjuvanted with CTA1-DD generated highly protective M2e-specific lung-resident Th17 T_{RM} cells (54). Moreover, immunized mice were protected against a potentially lethal challenge with H3N2 or H1N1 influenza virus strains, demonstrating effective cross-protection. These results demonstrate that induction of T_{RM} cells and their ability to protect against mucosal infections is influenced by the route of immunization. Therefore, the design of more effective vaccines against mucosal pathogens needs to move beyond the common approach of using injectable vaccines and should utilize appropriate routes of mucosal immunization to promote protective T_{RM} cells at the sites of infection.

ROLE OF ADJUVANTS AND ANTIGENS IN VACCINE-INDUCED T_{RM} CELLS

The choice of adjuvant can influence the induction of cellular immune response and formation of T_{RM} cells following vaccination. Stary et al. showed that genital infection with *C. trachomatis* induced protective immunity in the uterus, whereas immunization with UV-inactivated *C. trachomatis*, which favored generation of regulatory T cells, exacerbated subsequent infection (53). However, an experimental vaccine comprising UV-inactivated *C. trachomatis* complexed with charge-switching synthetic adjuvant particles was effective at inducing antigen-specific CD4 T_{RM} cells and long-term protection (53). It has been reported that IL-1β may act as an adjuvant for the induction of T_{RM} that mediate protective immune responses against influenza virus infection. Intranasal administration of a novel vaccine, based on recombinant adenoviral vectors (rAd) encoding influenza HA and nucleoprotein in combination with rAd-IL-1β promoted the generation of $CD103^+CD69^+$ T_{RM} cells that mediated protection against infection with homologous and heterologous influenza virus strains (55).

Current vaccines against whooping cough (pertussis) are administered parenterally, usually by intramuscular route; however, immunity is relatively short lived, especially after immunization with acellular pertussis (aP) vaccines, which is administered with alum as the adjuvant (56). Studies in a baboon model have shown that the current aP vaccine fails to prevent nasal colonization and transmission of *B. pertussis* (57). In contrast, immunization with an attenuated *B. pertussis* vaccine, BPZE1, protected baboons against nasopharyngeal colonization and disease induced by a highly virulent strain of *B. pertussis* (58). Since BPZE1 is replicating bacterium delivered by the intranasal route, it is likely to induce respiratory T_{RM} cells. Current parenterally delivered aP vaccines preferentially induce strong antibody and Th2-type responses, whereas experimental aP vaccines formulated with more potent adjuvants, such as TLR agonists, induce potent Th1 and Th17 responses in mice (59, 60). Therefore, it should also be possible to develop an intranasally delivered aP vaccine with an appropriate adjuvant that induces IL-17 and IFN-γ-secreting T_{RM} cells in the lungs and nasal tissue. It has been reported that the formation of CD8 T_{RM} cells in the nasal epithelium after immunization are key for limiting influenza viral spread to the lower respiratory track (61). Therefore, induction of respiratory T_{RM} cells by intranasal immunization appears to be an ideal approach for inducing long-term protection in the upper and lower respiratory tract.

IMPLICATIONS FOR NEW OR IMPROVING VACCINE DESIGN

In the vaccine field, the big questions include (1) whether CD4 T_{RM} cells are really important for long-term protective immunity in humans, (2) how antigen-specific T_{RM} cells can be optimally induced by vaccination, and (3) how antigen-specific T_{RM} cells can be detected and quantified after infection or vaccination in humans. Most of the vaccines in use today protect by induction of antibody responses that either neutralize viruses or bacterial toxins or opsonize bacteria for killing by phagocytic cells. However, there are other infectious diseases, such as HIV, malaria, and tuberculosis where we do not have an effective vaccine, and where T-cell responses may be more important in preventing or clearing the infection. Furthermore, there is a move away from killed and live attenuated vaccine to subunit vaccines, which are usually delivered by injectable routes. However, the first choice adjuvant alum, while capable of promoting the induction of antibody and Th2 responses is not very effective at inducing Th1 responses. In addition, injected alum-adjuvant vaccines do not appear to be capable of inducing T_{RM} cells. The current pertussis aP vaccine is a good example; it fails to induce Th1 cells (59) and protective immunity wanes rapidly after immunization in children (62). This is likely to reflect a failure to induce CD4 T_{RM} cells. Effort to develop more effective aP and other subunit vaccines need to focus on mucosal routes of immunization and adjuvants that induce T_{RM} cells, as well Th1 and Th17 cells that can be detected in the periphery. It was reported that subcutaneous priming followed by intranasal boosting with group A streptococcal C5a peptidase formulated with a cationic adjuvant induced persistent local immune response including IgA, Th17 cells, and T_{RM} cells (63). The "Prime and Pull" strategy may be a useful approach for eliciting both systemic and local immunity and immunological memory with subunit vaccines (64).

The vast majority of the published work on T_{RM} cells have been based on studies in mouse models. $CD69^+$ T_{RM} cells have also been identified in human tissues (65). However, since T_{RM} cells are in the tissue rather than the blood, one of the challenges in translating the mouse studies to humans is the difficulty in getting routine access to human mucosal tissue samples to study and quantify the induction of T_{RM} cells following infection or vaccination. This could be overcome by the identification of precursors of T_{RM} in the circulation as they migrate from lymph nodes to tissues. Peripheral memory CD8 T cells that express CX3CR1 have

been identified in mice (66). However, it has also been reported that intravascular CX3CR1^{+}KLRG1^{+} Th1 cells did not migrate into the lungs and were unable to control *M. tuberculosis* infection (67). Nevertheless, the design of new or improved vaccines that confer sustained sterilizing immunity at mucosal surface will be greatly facilitate by the identification of immunization approaches that induce potent pathogen-specific T_{RM} at the mucosal site of infection.

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Functional Heterogeneity of CD4$^+$ Tumor-Infiltrating Lymphocytes With a Resident Memory Phenotype in NSCLC

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Resident memory T cells (T_{RM}) inhabit peripheral tissues and are critical for protection against localized infections. Recently, it has become evident that CD103$^+$ T_{RM} are not only important in combating secondary infections, but also for the elimination of tumor cells. In several solid cancers, intratumoral CD103$^+$CD8$^+$ tumor infiltrating lymphocytes (TILs), with T_{RM} properties, are a positive prognostic marker. To better understand the role of T_{RM} in tumors, we performed a detailed characterization of CD8$^+$ and CD4$^+$ TIL phenotype and functional properties in non-small cell lung cancer (NSCLC). Frequencies of CD8$^+$ and CD4$^+$ T cell infiltrates in tumors were comparable, but we observed a sharp contrast in T_{RM} ratios compared to surrounding lung tissue. The majority of both CD4$^+$ and CD8$^+$ TILs expressed CD69 and a subset also expressed CD103, both hallmarks of T_{RM}. While CD103$^+$CD8$^+$ T cells were enriched in tumors, CD103$^+$CD4$^+$ T cell frequencies were decreased compared to surrounding lung tissue. Furthermore, CD103$^+$CD4$^+$ and CD103$^+$CD8$^+$ TILs showed multiple characteristics of T_{RM}, such as elevated expression of CXCR6 and CD49a, and decreased expression of T-bet and Eomes. In line with the immunomodulatory role of the tumor microenvironment, CD8$^+$ and CD4$^+$ TILs expressed high levels of inhibitory receptors 2B4, CTLA-4, and PD-1, with the highest levels found on CD103$^+$ TILs. Strikingly, CD103$^+$CD4$^+$ TILs were the most potent producers of TNF-α and IFN-γ, while other TIL subsets lacked such cytokine production. Whereas, CD103$^+$CD4$^+$PD-1^{low} TILs produced the most effector cytokines, CD103$^+$CD4$^+$PD-1^{++} and CD69$^+$CD4$^+$PD-1^{++} TILs produced CXCL13. Furthermore, a large proportion of TILs expressed co-stimulatory receptors CD27 and CD28, unlike lung T_{RM}, suggesting a less differentiated phenotype. Agonistic triggering of these receptors improved cytokine production of CD103$^+$CD4$^+$ and CD69$^+$CD8$^+$ TILs. Our findings thus provide a rationale to target CD103$^+$CD4$^+$ TILs and add co-stimulation to current therapies to improve the efficacy of immunotherapies and cancer vaccines.

Keywords: NSCLC, TRM, TILs, cytokines, exhaustion, differentiation, co-stimulation

INTRODUCTION

T cells are important mediators of tumor immunity and T cell infiltration of most types of solid tumors is a favorable prognostic marker (1, 2). Immunotherapy boosting T cell functionality in tumors is rapidly gaining a foothold as standard treatment. Unfortunately, durable responses are only observed in a minority of patients (3), which is most likely related to the highly immunosuppressive microenvironment of most tumors. Moreover, there is growing awareness that not only the degree of tumor infiltration but also the composition of T cell infiltrates varies substantially even between patients with the same cancer. As in healthy tissues, it is unlikely that all subsets of T cells are equally adapted to the physiological properties of the tumor microenvironments. Understanding the composition of tumor infiltrating lymphocytes (TILs) and defining the populations that contribute most to anti-tumor responses is essential to boost efficacy of immunotherapy.

In the past few years it became clear that immunity in tissues requires adaptation to the physiological properties of those tissues. In both mice and humans a specific subset of memory T cells permanently resides in tissues. Effector and memory T cells first enter tissues as part of an antigen-specific response and subsequently take up residency and become resident memory T cells (T_{RM}). Once established, T_{RM} are important for protecting barrier tissues against secondary infections (4). Due to their strategic location, T_{RM} can detect pathogens and kill infected cells at an early stage to control the spread of infection. As an effector mechanism T_{RM} produce effector molecules more rapidly than other memory T cells (5, 6). The rapid release of IFN-γ, TNF-α, and IL-2 primes the surrounding tissue and leads to the recruitment of auxiliary immune cells to the infected site (7, 8).

Different types of T_{RM} exist, residing in different tissues, but even within single organs strict spatial organization of T_{RM} subsets has been described (9, 10). As such, a subset of T_{RM} are specifically adapted for residence in epithelial tissues. These T_{RM} are traditionally characterized by the expression of CD69, which inhibits S1PR1 mediated egress from tissues (11), and CD103 (alpha subunit of αEβ7 integrin), which docks cells to epithelial E-cadherin (12, 13). Recently, a variety of novel markers have been revealed that characterize T_{RM}. These include the chemokine receptor CXCR6, important for development of T_{RM} (14), and CD49a (α subunit of α1β1 integrin), necessary for retention and cytotoxic function of T_{RM} (15, 16). Another hallmark of T_{RM} is the expression of a broad range of inhibitory receptors. T_{RM} often reside in delicate tissues, thus their activation appears to be strictly regulated to prevent immunopathology (5, 6, 17).

In line with the epithelial origin of most solid tumors, varying numbers of infiltrating T cells with an intraepithelial $CD103^+$ phenotype have been described. For several types of cancers, it is now appreciated that the presence of mainly $CD103^+CD8^+$ TILs is a positive prognostic marker (18–21). Among human NSCLC tumors with similar degrees of T cell infiltration, those with the greatest proportions of $CD103^+$ cells have the best prognosis. These $CD103^+CD8^+$ TILs share gene expression programs and phenotypic properties of T_{RM}, including the expression of CD69, CXCR6, and CD49a (21). T_{RM} characteristics of $CD4^+$ TILs are less explored. Although, the necessity of $CD4^+$ T cell help for the cytotoxic programming of $CD8^+$ T cells is widely appreciated (22, 23), they have also been described to suppress tumor growth through the secretion of IFN-γ or direct killing of tumor cells (24, 25). While $CD103^+CD8^+$ TILs isolated from NSCLC demonstrated greater cytotoxic capacity toward tumor cells than their $CD103^-$ counterparts (19), the functional characteristics of $CD103^+CD4^+$ TILs remain largely unexplored.

In this study we map the heterogeneity of $CD4^+$ and $CD8^+$ T cell infiltrates in human NSCLC and compare them with paired unaffected lung tissue. We investigated T_{RM} characteristics of TIL subsets and addressed the expression of various inhibitory receptors that can be targeted by checkpoint inhibition therapy. We demonstrated an increased number of $CD103^+CD8^+$ TILs in NSCLC compared to surrounding lung tissue. In contrast, numbers of $CD103^+CD4^+$ TILs were decreased. Although the highest expression of inhibitory receptors was found on $CD103^+$ TILs this was paradoxical to the superior cytokine production especially of $CD103^+CD4^+$ TILs. While TILs producing effector cytokines had lower PD-1 expression than TILs not producing cytokines, TILs with high PD-1 expression produced CXCL13, indicative of functionally distinct subsets within TILs. Furthermore, we found TILs to have a less differentiated phenotype than lung T_{RM} and that additional co-stimulation enhances cytokine production of some TIL subsets. Understanding the properties of TILs with T_{RM} attributes may have important implications for future cancer treatments.

RESULTS

Resident Memory Phenotypes in Paired Blood, Lung and Tumor Samples of NSCLC Patients

While $CD8^+$ T cells are in the spotlight of cancer immunotherapy, significant numbers of $CD4^+$ T cells can also be found in solid tumors. We determined the frequencies of $CD4^+$ and $CD8^+$ T cells among the total $CD3^+$ T cell pool in paired tumor, lung, and blood samples of 33 NSCLC patients. Included patients received a surgical resection of primary tumors as first line therapy without prior chemo- or radiotherapy. Blood was drawn from a central line at the start of surgery. We found comparable frequencies of $CD4^+$ and $CD8^+$ T cells in all three compartments (**Figure 1A**, general gating strategy in **Supplementary Figure 1**). Analysis of T_{RM} phenotypes was determined by the expression of CD69 and CD103. While $CD69^+$ T cells were virtually absent in peripheral blood, they dominated in the lung and tumor (**Figure 1B**). In the blood, the frequency of $CD103^+$ cells was low and as these cells lacked CD69 expression they cannot be defined as T_{RM} (data not shown). In contrast, both the lung and tumor compartments harbored high frequencies of $CD69^+CD103^+$ cells. As such, lung and tumor derived $CD4^+$ and $CD8^+$ T cells can be divided into three populations based on the expression

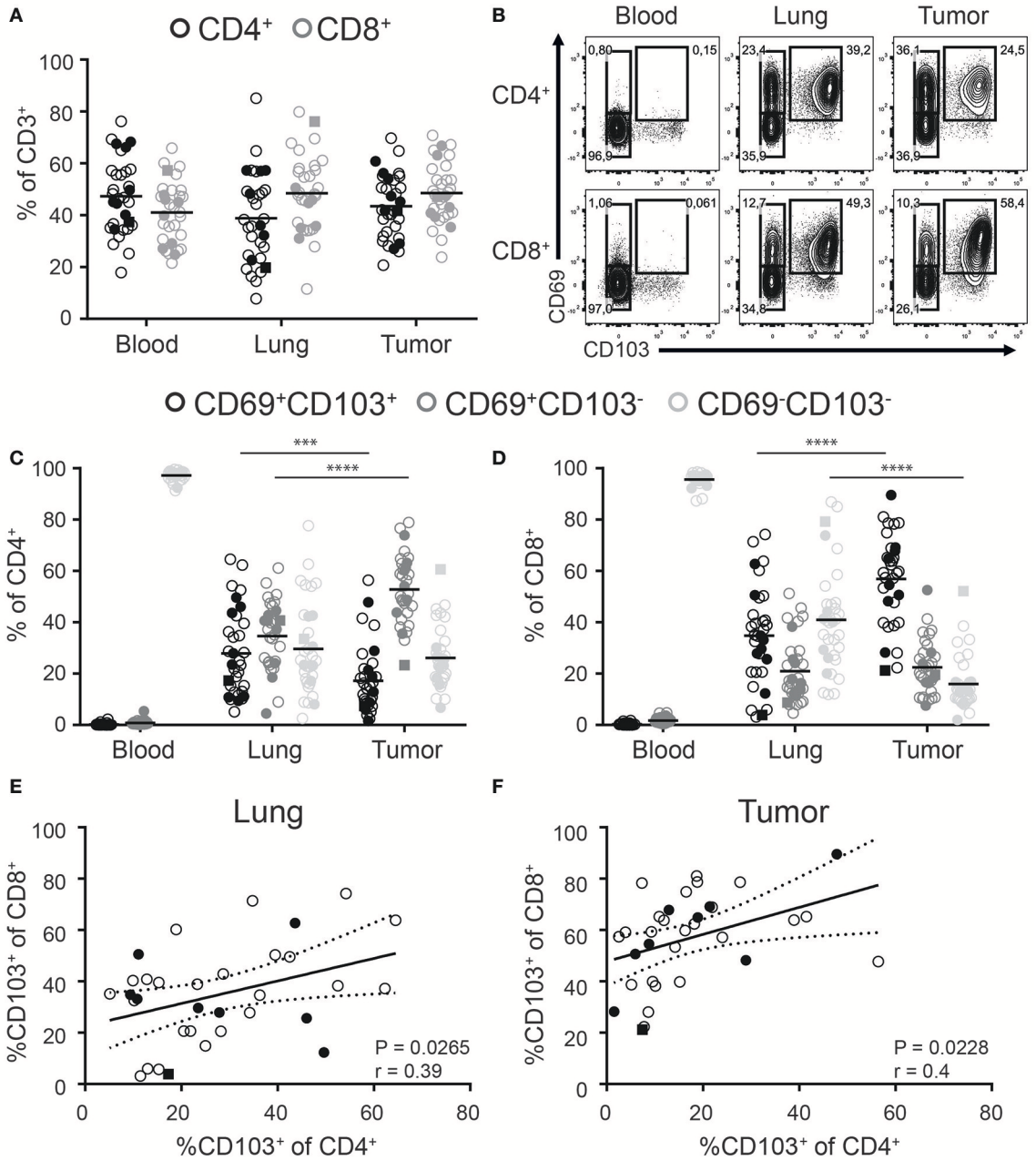

FIGURE 1 | Distribution of CD103 and CD69 expression on $CD4^+$ and $CD8^+$ T cells of paired blood, lung, and tumor samples. **(A)** Frequencies of $CD4^+$ (black circles) and $CD8^+$ (gray circles) cells of total $CD3^+$ T cells of paired blood, lung, and tumor tissue was analyzed by flow cytometry. **(B–D)** The expression of CD69 and CD103 was analyzed on paired blood, lung and, tumor $CD4^+$ and $CD8^+$ T cells. **(B)** Contour plots show representative examples of CD69 and CD103 expression on blood (left panels), lung (middle panels), and tumor (right panels) $CD4^+$ (top panels) and $CD8^+$ (bottom panels) T cells. **(C,D)** Frequencies of $CD103^+CD69^+$ (black circles), $CD103^-CD69^+$ (dark gray circles), and $CD103^-CD69^-$(light gray circles) cells of total blood, lung, and tumor $CD4^+$ **(C)** and $CD8^+$ **(D)** T cells were quantified. **(E,F)** Correlation between $CD103^+CD8^+$ and $CD103^+CD4^+$ lung **(E)** and tumor **(F)** T cells was determined. **(A–F)** $n = 33$. Open circles, solid circles, solid square indicate adeno-, squamous, and large cell carcinoma, respectively. **(A,C,D)** Quantifications are shown as dot plots with the horizontal line indicating the mean and each point represents a unique sample. **(E,F)** Correlation shown as X-Y graph where each point represents a unique sample. **(C,D)** $^{***}p < 0.001$, $^{****}p < 0.0001$; 2-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. **(E,F)** r, Pearson's rank coefficient; $p < 0.05$.

of CD69 and CD103 ($CD103^+CD69^+$, $CD103^-CD69^+$, and $CD103^-CD69^-$; **Figure 1B**). For the rest of paper we refer to the $CD103^+CD69^+$ and $CD103^-CD69^+$ tumor and lung populations as $CD103^+$ and $CD69^+$ TILs and T_{RM}, respectively, and tumor and lung $CD103^-CD69^-$ as $CD69^-$ TILs and $CD69^-$ T cells, respectively.

The percentage of $CD103^+CD8^+$ TILs was significantly increased compared to $CD103^+CD8^+$ lung T_{RM}. The increased abundance of $CD103^+CD8^+$ TILs was accompanied by a decreased percentage of $CD69^-CD8^+$ TILs (**Figure 1D**). On the other hand, the decreased frequencies of $CD103^+CD4^+$ TILs was compensated by more $CD69^+CD4^+$ TILs (**Figure 1C**). Of note, while we included patients with different types of NSCLC (24 × Adeno-, 8 × Squamous, and 1 × Large cell carcinoma), no differences were observed in the frequency of the different subsets (**Figure 1**: Adeno—open circles, squamous solid circles, large cell carcinoma solid square). We further found a correlation between the frequencies of $CD103^+CD8^+$ and $CD103^+CD4^+$ in both the lung and tumor (**Figures 1E,F**).

TIL Populations Are Enriched for T Cells With an Early Differentiated Memory Phenotype

A critical step in T_{RM} development is their recruitment into tissue where they undergo a process of maturation characterized by a loss of the co-stimulatory CD27 and CD28 receptors. We defined the differentiation stage of the different lung and tumor T cell subsets by analyzing the surface expression of CD45RA, CD28, CD27, and CCR7. While naïve T cells express all four markers, expression is lost stepwise by differentiating antigen-primed cells. Early, early-like, intermediate, late effector-type ($CD45RA^-$) and late effector-type ($CD45RA^+$) differentiated cells are described as, $CCR7^-CD27^+CD45RA^-CD28^+$, $CCR7^-CD27^-CD45RA^-CD28^+$,$CCR7^-CD27^+CD45RA^-CD28^-$, $CCR7^-CD27^-CD45RA^-CD28^-$, and $CCR7^-CD27^-CD45RA^+CD28^-$, respectively (26–28). In accordance with our previous studies (5, 6), lung and tumor T cells did not express CCR7 (**Supplementary Figure 2A**). As such, there were barely any undifferentiated naïve ($CD45RA^+CD27^+CD28^+$) T cells in the lung or tumor (**Figures 2A–D**). In the lung, $CD103^+$ T_{RM} harbored mainly late differentiated $CD28^-CD45RA^-CD27^-$ cells for both $CD4^+$ and $CD8^+$ lineages (**Figures 2C,D**; **Supplementary Figure 2B**). On the other hand, large fractions (40–50%) of lung $CD69^+$ T_{RM} were early or intermediate differentiated. The differentiation profile of lung $CD69^-$ T cells was more variable but mainly comprised of intermediate to late differentiated cells. Compared to lung T cell subsets, all TIL subsets contained less differentiated cells (**Figures 2C,D**). The largest differences were observed for the $CD4^+$ TILs. $CD103^+CD4^+$ TILs contained more $CD27^+CD45RA^-CD28^+$ early differentiated cells, while these cells were virtually absent in $CD103^+CD4^+$ T_{RM}. This pattern was even more pronounced for the $CD69^+CD4^+$ and $CD69^-CD4^+$ subsets. $CD103^+CD8^+$ TILs had higher expression of CD27 than lung $CD103^+CD8^+$ T_{RM}. In line with the $CD4^+$ TILs, the strongest decrease in late differentiated cells was observed in the $CD69^+CD8^+$ and $CD69^-CD8^+$ TIL compartments. Of note, we also did not find differences in the phenotype of the T_{RM} or TILs between adenocarcinoma and squamous carcinoma (**Supplementary Figures 2C,D**). In summary, both $CD4^+$ and $CD8^+$ TILs, regardless of phenotype, contained less late differentiated cells compared to their lung equivalents.

$CD103^+$ TILs Express Common T_{RM} Homing and Adhesion Molecules

Recently, homing and adhesion molecules CXCR6 and integrin CD49a (α subunit of α1β1 integrin), were found in numerous T_{RM} core signatures and promote formation and retention of T_{RM} (14, 17, 29). Although $CD8^+$ TILs were previously demonstrated to express CXCR6 (21), it remains unclear if this chemokine receptor defines TILs with a T_{RM} phenotype in tumors. In line with the expression pattern in lungs, we found that $CD4^+$ and $CD8^+$ TILs with a T_{RM} phenotype were enriched for $CXCR6^+$ cells (**Figures 3A,C**). While CXCR6 was uniformly expressed by almost all $CD103^+$ TILs, roughly half of the $CD69^+$ TILs also expressed this chemokine receptor. Also in tumors CXCR6 expression appeared to define T_{RM}, as $CD69^-$ TILs barely expressed CXCR6, comparable with lung $CD69^-$ T cells. Similarly to the CXCR6 expression, expression of CD49a was highest in $CD103^+$, intermediate in $CD69^+$, and absent in $CD69^-$ TILs (**Figures 3B,D**). This was the case for both $CD4^+$ and $CD8^+$ TILs, albeit the CD49a expression was more uniform on $CD8^+$ cells.

Shared Expression of Transcription Factors by T_{RM} and TILs With a T_{RM}-Like Phenotype

In both human and mice, T_{RM} express a different repertoire of transcription factors when compared to other memory and effector T cells (5, 6, 30). Among the most differentially expressed transcription factors are T-bet and Eomes. Downregulation of both T-box transcription factors is required T_{RM} development (31). Accordingly, lung $CD103^+CD4^+$, $CD69^+CD4^+$, and $CD103^+CD8^+$ T_{RM} expressed low levels of T-bet and Eomes (**Figures 4A–D**). In contrast, a substantial population of $CD69^+CD8^+$ T_{RM} expressed Eomes, while Tbet expression was similar to that of the other T_{RM} subsets. Of note, while T-bet expression was lower than in blood effector T cells, it was higher than blood-derived naïve T cells (**Supplementary Figure 3A**). Lung $CD69^-CD4^+$ and $CD69^-CD8^+$ subsets expressed the highest levels of T-bet and Eomes, similar to blood effector T cells (**Figures 4A–D**, **Supplementary Figure 3A**). TILs with a T_{RM} phenotype demonstrated comparable T-bet and Eomes expression patterns as their T_{RM} counterparts. However, Eomes expression was decreased in $CD69^-CD4^+$ TILs compared to lung $CD69^-CD4^+$ T cells, which fits with the decreased number of late differentiated cells in this subset, observed above. In line with the less differentiated phenotype of the TILs and the requirement of T_{RM} to downregulate T-box transcription factors, we determined whether TILs expressing CD27 also expressed T-bet and Eomes. Interestingly, $CD8^+$ TILs that expressed CD27 also expressed Eomes. However, we did not find this pattern for $CD4^+$ TILs, suggesting that there is correlation between the downregulation of CD27 and Eomes in $CD8^+$ TILs but not in $CD4^+$ TILs (**Supplementary Figure 3B**). We also determined Foxp3 expression in $CD4^+$ TILs with a T_{RM} phenotype and

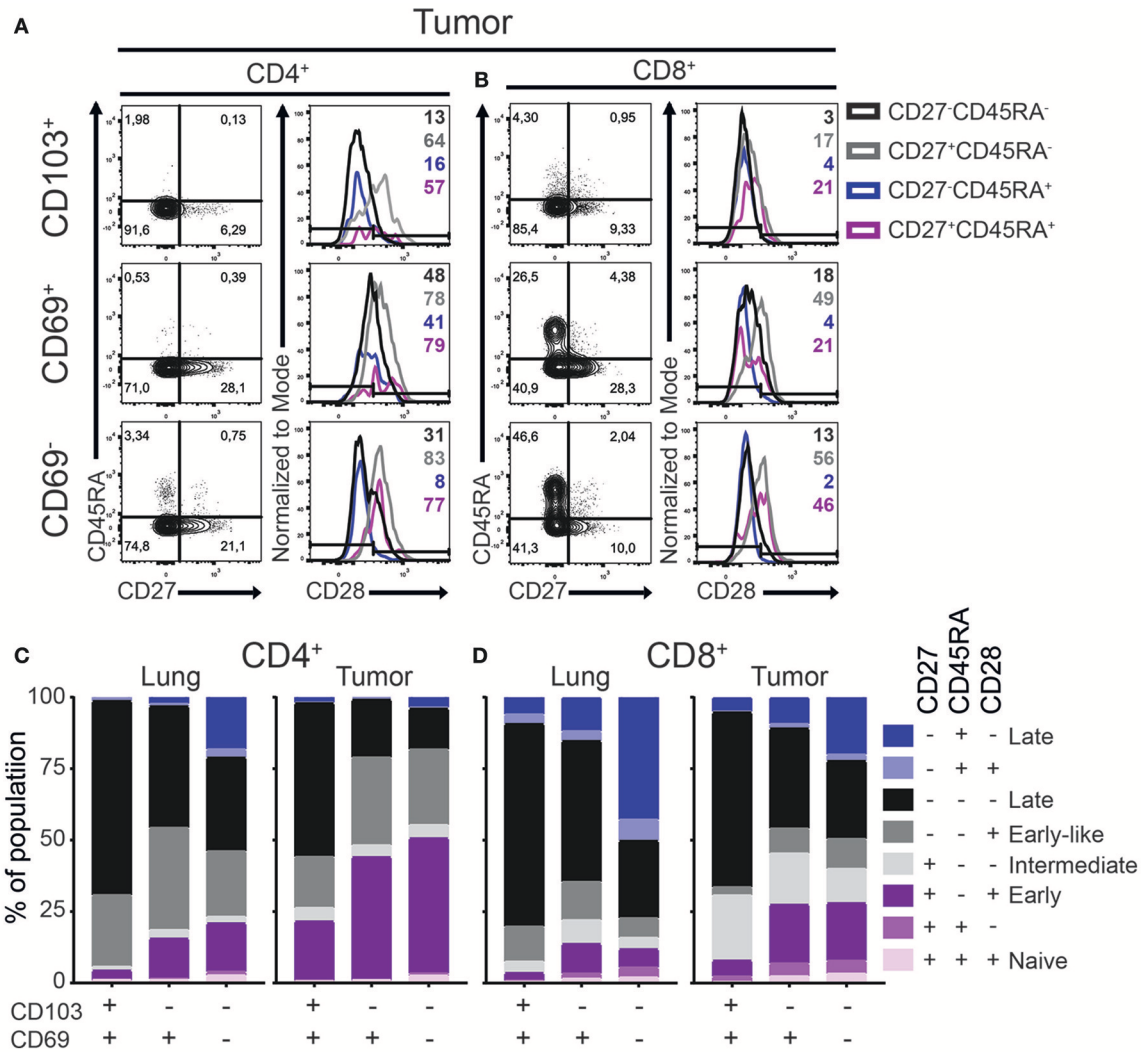

FIGURE 2 | Differentiation status of lung T_{RM} and TILs. **(A–D)** The expression of CD45RA, CD27, and CD28 on $CD4^+$ and $CD8^+$ lung T_{RM} and TILs was determined. **(A,B)** The expression of CD27, CD45RA, and CD28 tumor $CD103^+$ (top panels), $CD69^+$ (middle panels), and $CD69^-$ (bottom panels) $CD4^+$ **(A)** and $CD8^+$ **(B)** T cells shown by representative contour plots (CD45RA on y-axis, CD27 on x-axis) and histograms overlays (maximum set to 100%) show the expression of CD28 on the different subsets (black $CD27^-CD45RA^-$, gray $CD27^+CD45RA^-$, blue $CD27^-CD45RA^+$, purple $CD27^+CD45RA^+$). **(C,D)** The frequencies of $CD27^+CD45RA^+CD28^+$ (light purple), $CD27^+CD45RA^+CD28^-$ (medium purple), $CD27^+CD45RA^-CD28^+$ (dark purple), $CD27^+CD45RA^-CD28^-$ (light gray), $CD27^-CD45RA^-CD28^+$ (medium gray), $CD27^-CD45RA^-CD28^-$ (black), $CD27^-CD45RA^+CD28^+$ (light blue), $CD27^-CD45RA^+CD28^-$ (dark blue) of $CD103^+$, $CD69^+$, and $CD69^-$ lung $CD4^+$ (**C**; left bar graph), tumor $CD4^+$ (**C**; right bar graph), lung $CD8^+$ (**D**; left bar graph), and tumor $CD8^+$ (**D**; right bar graph). **(C,D)** The quantifications are shown as bar graphs with the mean. $n = 15$.

found that most regulatory T cells (T_{reg}) were found in the $CD4^+CD69^+$ TIL compartment (**Supplementary Figure 3C**).

Granzyme B (GZMB) Expression by T_{RM} and TILs

Since both T-bet and Eomes are important for effector cell differentiation and function (32), we determined granzyme B (GZMB) expression among the different T cell subsets. In line with the observed T-bet and Eomes expression, the frequency of $GZMB^+$ cells was highest in lung $CD69^-$ T cells (**Figures 4A,E**). However, there was a strong decrease of GZMB expression in $CD69^-$ TILs compared to lung T cells. As for the $CD69^+$ TIL subsets, GZMB expression resembled the levels of their lung counterparts. Most $CD103^+CD4^+$ and $CD103^+CD8^+$ lung T_{RM} lacked expression of GZMB, yet there was a significant increase of $GZMB^+$ cells in $CD103^+CD8^+$ TILs. Overall, GZMB expression patterns were similar to those of T-bet and Eomes in both lung T_{RM} and TILs.

$CD103^+$ TILs Expressed the Highest Levels of Inhibitory Receptors

A shared feature of T_{RM} in mice and human is the expression of multiple inhibitory receptors (30). These receptors are thought to help protect against excessive T_{RM} activation and subsequent

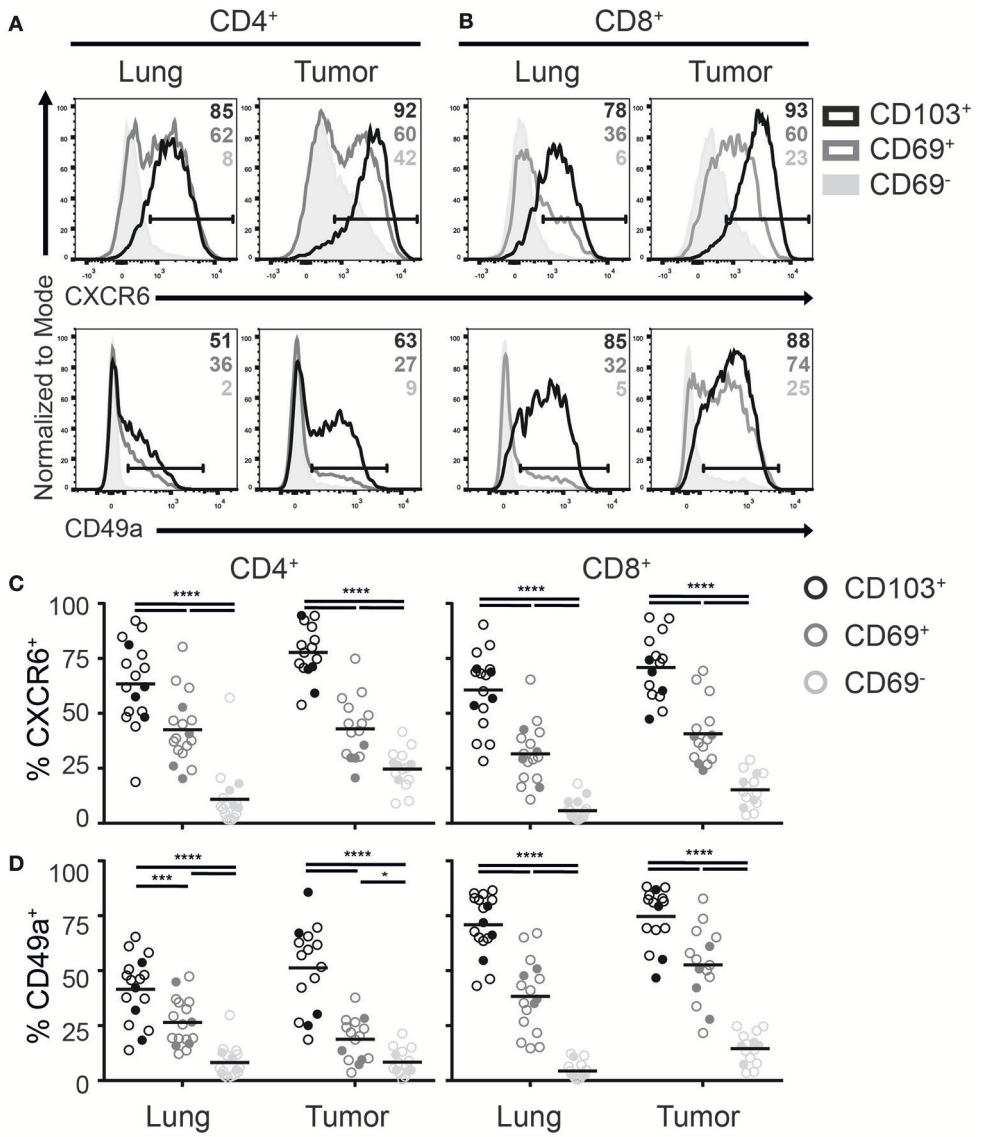

FIGURE 3 | Expression of T_{RM} homing molecules by TILs. **(A-D)** The expression of chemokine receptor CXCR6 and integrin CD49a were analyzed on $CD4^+$ and $CD8^+$ T_{RM} and TILs. The expression of CXCR6 (top panels) and CD49a (bottom panels) on lung (left panels) and tumor (right panels) $CD4^+$ **(A)** and $CD8^+$ **(B)** T cell subsets is shown by representative histogram overlays (maximum set to 100%) ($CD103^+$ black, $CD69^+$ dark gray, $CD69^-$ solid light gray). The frequencies of $CXCR6^+$ **(C)** and $CD49a^+$ **(D)** $CD103^+$ (black circles), $CD69^+$ (dark gray circles), and $CD69^-$ (light gray circles) cells of lung and tumor $CD4^+$ T cells (left graphs) and $CD8^+$ T cells (right graphs). **(C,D)** The quantifications are shown as dot plots with the horizontal line indicating the mean and each point represents a unique sample. $n = 15$–17. Open circles and solid circles indicate adeno- and squamous carcinoma, respectively. $^*p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$; 2-way ANOVA with Tukey's multiple comparisons test.

immunopathology of delicate tissues. In the tumor environment, upregulation of inhibitory receptors, such as PD-1, have also been linked to exhaustion (33). Paradoxically, PD-1 expression has also been described as a favorable prognostic marker in several cancers, in which it defines tumor-specific $CD8^+$ T cells (34, 35). As several inhibitory molecules are targeted by immunotherapy, we investigated the expression of PD-1, CTLA-4, and 2B4 among the different TIL populations. PD-1 was broadly expressed by $CD4^+$ and $CD8^+$ T_{RM} and TILs (**Figure 5A**). The highest frequencies and levels of PD-1 expression were found on $CD103^+CD4^+$, $CD69^+CD4^+$, and $CD103^+CD8^+$ TILs followed by $CD69^+CD8^+$ TILs (**Figures 5B,C**). We found that the expression pattern of CTLA-4 was comparable to that of PD-1 (**Figures 5D–F**). Interestingly, $CD4^+$ TILs expressed higher CTLA-4 levels than their $CD8^+$ counterparts. Expression of 2B4 appeared different. While 2B4 is associated with T cell exhaustion, it functions differently from classical inhibitory receptors and can also act as a co-stimulatory molecule (33, 36).

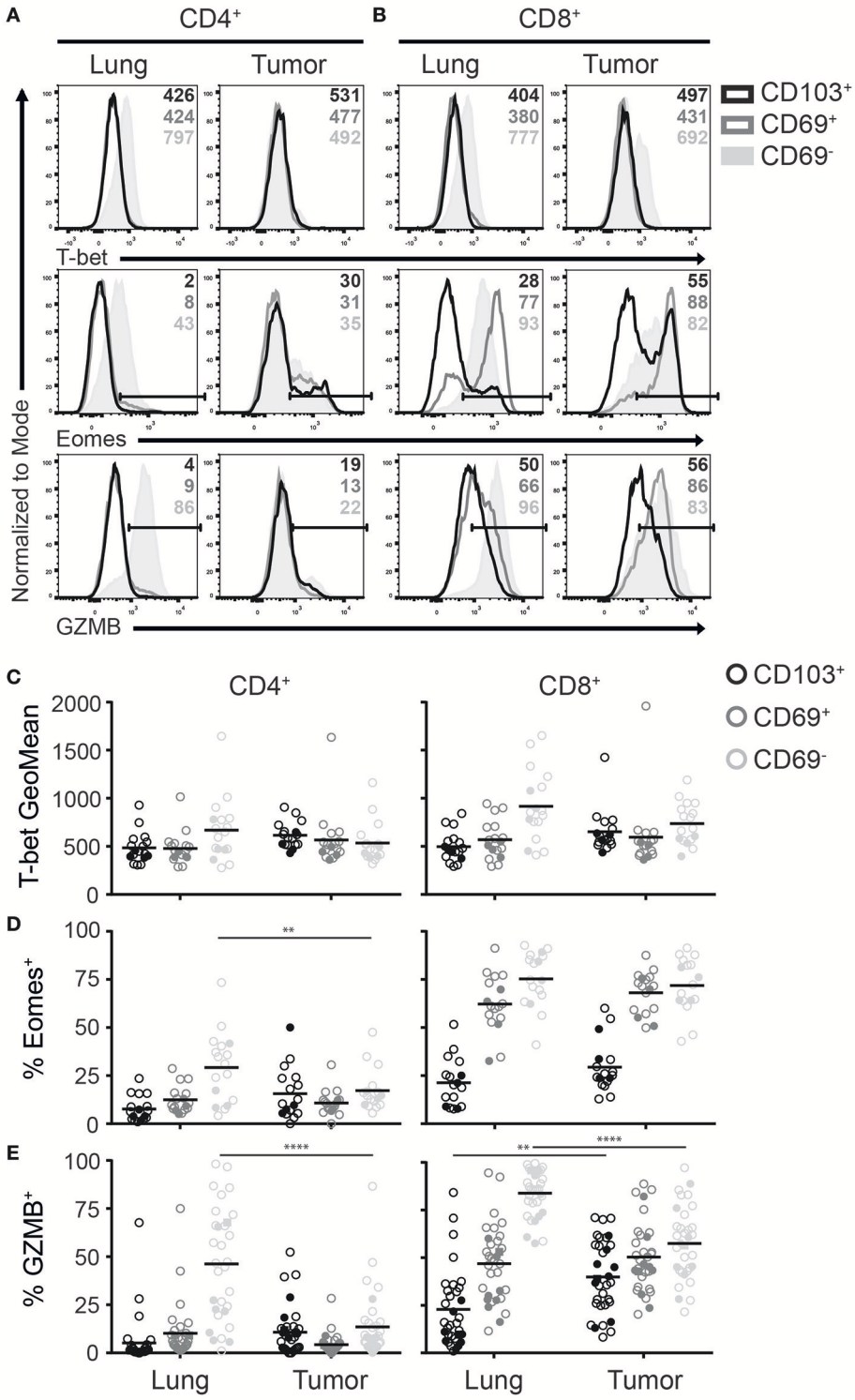

FIGURE 4 | Expression of T-bet, Eomes and GZMB on T_{RM} and TILs. **(A–D)** The expression of T-bet, Eomes, and GZMB was analyzed on $CD4^+$ and $CD8^+$ T_{RM} and TILs. The expression of T-bet (top panels), Eomes (middle panels), and GZMB (bottom panels) on lung (left panels) and tumor (right panels) $CD4^+$ **(A)** and $CD8^+$ **(B)** T cells is shown by representative histogram overlays (maximum set to 100%) ($CD103^+$ black, $CD69^+$ dark gray, $CD69^-$ solid light gray). The expression of T-bet (geometric mean fluorescence intensity; GeoMFI) **(C)** and frequencies of $Eomes^+$ **(D)** and $GZMB^+$ **(E)** $CD103^+$ (black circles), $CD69^+$ (dark gray circles), and $CD69^-$ (light gray circles) cells of lung and tumor $CD4^+$ (left graphs) and $CD8^+$ (right graphs) T cells. **(C–E)** The quantifications are shown as dot plots with the horizontal line indicating the mean and each point represents a unique sample. $n = 17$. Open circles and solid circles indicate adeno- and squamous carcinoma, respectively. $^{**}p < 0.01$, $^{****}p < 0.0001$; 2-way ANOVA with Tukey's multiple comparisons test.

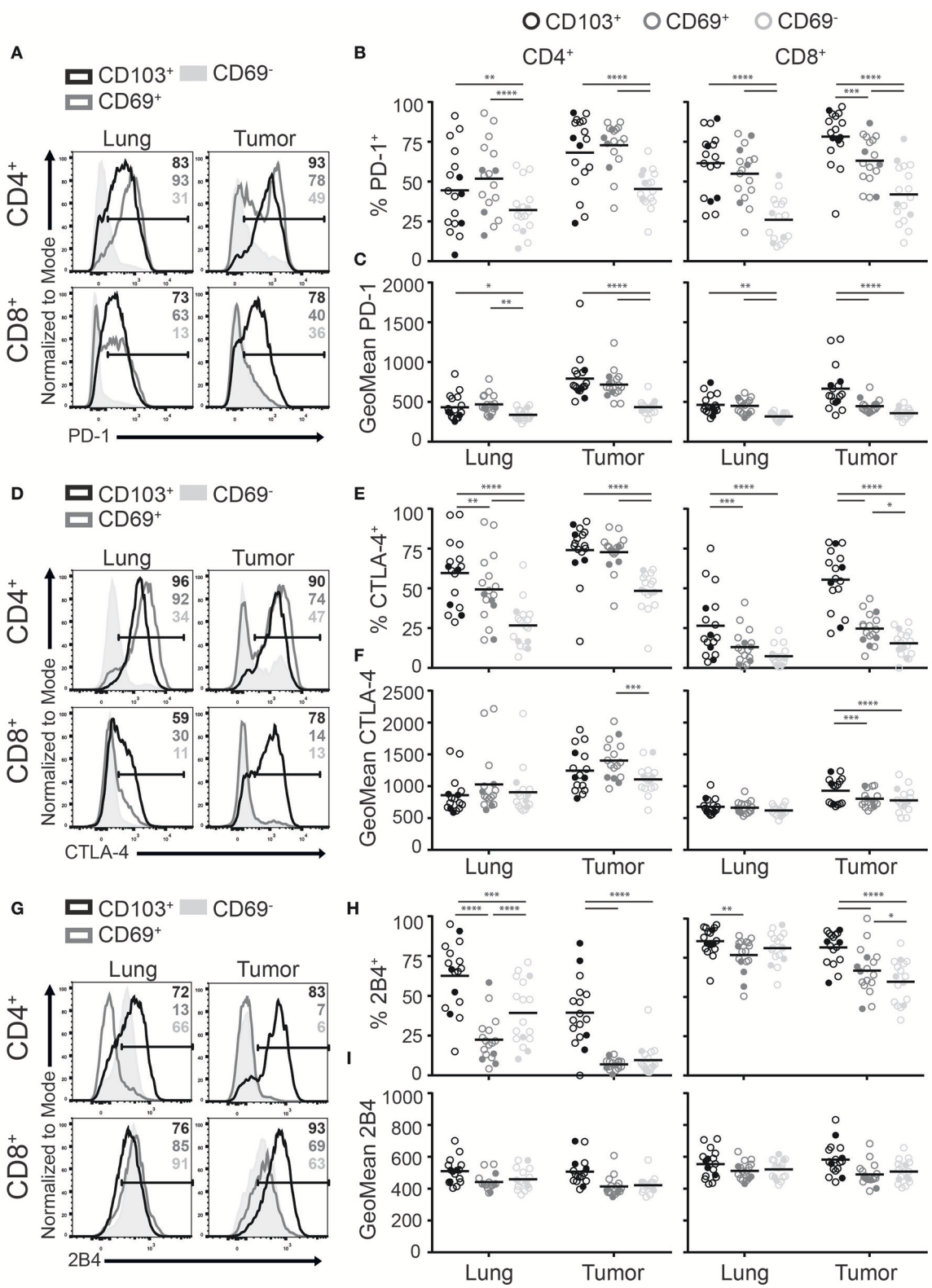

FIGURE 5 | CD103+ TILs express the highest levels of inhibitory receptors. **(A–I)** The expression of inhibitory receptors PD-1, CTLA-4, and 2B4 was analyzed on CD4+ and CD8+ T_{RM} and TILs. The expression of PD-1 **(A)**, CTLA-4 **(D)**, and 2B4 **(G)** on lung (left panel) and tumor (right panel) on CD4+ (top panel) and CD8+ (bottom panel) T cells is shown by representative histogram overlays (maximum set to 100%) (CD103+ black, CD69+ dark gray, CD69− solid light gray). The

(Continued)

FIGURE 5 | frequencies and geoMFI of PD-1$^+$ **(B,C)**, CTLA-4$^+$ **(E,F)** and 2B4$^+$ **(H,I)** were quantified for CD103$^+$ (black circles), CD69$^+$ (dark gray circles), and CD69$^-$ (light gray circles) cells of lung and tumor CD4$^+$ (left graphs) and CD8$^+$ (right graphs) T cells. **(B,C,E,F,H,I)** The quantifications are shown as dot plots with the horizontal line indicating the mean and each point represents a unique sample. $n = 17$. Open circles and solid circles indicate adeno- and squamous carcinoma, respectively. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; 2-way ANOVA with Tukey's multiple comparisons test.

Though virtually all lung CD8$^+$ T cells were 2B4$^+$, 2B4 was also expressed by most CD103$^+$CD4$^+$ T_{RM} and some CD69$^-$CD4$^+$ T cells. In the tumor, frequencies of 2B4$^+$ cells were again highest on CD103$^+$ TILs. In comparison to the lung, CD69$^+$ and CD69$^-$ TILs expressed low or decreased 2B4 levels for the CD4$^+$ and CD8$^+$ populations, respectively (**Figures 5G,H**). Expression levels of 2B4 were comparable between the lung and tumor (**Figure 5I**). Overall, CD103$^+$ TILs expressed the most inhibitory receptors.

CD103$^+$CD4$^+$ TILs Are the Most Potent Cytokine Producers in Tumors

The main obstacle faced by TILs is exhaustion induced by repeated stimulation and subsequent loss of T cell receptor responsiveness. A common feature of exhausted T cells is a step-wise loss of the capacity to produce multiple cytokines upon activation (37, 38). Thus, we set out to test the functionality of the different CD4$^+$ and CD8$^+$ T_{RM} and TIL subsets. We stimulated T_{reg}-depleted T cells with plate-bound agonistic αCD3 and soluble αCD28 antibodies and determined cytokine production. The majority of CD4$^+$ T_{RM} and TILs upregulated CD40L and/or CD137 upon activation. CD8$^+$ T_{RM} and TIL activation was restricted to CD137 upregulation (**Supplementary Figures 4A,C**). In terms of cytokine production, CD103$^+$CD4$^+$ TILs produced significantly more TNF-α and IFN-γ than CD69$^+$CD4$^+$ and CD69$^-$CD4$^+$ TILs (**Figures 6A,B**; **Supplementary Figures 4B,C**). No differences in cytokine production were observed between CD103$^+$CD8$^+$ and CD69$^+$CD8$^+$ TIL and T_{RM} fractions. Cytokine production of CD103$^+$CD4$^+$ TILs also exceeded that of all CD8$^+$ TIL populations.

PD-1 Expression Delineates Between Functionally Distinct Subsets of CD4$^+$ TILs

As we demonstrated the expression of PD-1 to be highest on TILs with a T_{RM} phenotype and CD103$^+$CD4$^+$ TILs to be the best cytokine producers, we investigated the relationship between PD-1 expression and cytokine production. To do so, we determined the expression of PD-1 (geometric mean fluorescence intensity) on T_{RM} and TILs that produced cytokines (positive for TNF-α and/or IFN-γ) and TILs that did not produce cytokines (TNF-α$^-$IFN-γ$^-$; **Figure 6C**; **Supplementary Figure 4D**). For lung CD4$^+$ and CD8$^+$ T_{RM}, there was no differential expression of PD-1 between the cytokine producing or non-producing cells. On the other hand, within CD103$^+$CD4$^+$, CD69$^+$CD4$^+$, and CD103$^+$CD8$^+$ TIL populations, significantly lower expression of PD-1 was observed for the cytokine producers. Recently, PD-1^{++}CD4$^+$ TILs in breast cancer and PD-1^{++} CD8$^+$ TILs in NSCLC were shown to produce CXCL13 (39, 40). Therefore, we determined CXCL13 expression in the different T_{RM} and TIL subsets. Also in the CD4$^+$ T cells, expression of CXCL13 appeared to be biased to the tumor fraction. A high percentage of CD4$^+$ TILs expressed CXCL13 in 4 out of 4 tested samples while only in 1 out of 4 lung samples expression was detected. Strikingly CXCL13 was only expressed by TIL with a T_{RM} phenotype. The highest numbers of CXCL13$^+$ cells were detected in the CD4$^+$ lineage (**Figures 6D,E**). Furthermore, CXCL13 was solely expressed by PD-1^{++} TILs (**Figures 6D,F**). Thus, we found that PD-1 expression defines functionally distinct subsets of CD4$^+$ TILs, effector cytokine producer PD-1^{low} and CXCL13 producing PD-1^{++} TILs.

Co-stimulation Increases Cytokine Production of TILs

Adoptive transfer and vaccination strategies to treat cancer have demonstrated that CD4$^+$ T cell help, through co-stimulation, is required for optimal cytotoxic CD8$^+$ T cell responses in tumors. Administration of co-stimulation in combination with PD-1 therapy improved the cytokine production of TILs in tumor-bearing mice (22). We next assessed whether CD28 and CD27 co-stimulation in addition to TCR triggering could boost cytokine production of T_{reg}-depleted TILs. CD103$^+$CD4$^+$ TILs, but not other CD4$^+$ TILs, mainly responded to CD28 co-stimulation by producing more IFN-γ and/or TNF-α (**Figures 7A–C**). However, agonistic stimulation of CD27 did not add to this, which could be explained by higher CD28 than CD27 expression by the CD103$^+$CD4$^+$ TILs. While CD103$^+$CD8$^+$ TILs appeared non-responsive to co-stimulation, agonistic CD28 stimulation boosted TNF-α production by CD69$^+$CD8$^+$ TILs (**Figure 7E**). The addition of CD27 co-stimulation further enhanced TNF-α and/or IFN-γ production (**Figures 7D–F**). We did not find differences between adenocarcinoma and squamous carcinoma samples (**Supplementary Figure 5**). These data suggest that therapeutic efficacy of cancer immunotherapy targeting specific TIL populations may improve by providing agonistic stimulation of co-stimulatory molecules.

DISCUSSION

In this study, we investigated the phenotype of tumor infiltrating T cells in NSCLC. We phenotypically characterized CD4$^+$ and CD8$^+$ TILs and directly compared these with T cell populations in the surrounding lung tissue. While adaptive immune responses that protect against tumors are typically attributed to CD8$^+$ T cells, several studies provide evidence that CD4$^+$ T cells also play a central role (41). As CD4$^+$ T cells exhibit phenotypic and functional heterogeneity, different subsets are expected to play different and even opposing roles in the tumor environment. While accumulation of CD4$^+$ T_{reg} within tumors is associated with worse prognoses in

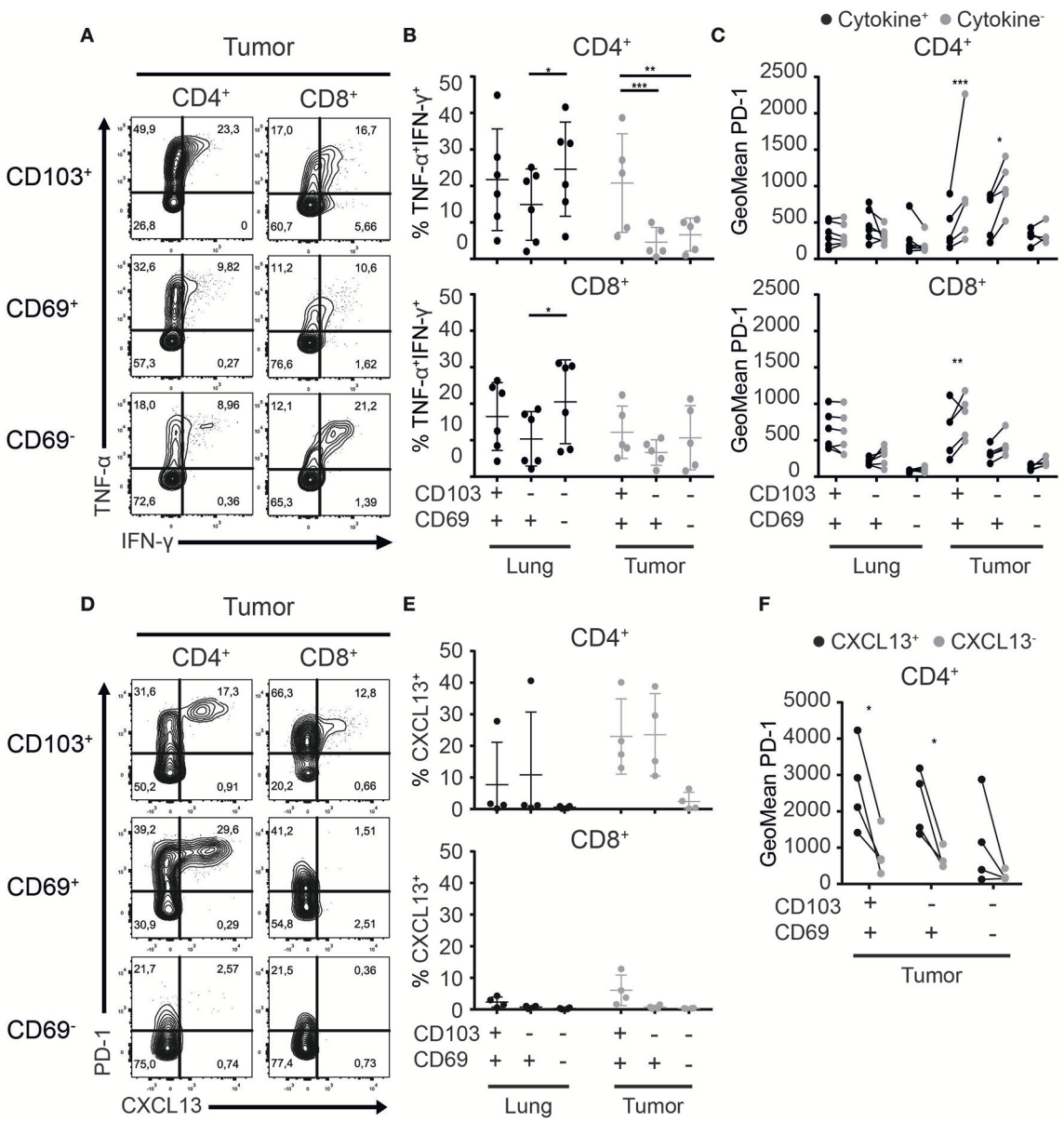

FIGURE 6 | Cytokine and chemokine production of T_{RM} and TILs. **(A–C)** Cytokine production by lung T_{RM} and TILs was determined after overnight αCD3/αCD28 stimulation. **(A)** The production of TNF-α and IFN-γ by $CD103^+$ (top panels), $CD69^+$ (middle panels), and $CD69^-$ (bottom panels) tumor $CD4^+$ (left panels) and $CD8^+$ (right panels) TILs shown by representative contour plots (TNF-α on y-axis, IFN-γ on x-axis). **(B)** $TNF\text{-}\alpha^+IFN\text{-}\gamma^+$ $CD103^+$, $CD69^+$, and $CD69^-$ cells of lung and tumor $CD4^+$ (top graph) and $CD8^+$ (bottom graph) T cells. **(C)** PD-1 expression (geometric mean fluorescence intensity; GeoMFI) was quantified on cytokine$^+$ ($TNF\text{-}\alpha^+$ and/or $IFN\text{-}\gamma^+$) (black circles) and cytokine$^-$ ($TNF\text{-}\alpha^-IFN\text{-}\gamma^-$) (gray circles) $CD103^+$, $CD69^+$, and $CD69^-$ lung and tumor $CD4^+$ (top graph) and $CD8^+$ (bottom graph) T cells. **(D)** The expression of CXCL13 was determined by flow cytometry in $CD103^+$ (top panels), $CD69^+$ (middle panels), and $CD69^-$ (bottom panels) tumor $CD4^+$ (left panels) and $CD8^+$ (right panels) TILs and is shown by representative contour plots (PD-1 on y-axis, CXCL13 on x-axis). **(E)** $CXCL13^+$ of $CD103^+$, $CD69^+$, and $CD69^-$ cells was quantified in lung and tumor $CD4^+$ (top graph) and $CD8^+$ (bottom graph) T cells. **(F)** PD-1 expression (GeoMFI) was quantified on the $CXCL13^+$ (black circles) and $CXCL13^-$ (gray circles) $CD103^+$, $CD69^+$, and $CD69^-$ $CD4^+$ TILs. $n = 4$–6 paired lung-tumor samples; all adenocarcinoma. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; 2-way ANOVA with Tukey's multiple comparisons test.

many cancers (42), $CD4^+$ T helper cells are required to optimize cytotoxic $CD8^+$ T cell responses against tumor cells (43). In addition, $CD4^+$ T cells were demonstrated to mediate tumor-antigen-mediated killing of tumor cells, highlighting the importance to understand the functional heterogeneity of the different T cell subsets in tumors (24, 44, 45).

While numerous studies have reported the presence of T_{RM}-like $CD8^+$ T cells in solid tumors to be a favorable prognosis, the role of $CD4^+$ TILs with a shared phenotype is unclear. We

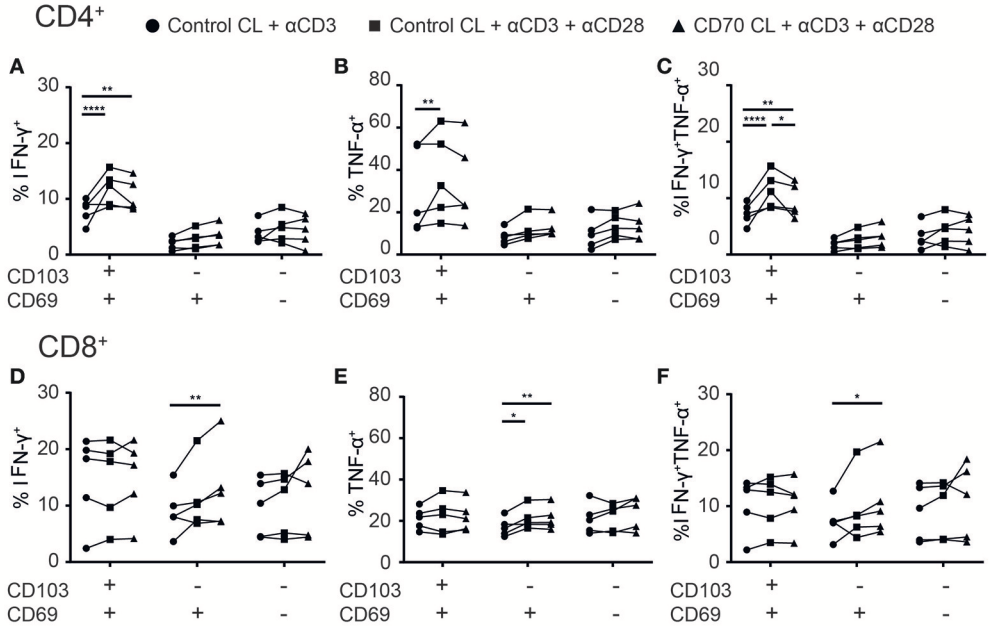

FIGURE 7 | Co-stimulation enhances cytokine production of TILs. **(A–F)** The effect of co-stimulation on cytokine production of TILs was assessed with overnight stimulation. **(A–C)** The frequencies of IFN-γ^+ **(A)**, TNFα^+ **(B)**, or TNF-α^+IFN-γ^+ **(C)** CD103$^+$, CD69$^+$, and CD69$^-$ CD4$^+$ TILs stimulated with αCD3 with control cell line (CL) (black circles), αCD3 and αCD28 with control CL (black squares), or αCD3 and αCD28 with CD70-expressing CL (black triangles) was quantified. **(D–F)** The frequencies of IFN-γ^+ **(D)**, TNFα^+ **(E)**, or TNF-α^+IFN-γ^+ **(F)** CD103$^+$, CD69$^+$, and CD69$^-$ CD8$^+$ TILs stimulated with αCD3 with control CL (black circles), αCD3 and αCD28 with control CL (black squares), or αCD3 and αCD28 with CD70-expressing CL (black triangles) was quantified. $n = 5$. $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$; 2-way ANOVA with Tukey's multiple comparisons test.

demonstrated for the first time a positive correlation between CD103$^+$CD8$^+$ and CD103$^+$CD4$^+$ TILs in NSCLC. Our findings are supported by the observation that CD8$^+$ TILs from CD103-rich tumors expressed transcripts linked to CD4$^+$ T cell-mediated help, while CD8$^+$ TILs from CD103-poor tumors did not (21). As CD4$^+$ T cell help was demonstrated to be required for guiding CD8$^+$ T_{RM} formation in the lungs by regulating the entry of T_{RM} precursors to the lung mucosa (46), it is tempting to speculate that a similar role applies in NSCLC. A key mechanism to attract T_{RM} precursors into the tissue is IFN-γ production by CD4$^+$ T cells (46, 47). IFN-γ induces the production of chemokines by the tissue and boosts the expression of adhesion molecules by the vasculature which result in higher T cell infiltration (7, 8). In the tumor, we found the best producers of IFN-γ to be CD103$^+$CD4$^+$ TILs. While adapted to the metabolic requirements in tissues, such specialization may also provide CD103$^+$CD4$^+$ T cells with an advantage over other CD4$^+$ T cell subsets in malignant niches. Strategies designed to boost anti-tumor CD8$^+$ CTL responses may therefore benefit from taking into account the CD4$^+$ subset that appears most effective for their generation.

Once in the tissue, CD8$^+$ T_{RM} maturation is believed to be independent of CD4$^+$ T cell help. T_{RM} maturation is driven by local inflammatory stimuli that induce the expression of CD69 and CD103 (48). In the healthy tissue many of these signals are provided by local macrophages and dendritic cells, which were demonstrated to be crucial for full maturation of especially CD4$^+$ T_{RM} (49–51). It remains to be investigated if the increased frequency of phenotypically less-differentiated T_{RM}-like cells in NSCLC may be the result of the tumor environment that suppresses dendritic cell function (52). Full maturation of CD103$^+$ T_{RM} requires TGF-β signaling (31). Several lung tumors are described to express high levels of TGF-β (53), which may explain the high level of CD103$^+$CD8$^+$ TILs in NSCLC. At apparent odds, we found the frequencies of CD103$^+$CD4$^+$ TILs to be decreased relative to the surrounding lung tissue. The altered ratio between CD103$^+$CD8$^+$ and CD103$^+$CD4$^+$ TILs in NSCLC may be a result of different requirements for their maintenance. While CD8$^+$ T_{RM} maintenance was described to be independent of persistent antigen (54), whether antigen presence is required for the maintenance of CD103$^+$CD4$^+$ T_{RM} remains unclear. If CD103$^+$CD4$^+$ T_{RM} maintenance is antigen-dependent, this may be a major hurdle for CD103$^+$CD4$^+$ TILs as many tumors express little or no MHC class II molecules (55). Strikingly, tumor cells upregulate MHC class Il molecules and consequently their cytotoxicity in response to IFN-γ. Moreover, adoptive transfer of Th1-like CD4$^+$ T cells was found to protect against tumors lacking MHC class II expression (24, 44, 56). Our data suggest the CD103$^+$CD4$^+$ TILs to be the best candidates for such therapies.

In NSCLC, IFN-γ-responsive gene expression signatures are associated with favorable prognosis (57). In light of our findings, a prominent role for CD103$^+$CD4$^+$ TILs seems possible as they were the most potent intratumoral cytokine

producing T cell subset, despite the high expression of PD-1 and CTLA-4. These $CD103^+CD4^+$ TILs also expressed 2B4 while other $CD4^+$ TILs did not. While generally 2B4 is considered an inhibitory receptor, it functions differently from typical inhibitory receptors and has also been demonstrated to act as a co-stimulatory molecule depending on the availability of intracellular SAP protein (58). Therefore, 2B4 may be playing a different role on $CD103^+CD4^+$ TILs. On the other hand, we found that PD-1 expression delineates between effector cytokine and CXCL13 producing $CD4^+$ TILs. TILs with high PD-1 expression are classically thought to be exhausted since they do not produce effector cytokines. This raised the question of how $PD\text{-}1^{++}CXCL13^+$ cells act in the tumor environment. Our data suggests that $CXCL13^+$ TILs are functionally adapted to the tumor environment rather than being exhausted. As a mechanism, intratumoral CXCL13 production may serve to recruit $CXCR5^+$ T follicular helper cells (Tfh) or B cells. Recently, it was shown that $PD\text{-}1^{++}CD8^+$ TILs are localized within tertiary lymphoid structures (TLS) in tumors and may be important for the formation of TLS (40). As such, PD-1 has also been shown to control the positioning and function of Tfh, which also produce CXCL13 (39, 59). Therefore, these $PD\text{-}1^{++}CD4^+$ TILs may also be located within TLS and contribute to the formation of TLS in tumors of NSCLC. It has been shown that in chronic viral infections, a subset of memory $CD8^+$ T cells with an "exhausted" phenotype retain their effector function through TCF-1 (60), indicating that these phenotypically exhausted T cells contain diverse subsets. Our data reveal the functional heterogeneity within these "exhausted" $CD4^+$ TILs, suggesting that not all of these TILs are exhausted but functionally distinct from the effector cytokine producing TILs.

Agonistic activation of co-stimulatory CD27 and CD28 boosted cytokine production of the $CD103^+CD4^+$ TILs. However, $CD69^+CD4^+$ TILs expressed identical levels of PD-1 and higher levels of CD27 and CD28, but cytokine production was not boosted with additional co-stimulation. If parallels may be drawn with differentiation of circulating T cells associated with a step-wise loss of CD27 and CD28, our data suggests these $CD69^+CD4^+$ cells are less differentiated and adapted to the tissue niche. On the other hand, while $CD69^-CD4^+$ TILs have lower expression of inhibitory receptors, this subset was not able to produce effector cytokines to the same extent as $CD103^+CD4^+$ TILs. However, $CD69^-CD4^+$ TILs mainly consists of early and early-like differentiated cells, which could indicate that these cells are recent emigrants and are yet to fully differentiate. This is also supported by the lower expression of co-inhibitory molecules which suggest they are not yet exhausted by the tumor microenvironment. Recently, it has been suggested that these phenotypically "exhausted" TILs are in a stage of differentiation rather than exhausted and that this state of "exhaustion" may be reversible (61–63). Overall, both $CD4^+$ and $CD8^+$ TILs expressed high levels of CD27 and CD28, suggestive of cells in an early stage of differentiation. Perhaps the addition of co-stimulation to current cancer vaccines and immunotherapies could push the differentiation of TILs into optimal cytotoxic effector cells and enhance the efficacy of cancer therapies.

While IFN-γ production may directly inhibit tumor growth in synergy with TNF-α (64, 65), it remains to be investigated whether $CD103^+CD4^+$ TILs are equally equipped to kill cancer cells as their $CD103^+CD8^+$ counterparts (19). CD49a expression by $CD103^+CD4^+$ and $CD103^+CD8^+$ TILs may allude to this, as CD49a expressing $CD103^+CD8^+$ TILs were the most potent killers of tumor cells in a mouse model of melanoma and CD49a defines cytotoxic $CD8^+$ T_{RM} in skin (16, 66, 67). As such, strategies to identify $CD4^+$ T cells that can directly target tumor cells may focus on $CD103^+CD4^+$ T cells. Therapeutic manipulation of such reactivity could be a highly attractive strategy.

MATERIALS AND METHODS

Subjects

Lung and tumor tissue samples were obtained from a total of 33 non-small cell carcinoma (NSCLC) patients. The patients received a surgical resection of primary tumors as first line therapy without prior chemo- or radiotherapy. Blood was drawn from a central line at the start of surgery. Patients included were stages AJCC between IA1 and IIIA. The exclusion criteria included history of asthma or a recent lower respiratory tract infection. The patients were recruited from Onze Lieve Vrouwe Gasthuis (OLVG), Amsterdam, the Netherlands. A list of the age, gender, pathology of the patients used in this study are listed in **Supplementary Table 1**.

Study Approval

Written informed consent was given by all of the patients and donors before inclusion into the study. The Ethical Review Board (ERB) of the METC/CCMO of the OLVG approved the study under the MEC-U number NL52453.100.15 according to the Declaration of Helsinki.

Isolation of Mononuclear Cells From Peripheral Blood and Lung Tissue

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood samples with standard density gradient techniques. For the lung material, after the lobectomy the pathologist cuts off a piece of peripheral normal looking lung tissue farthest away from the tumor. For the tumor material, the pathologist cuts off a piece of the tumor. Lung mononuclear cells (LMC) and tumor mononuclear cells (TMC) were isolated from the tissues as previously described (68, 69). In short, the tissue was cut into small pieces and incubated for 1 h at 37°C in digestion medium [RPMI with 20 mM Hepes, 10% fetal calf serum (FCS), 50 U/ml DNAse type I (Sigma-Aldrich), 300 U/ml collagenase type 4 (Worthington)] while shaking. Before and after the digestion, the tissue was dissociated using gentleMACS Tissue Dissociator (Miltenyi). The digested tissue was passed through a flow-through chamber to achieve a single cell suspension. To isolate mononuclear cells from the cell suspension standard density gradient techniques were used. LMC, TMC, and PBMC samples were cryopreserved in liquid nitrogen until further analysis.

Flow Cytometry Analysis

PBMC or LMC were labeled with combinations of the following antibodies: anti-CD4, anti-CD3, anti-CD8, anti-CD27, anti-CD45RA, anti-CD69, anti-CD103, anti-CD49a, anti-CXCR6, anti-CD28, anti-CCR7, anti-PD-1, and anti-2B4. Near-IR fixable dye (Invitrogen) was used to exclude dead cells from the analysis. For intracellular staining the following antibodies were used: anti-CTLA4, anti-Eomes, anti-Tbet, anti-IFNγ, anti-GZMB, anti-CD40L, anti-CD137, anti-IL-2, anti-TNFα, and anti-CXCL13. The cells were labeled according to manufacturer's instructions. For the intracellular staining the cells with fixed and permeabilized using the Foxp3/Transcription Factor Staining kit (eBioscience). All samples were measured in PBS 0.5% FCS with a LSR Fortessa (BD) or FACSymphony (BD) and the analysis was performed using FlowJo Version 10 software. See **Supplementary Table 2** for the full list of antibodies used in this manuscript.

In vitro Stimulation Assays

Cytokine production by lung and tumor T cells was determined by incubating TMC with platebound αCD3 (HIT3A; eBioscience) and soluble αCD28 (s.28; CLB) overnight at 37°C in the presence of Brefeldin A (eBioscience). T_{reg} were depleted by MACS (Miltenyi) isolation CD25$^+$ cells from the LMC and TMC samples before the stimulation according to manufacturer's protocol. To determine the effects of co-stimulation on TILs, TILs were incubated with only soluble αCD3 (HIT3A; eBioscience) with control cell line, soluble αCD3 and αCD28 (s.28; CLB) with control cell line, or soluble αCD3 (HIT3A; eBioscience) and αCD28 (s.28; CLB) with a CD70-expressing cell line. The cell lines were made by cloning CD70 cDNA into pMX-IRES-GFP vector using EcoRI and NotI restriction enzymes (NEB). Retroviral packaging by transfection of either pMX-IRES-GFP empty vector or pMX-hCD70-IRES-GFP together with pCL-ECO into Phoenix-ECO packaging cells using polyethyleminine. Supernatants containing retrovirus was collected 48 h after transfection and used for retroviral transduction of mouse NIH3T3 cells. Transduced NIH3T3 cells were sorted on GFPhigh (pMX-IRES-GFP) or GFPhighCD70high (pMX-hCD70-IRES-GFP) expression using a MoFlo Astrios cell sorter (Beckman Coulter).

Statistics

To determine the significance of our results, we used 2-way ANOVA and Tukey's multiple comparisons test with GraphPad Prism 6. p-value of < 0.05 was considered statistically significant ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$).

AUTHOR CONTRIBUTIONS

AO and PH designed the project and experiments. All of the authors performed experiments and/or collected tissue and blood samples. All authors contributed to the interpretation and discussion of data. AO and PH wrote the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02654/full#supplementary-material

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Targeting Resident Memory T Cells for Cancer Immunotherapy

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A novel population of memory CD8$^+$ T cells called resident memory T cells (T_{RM}) has been identified based on their phenotype (CD103, CD69) and on their local tissue residency without recirculating in the blood. These cells have been implicated in protective immune response against pathogens in both animal models and humans. Their role in cancer is just emerging as a key player in tumor immunosurveillance. Many properties of these cells suggest that they could control tumor growth: (i) they respond much faster to reexposure to cognate antigen than circulating memory cells, (ii) they express high levels of cytotoxic molecules, and (iii) they are enriched in tumor-specific T cells in close contact with tumor cells. T_{RM} are present in many human cancers and are associated with a good clinical outcome independently of the infiltration of CD8$^+$ T cells. It has been recently shown that the efficacy of cancer vaccines depends on their ability to elicit T_{RM}. In adoptive cell therapy, the transfer of cells with the ability to establish T_{RM} at the tumor site correlates with the potency of this approach. Interestingly, T_{RM} express immune checkpoint molecules and preliminary data showed that they could expand early during anti-PD-1 treatment, and thus be considered as a surrogate marker of response to immunotherapy. Some cues to better expand these cells *in vivo* and improve the success of cancer immunotherapy include using mucosal routes of immunization, targeting subpopulations of dendritic cells as well as local signal at the mucosal site to recruit them in mucosal tissue.

Keywords: resident memory T cells, cancer vaccine, immune checkpoint molecule, mucosal route of vaccination, immunotherapy

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INTRODUCTION

After studying herpes simplex virus infection and lymphocytic choriomeningitis virus infection, two groups reported that memory T cells remain in dorsal root ganglia and small intestines, respectively, without recirculating through the blood (1, 2). These cells were called tissue-resident memory T cells (T_{RM}). These T_{RM} cells may persist for a long time and represent one of the main lymphocyte populations in adults (3, 4).

T_{RM} cells originate from a common KLRG^{neg} memory precursor cell that also gives rise to circulating central and effector memory CD8 T cell populations (5). These cells share TCR repertoires (6).

T_{RM} cells from different tissues were transcriptionally related (5) with a core marker (CD69, CD103, and CD49a) both in mice and humans. However, subpopulations of T_{RM} differing by the expression of these markers and exhibiting additional markers also exist. For example, CD49a is expressed by

only 15% of T cells from the human skin. The chemokine receptor CCR8 and the CD8αα homodimer are expressed only in skin T_{RM} cells, while the aryl hydrocarbon receptor (AHR) is expressed in gut and skin T_{RM}, but not in lung T_{RM} (5, 7). This phenotypic heterogeneity extends to functional heterogeneity even within a same organ. For example, it has been shown that the airway T_{RM} has a poor *in vivo* proliferative and cytolytic ability, when they were compared with lung T_{RM}, while IFNγ are produced faster by CD8 T_{RM} compared to systemic effector $CD8^+$ T cells (8). In addition, T_{RM} in the airway has a short half-life (less than 1 month) whereas T_{RM} in lung parenchyma may persist for several months or years (9).

T_{RM} cells express high levels of protein associated with tissue retention, such as RGS-1 and RGS-2, both known as G protein-coupled inhibitors. By contrast, they display low levels of sphingosine-1-phosphate receptor 1 (S1PR1) and CCR7 (5, 10), which are indispensable for tissue exit. Various molecules expressed by T_{RM} may explain their long survival in tissue. Indeed, anti-apoptotic factors such as Bcl-2 could be detected in T_{RM} (5). In the presence of exogenous free fatty acids (FFAs), $CD8^+$ T_{RM} cells exhibited high levels of mitochondrial oxidative metabolism. This feature was not observed in central memory $CD8^+$ T cells. *Fabp4* and *Fabp5* (*Fabp4*/*Fabp5*) proteins favor FFA uptake by $CD8^+$ T_{RM} cells. Their specific deficiency on T cells decreased the survival of T_{RM} *in vivo* (11).

Downregulation of T-bet, likely induced by TGF-β and T-box proteins Eomesodermin, is required for T_{RM} differentiation, but residual levels of T-bet for maintaining IL-15R are crucial for long-term T_{RM} function and survival in the skin, kidney, and salivary gland (12). However, IL-15 is not required for their maintenance in the small intestine or female reproductive tract (FRT) (5).

Aryl hydrocarbon receptor and Notch activity are also required for the maintenance of $CD103^+$ T_{RM} cells (13, 14). Recent studies by Milner et al. identified the transcription factor Runx3 as a master regulator for inducing and maintaining $CD8^+$ T_{RM} by reducing T_{RM} apoptosis (15).

In addition, in some tissue localizations (e.g., brain or lung), the presence of antigen is required for T_{RM} establishment (16, 17). By contrast, local inflammatory signal without antigenic stimulation may favor systemic $CD8^+$ T cells to adopt T_{RM}-like characteristics in skin, nasal tissue, and FRT (18).

T_{RM} have all the features of memory $CD8^+$ T cells ($CD45RA^-$ $CD62L^-CD28^-CD27^-CCR7^-$) (19, 20). It has been clearly established that, at least in some tissues, T_{RM} cells might persist without the secondary recruitment of systemic effector memory T cells (21).

PROPERTIES OF T_{RM} THAT MAY EXPLAIN THEIR ROLE IN A TUMOR CONTEXT

Various studies have shown that T_{RM} cells respond much faster to reexposure to cognate antigen than circulating memory cells [either TEM (effector memory T cells) or TCM (central memory T cells)] (22, 23). In addition, T_{RM} underwent *in situ* division after local antigen challenge, triggered the recruitment of innate immune cells and recirculating memory T cells and thus regulated local immunosurveillance (22–24).

T_{RM} cells in non-small cell lung cancer (NSCLC) are preloaded with preformed mRNA encoding inflammatory cytokines (granzyme B, IFN-γ, and TNF) and with cytotoxic molecules (13). In ovarian cancer, $CD103^+$ tumor-infiltrating lymphocytes (TILs) uniformly express TIA-1, a marker of potential cytotoxicity (25). In liver cancer, T_{RM} express high levels of perforin (26). CD49a expression has been demonstrated to characterize T_{RM} cells poised with cytotoxic function in the human epidermis (27).

In some tissues such as the brain or the lung, local antigen presentation is required to drive T_{RM} cell formation (17). In addition, $CD103^+$ TILs express high levels of PD-1 (25), which has been reported to be a marker of antitumor TILs in melanoma (28). Indeed, after their sorting based on their expression of PD-1, $CD8^+$ T cells that expressed this inhibitory receptor in melanoma patients identified those that preferentially recognized tumor cells (28, 29). From these results, it thus appears that in many localizations, T_{RM} may represent antitumor-specific T cells.

In healthy tissues such as the lungs, the skin, the reproductive tract, and the gut, T_{RM} cells localize within the epithelial layer. $CD103^+$ TILs were preferentially localized in epithelial regions of tumors in close contact with tumor cells, likely due to the natural interaction between CD103, and its ligand, E-cadherin, expressed by tumor cells, may explain that $CD103^+$ TIL were rather found in close contact with the tumor cells rather than in the stroma (25, 30).

Finally, it has been shown that T_{RM} represent an effective *in situ* first line of defense to tissue-specific infections and are implicated in protective immune responses against many pathogens in both animal models and humans. It is thus tempting to extrapolate their role from infectious models to cancer (31).

T_{RM} IN THE NATURAL COURSE OF TUMOR

T_{RM} are present in many human cancers (NSCLC, ovarian cancer, bladder cancer, endometrial cancer, melanoma, etc.).

Overall, they are associated with a good clinical outcome (19, 32). Interestingly, the impact of T_{RM} on survival was independent of the infiltration of $CD8^+$ T cells. Indeed, we have shown in a multivariate analysis (33) that intratumoral $CD103^+CD8^+$ T cells correlate with a better survival in NSCLC patient (33). Confirming our results, a greater number of intratumoral T_{RM} cells correlated with a better survival in lung cancer, cervical cancer, and melanoma, independently of that conferred by total $CD8^+$ T cells (34–36).

Finally, intratumoral $CD8^+$ T cells not expressing CD103 were associated with poor prognosis, as observed in tumors not infiltrated by $CD8^+$ T cells (25).

The localization of T_{RM} inside the tumor may be a parameter to take into account to assess their impact on the control of the tumor. Indeed, intraepithelial CD103 but not intra-stromal CD103 correlated with better overall survival and absence of relapse in a basal-like subtype of breast cancers (30). In many of these studies, the CD103 marker was analyzed and not really T_{RM}

(CD103$^+$CD8$^+$ T cells). Since CD103 is also expressed by CD4$^+$ T cells, innate lymphoid cells, NK cells, and dendritic cells (DCs), it could introduce a bias in the interpretation of the results.

Interestingly, the genetic variability of TCRs from resident memory T cells between different metastatic lesions from the same patient was greater than the variance in mutational or neoepitope load in tumor cells (37). This absence of equilibration between tissue-resident TCR within individual metastases may affect the clinical results of immunotherapy at the various sites and explain mixed clinical response.

ROLE OF T_{RM} IN THE EFFICACY OF CANCER VACCINE

Using a model of orthotopic head and neck or lung cancer, we showed that only the intranasal route of immunization elicited local T_{RM}. By means of parabiosis experiment or the use of the FTY720 inhibitor, which downregulates the S1PR1 receptor and blocks the recruitment of circulating memory T cells in the tissue, we demonstrated that the T_{RM} alone could partially control the growth of the tumor (33, 38). It was also reported that an intravaginal boost with an HPV vaccine after a systemic (intramuscular) prime was more efficient at eliciting local cervical T_{RM} cells, which led to a better overall mouse survival after a tumor challenge than that observed with an intramuscular boost (39).

In melanoma patients vaccinated with a mixture of Melan-A peptide combined with Montanide and CpG, the ability to elicit anti-Melan A CD8$^+$ T cells expressing VLA-1, a surrogate marker of T_{RM}, was correlated with better survival (40).

Treatment of breast DCs with β-glucan—a ligand of dectin-1 reprogrammed DC with an upregulation of ITGB8, an integrin which binds the latent domain (LAP) of TGF-β, and which after its cleavage constitutes the main mechanism of TGF-β activation *in vivo*. Administration of DC treated with β-glucan curdlan or its direct intratumoral delivery induced intratumoral antitumor CD8$^+$ T cells expressing CD103, which inhibit tumor progression in a humanized mouse model of breast cancer (41).

While these examples strongly suggest the role of T_{RM} in the protection generated by cancer vaccine, it has to be kept in mind that FTY720 experiments showed that the recruitment of circulating effector memory T cells increased the efficacy of T_{RM} after mucosal vaccine (33). Conversely, Dr. Sancho's group reported that, while both T_{RM} cells and circulating memory T cells play a role in tumor immunosurveillance, the presence of T_{RM} cells improves vaccine efficacy (42).

ROLE OF T_{RM} IN ADOPTIVE T CELL THERAPY

Mucosal CD103$^+$CD8$^+$ T cells elicited by reprogrammed DC with β-glucan curdlan can reject an established tumor and this effect is inhibited by the blockade of CD103 (41).

The establishment of T_{RM} cell populations in various normal tissues and in cancer required the expression of Runx3 (15). In a preclinical model of melanoma, CD8$^+$ TIL not expressing Runx-3 did not accumulate in tumor microenvironment, resulting in uncontrolled tumor growth and low survival. By contrast, when antitumor CD8$^+$ T cells that overexpress Runx3 were transferred *in vivo*, tumor growth was inhibited, and mice survival improved (15). Thus, the adoptive T cell therapy of T_{RM} seems a promising strategy.

ROLE OF T_{RM} IN CANCER IMMUNOTHERAPY BASED ON THE BLOCKADE OF IMMUNE CHECKPOINT MOLECULES

T_{RM} from healthy organs (brain, gut, lung, and skin) or localized in tumors (NSCLC, melanoma, etc.) express higher amounts of inhibitory receptors (PD-1, Tim-3, CTLA-4, NKG2A, BTLA, LAG-3, SPRY1, adenosine receptor A2AR, CD39, CD101, and 2B4) and costimulatory molecules (CD27, ICOS, SIRPG, and CD137) than peripheral memory CD8$^+$ T cells or CD8$^+$CD103^{neg} TIL (5, 13, 20, 34, 37).

However, depending on the tumor localization, the profile of immune checkpoint molecules detected on T_{RM} may vary. For example, T_{RM} derived from NSCLC and melanoma did not express membrane CTLA-4 (19, 35), and in ovarian cancer, PD-1$^+$CD103$^+$CD8$^+$ T cells exhibited a weak expression of other exhaustion-associated markers, such as CTLA-4, LAG-3, and TIM-3 (32).

TCGA analysis of cervical cancer data shows that *CD103* (*ITGAE*) expression correlates with the usual T cell genes such as *CD8A*, but more interestingly also with T cell activation and exhaustion markers such as CTLA-4, CD137, PD-1, and PD-L1 (36).

Transcriptomic analysis of T_{RM} also reported the expression of genes with well-recognized inhibitory functions in T cells, such as the dual specificity phosphatase DUSP6, which turns off MAP kinase signaling, as well as IL-10 (20). However, despite high expression of checkpoint inhibitors, several arguments show that T_{RM} cells from infected organs or tumors are not terminally exhausted. Indeed, T_{RM} in the hepatitis B virus-infected human liver co-express PD-1 and CD39 at high levels, but they readily produce IFN-γ, TNFα, and IL-2 after *in vitro* stimulation (26).

In addition, when T_{RM} cells sorted from lung carcinomas were co-cultured with autologous tumor cells, their cytotoxic activity was enhanced in the presence of anti-PD-1 mAb (19).

In a preclinical model, administration of anti-PD-1 antibody concomitantly with Tcm transfer (which converts to T_{RM}) in a tumor therapy setting inhibited the growth of s.c. MC38-OVA tumor and i.d. B16-OVA tumor when compared with the adoptive T cell therapy with Tcm cells only. Interestingly, after anti-PD-1 therapy, the number and frequency of TIL with a T_{RM} phenotype were increased more than 10-fold within the CD45$^+$ cells in both tumor settings (42).

In humans, tumor-resident CD8$^+$ T cells significantly expanded early during anti-PD-1 treatment (35). There was a significant difference in their numbers (T_{RM}) early during treatment between those who responded to the treatment and those who did not respond (35). In line with these results, Wei et al. showed that

T cell clones that expanded during anti-PD-1 treatment expressed high levels of CD69, PD-1, LAG-3, and CD45RO, an identical phenotype to the tumor-resident CD8+ T cell population (43).

CUES TO ELICIT T_{RM} TO IMPROVE CANCER IMMUNOTHERAPY

From these results, it is clear that T_{RM} are involved in the efficacy of different cancer immunotherapy strategies. A field of future investigation will rely on the development of new strategies to induce and amplify T_{RM} (**Figure 1**).

Route of Immunization

Compelling experiments demonstrate the crucial role of the route of vaccination to elicit tissue-resident memory T cells both during natural infection and after vaccine administration.

Indeed, various vaccine studies showed that intravaginal immunization or a systemic prime followed by a mucosal vaginal boost maximized the induction of genital T_{RM} (31). Intranasal vaccination with a recombinant cytomegalovirus vector encoding the respiratory syncytial virus (RSV) matrix (M) or with BCG protein also generated robust and durable tissue-resident effectors that were undetectable after intraperitoneal or subcutaneous vaccination (44, 45).

Local Signal to Favor the Recruitment of T_{RM}

In mice, cancer vaccine synergizes with local radiation to favor the recruitment of intratumoral antitumor CD8+ T cells, some of them exhibiting a T_{RM} phenotype (36, 46).

Local injection of Toll-like receptor agonists or of selected chemokines *via* the modification of the expression of selectins, integrins, and chemokines could also enhance the recruitment of CD8+ T cells in the tissue and at local tumor site. This concept has been assessed *in vivo* by the "prime and pull" strategy, which comprises two steps: conventional systemic immunization to induce T-cell responses in the blood (prime), followed by secondary recruitment of effector T cells by means of local chemokine injection into the mucosal genital tract (pull). This prime-pull strategy succeeded in establishing a long-term residency and thus favored protective immunity. In mice, this prime and pull strategy was shown to decrease the diffusion of infectious herpes simplex virus 2 (HSV-2) into the sensory neurons and to be efficient to control clinical disease (18). In line with these results, after systemic administration of a vaccine, an intravesical administration of Ty21, a live bacterium used against typhoid fever or an intravaginal administration of CpG resulted in the accumulation of local specific CD8+ T cells and led to tumor regression (47, 48).

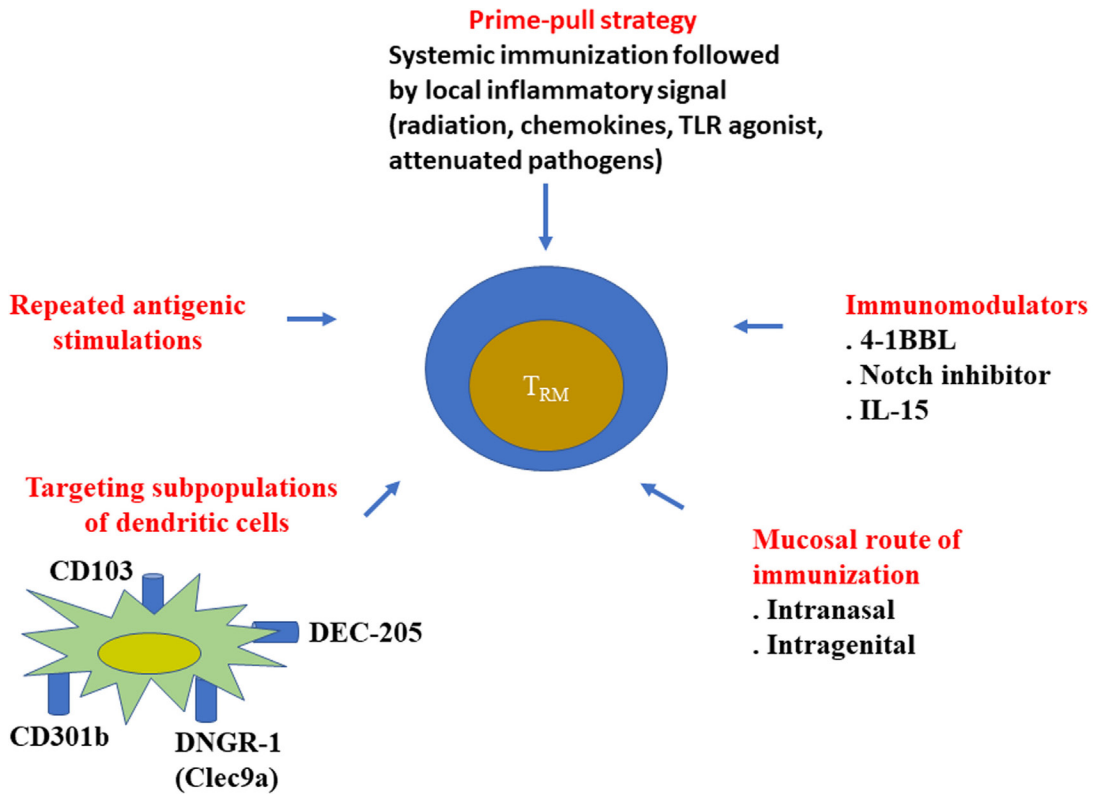

FIGURE 1 | Various strategies to elicit resident memory T cells. Various approaches have been proposed to better elicit resident memory CD8+ T cells, especially during vaccine administration. It has been well demonstrated that mucosal routes of immunization (intranasal, intragenital, etc.) better induce local CD8+ T_{RM}, than the conventional systemic route (intramuscular, subcutaneous, etc.). Local inflammatory signal after a systemic vaccination (prime-pool protocol) could also recruit CD8+ T cells in the tissue. However, the phenotype of the recruited cells was not always checked to determine if they represented *bona fide* T_{RM}.

This prime-pull strategy is thus an attractive strategy, but the phenotype of these intratissular-recruited $CD8^+$ T cells has not been fully established. In addition, it has not been reported whether these cells represent *bona fide* T_{RM}.

Targeting DCs to Elicit T_{RM}

Optimal generation of T_{RM} cells requires $CD103^+$ DCs in non-lymphoid tissues, which are dependent on the transcription factor BATF3 for their development, as well as mouse $CD8\alpha^+$ DCs in lymphoid organs (49). CLEC9A (DNGR-1) and DEC-205 are highly expressed by $CD103^+$ DC and CD8αDC. Intranasal delivery of targeting antibodies (DEC-205 or CLEC9A) proved highly protective against lethal influenza challenge (50). This protection is based on both the initiation of T-cell priming in the lung and the enhancement of local presentation and differentiation of T_{RM} cell (50).

$CD301b^+$ DCs also promote $CD8^+$ T cells with a T_{RM} phenotype which control genital HSV-2 infection (51).

In humans, lung-resident $CD1c^+$ DCs drove CD103 expression on effector $CD8^+$ T cells by displaying membrane-bound TGF-β1 (52).

Immunomodulators

Intranasal delivery of 4-1BBL in combination with an adenovirus encoding an influenza nucleoprotein to naïve mice elicits systemic effector memory $CD8^+$ T-cell expressing IL-7Rα, as well as an intraparenchymal lung $CD69^+CD8$ T_{RM} population, which comprised both $CD103^+$ and $CD103^{neg}$ cells. Moreover, physiologically, during primary influenza infection, T cells deficient for 4-1BB do not differentiate into lung-resident T_{RM} population (53).

Formalin inactivated RSV plus CpG plus L685,458, an inhibitor of Notch signaling, promoted protective $CD8^+$ lung tissue-resident memory T cells (54).

IL-15 complexes delivered locally to mucosal tissues without reinfection are an effective strategy to enhance establishment of tissue-resident memory CD8 T cells within mucosal tissues (55).

Our group showed that cancer vaccine administered by the intranasal route in combination with an anti-TGFβ decreased the number of T_{RM} without having any impact on T effector cells, and partially inhibited the protective effect of the vaccination (33).

Repeated Antigenic Stimulation

We and other showed that the density of T_{RM} in tissues and tumors progressively increased after each immunization (33). Prime boost immunization with recombinant adenovirus expressing HPV16 E7 protein *via* a homologous (intravaginal) or heterologous (intramuscular followed by intravaginal) route of immunization elicited more T_{RM} in the cervicovaginal mucosa than did a single priming by the intravaginal route (56). Multiple infections also result in more widespread or global T_{RM} dissemination (21).

CONCLUSION

In the recent past, T_{RM} have been emerging as having an important role in cancer immunotherapy based on cancer vaccine, adoptive cell therapy, and the blocking of the interaction of immune checkpoint molecules with their ligands. In the next few years, it will be necessary to better distinguish subpopulations of T_{RM} in different tissues with different phenotypes and functions. The vast majority of studies focus(ed) on $CD8^+$ T_{RM}. Further analysis of $CD4^+$ T_{RM} with phenotype and function that may be different from $CD8^+$ T_{RM} should be performed. Optimization of immunotherapy strategies to induce these T_{RM} is already the subject of ongoing work. Their role as a biomarker of responses to immunotherapy is also being evaluated based on preliminary encouraging results.

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REVIEW
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The Emerging Role of CD8+ Tissue Resident Memory T (T_{RM}) Cells in Antitumor Immunity: A Unique Functional Contribution of the CD103 Integrin

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Cancer immunotherapy is aimed at stimulating tumor-specific cytotoxic T lymphocytes and their subsequent trafficking so that they may reach, and persist in, the tumor microenvironment, recognizing and eliminating malignant target cells. Thus, characterization of the phenotype and effector functions of CD8+ T lymphocytes infiltrating human solid tumors is essential for better understanding and manipulating the local antitumor immune response, and for defining their contribution to the success of current cancer immunotherapy approaches. Accumulating evidence indicates that a substantial subpopulation of CD3+CD8+ tumor-infiltrating lymphocytes are tissue resident memory T (T_{RM}) cells, and is emerging as an activated tumor-specific T-cell subset. These T_{RM} cells accumulate in various human cancer tissues, including non-small-cell lung carcinoma (NSCLC), ovarian and breast cancers, and are defined by expression of CD103 [α_E(CD103)β_7] and/or CD49a [α1(CD49a)β1] integrins, along with C-type lectin CD69, which most likely contribute to their residency characteristic. CD103 binds to the epithelial cell marker E-cadherin, thereby promoting retention of T_{RM} cells in epithelial tumor islets and maturation of cytotoxic immune synapse with specific cancer cells, resulting in T-cell receptor (TCR)-dependent target cell killing. Moreover, CD103 integrin triggers bidirectional signaling events that cooperate with TCR signals to enable T-cell migration and optimal cytokine production. Remarkably, T_{RM} cells infiltrating human NSCLC tumors also express inhibitory receptors such as programmed cell death-1, the neutralization of which, with blocking antibodies, enhances CD103-dependent TCR-mediated cytotoxicity toward autologous cancer cells. Thus, accumulation of T_{RM} cells at the tumor site explains the more favorable clinical outcome, and might be associated with the success of immune checkpoint blockade in a fraction of cancer patients.

Keywords: CD8 tissue resident memory T (T_{RM}) cells, CD103 integrin, cytotoxic T lymphocytes, onco-immunology, cancer immunotherapy

Abbreviations: CTL, cytotoxic T lymphocytes; CTLA, cytotoxic T-lymphocyte-associated antigen; PD-1, programmed cell death-1; IFN, interferon; LFA-1, lymphocyte-function-associated antigen-1; mAb, monoclonal antibodies; NSCLC, non-small-cell lung carcinoma; MHC-I, major histocompatibility complex class I; TCR, T-cell receptor; TIL, tumor-infiltrating lymphocytes.

INTRODUCTION

$CD8^+$ T lymphocytes play an essential role in defense against cancers through recognition by T-cell receptors (TCR) of specific antigenic peptides presented on the surface of malignant cells by major histocompatibility complex class I (MHC-I) molecules, and elimination of the tumor target, mainly by releasing the content of cytolytic granules containing perforin and granzymes. To destroy their target, cytotoxic T lymphocytes (CTL) must first migrate to the tumor site, infiltrate the tumor tissue, and interact with the cancer cell, to finally trigger effector functions leading to transformed cell eradication. Integrins and their ligands (1) play a crucial role in promoting antitumor T-cell activities by regulating T-cell migration and retention within the tumor, adhesion to antigen-presenting cells and co-stimulation resulting in CTL activation and functions (2). Cytokines and chemokines are also involved in coordinating circulation, homing, retention, and activation of T lymphocytes. Although some of them are known to contribute to tumor cell proliferation and dissemination by inhibiting tumor-specific T-cell responses, others promote infiltration and activation of T lymphocytes in a hostile tumor ecosystem, resulting in tumor cell destruction (3). In this regard, TGF-β, abundant in the tumor microenvironment, was reported to be an immunosuppressive factor used by malignant cells to escape from the immune response (4). This cytokine inhibits expression of lymphocyte-function-associated antigen-1 (LFA-1, also known as $\alpha_L\beta_2$ or CD11a) integrin and LFA-1-mediated T-cell functions (5). Paradoxically, this cytokine induces CD103 (also known as $\alpha_E\beta_7$ or HML-1) integrin expression on activated intraepithelial $CD8^+$ T lymphocytes, and enhanced CD103-dependent T-cell adhesion and signaling (6, 7).

LFA-1 and CD103 are the predominant integrins expressed by intraepithelial T lymphocytes (IEL) and $CD8^+$ tumor-infiltrating lymphocytes (TIL). While the contribution of LFA-1 and its ligand ICAM-1 (CD54) to TCR-mediated CTL activities is well documented (8), much less is known about the role of CD103 and its ligand, the epithelial cell marker E-cadherin, to T-cell-mediated cytolytic activity. CD103 has been associated with cytotoxicity of $CD8^+$ T cells in several human pathologies, including graft-versus-host disease (GVHD) (9), allogeneic transplant rejection (10–12), autoimmune diseases (13, 14), and cancer (6, 15). This integrin, together with the activation marker CD69 and the integrin CD49a [also known as $\alpha_1\beta_1$ or very late antigen-1 (VLA-1)], defines a recently identified subtype of $CD8^+$ T lymphocytes called "tissue-resident memory T (T_{RM}) cells," possibly endowed with potent cytotoxic activities. Moreover, there is an emerging consensus that T_{RM} cells frequently accumulate in multiple human tumors, especially of epithelial origin, and play an essential role in tumor-specific T-cell responses and, likely, in control of malignant diseases. T_{RM} cells are also surrogate markers of the efficacy of cancer vaccines (16, 17), and a low number of this T-cell subset among TIL may correlate with failure of immune checkpoint blockade therapy in most cancer patients. In this review, we focus on $CD8^+$ T_{RM} cells accumulating in human solid tumors, mainly non-small-cell lung carcinoma (NSCLC), and current insight implicating CD103 integrin in regulating T_{RM} functions and CTL-mediated antitumor immune responses, with potential prognosis and immunotherapeutic applications.

PHENOTYPIC AND MOLECULAR FEATURES OF T_{RM} CELLS IN TUMORS

It is now generally agreed that a population of T_{RM} cells accumulates in tumors of epithelial origin, such as ovarian, pancreatic, colorectal, and lung tumors (15, 18–20), as well as those of non-epithelial origin, including malignant glioma and melanoma (21, 22). These T_{RM} cells express a broad range of integrins and chemokine receptors, probably involved in their migration to the tumor site, and may interfere with their egress from the tumor tissue. Transcriptional studies pointed to expression of CXCR3 and CXCR6 by T_{RM} cells infiltrating human lungs (23). Intratumoral T_{RM} cells express high levels of CCR5 and CCR6 chemokine receptors that may confer T-cell homing to the inflammatory tumor microenvironment (15). Moreover, CCR5 is recruited at the immune synapse formed between T cells and tumor target cells upon interaction of CD103 with E-cadherin, promoting retention of T_{RM} cells at the tumor site by inhibiting their sensitivity to a CCL5 chemotactic gradient (7). By contrast, T_{RM} cells do not express CX3CR1, a chemokine receptor that mediates transmigration through the endothelium, supporting the hypothesis that this T-cell population has reached its final destination and does not need to exit from the lung tissue (23). Lung tumor T_{RM} lack expression of lymph node homing receptors CCR7 and CD62L, as well as the receptor for sphingosine 1-phosphate, S1PR1 (15), which mediates the egress of T cells from lymphoid organs (24). Indeed, downregulation of SIPR1 appears to be a prerequisite for retention of $CD8^+CD103^+$ T_{RM} cells in peripheral tissues (25, 26).

With regard to adhesion/costimulatory molecules, the expression profile of intratumoral T_{RM} cells seems to be compatible with their capacity to reside in tumor tissue and their inability to recirculate in the bloodstream. In melanoma, $CD8^+$ T_{RM} cells were found to co-express CD69, CD103, and VLA-1 (CD49a or $\alpha_1\beta_1$ integrin), with the latter reported to cause long-term retention of activated T cells in peripheral tissues (27). Human lung tumor $CD8^+$ T_{RM} cells are characterized by downregulation of CD28 and upregulation of CD69 and CD103 and CD49a integrins, which are most likely induced by TGF-β in the tumor microenvironment (15, 28). TGF-β plays a pivotal role in formation and maintenance of T_{RM}, at least in part *via* induction of CD103. Indeed, TGF-β is directly involved in CD103 expression in tumor-specific T cells upon engagement of TCR with specific tumor peptide–MHC-I complexes (7), through binding of Smad2/3 and NFAT-1 transcription factors to promoter and enhancer elements of the *ITGAE* gene, which encodes the CD103 (α_E) subunit (29). This cytokine is also involved in dampening expression of the LFA-1 integrin on TIL, thus participating in T-cell residency within the tumor (15, 30). In LCMV chronic infection, but not acute infection, TGF-β signaling inhibits migration of $CD8^+$ effector T lymphocytes from the spleen to the gut by dampening expression of integrin $\alpha_4\beta_7$ during the formation phase of T_{RM} cells (31). Consequently, $CD8^+$ $Tgfbr2^{-/-}$ T cells migrate normally to the intestine, but their retention in the gut epithelium is impaired. In contrast,

TGF-β signaling does not impact $\alpha_4\beta_7$ integrin expression and T-cell migration to the gut after acute bacterial infection (32). Moreover, E-cadherin, which is downregulated by TGF-β in cancer cells during epithelial-to-mesenchymal transition [for a review see Ref. (33)], appeared to promote accumulation of a subset of $CD8^+$ memory T cells in murine submandibular glands by a mechanism independent of CD103 (34). This cytokine has been identified as a potential therapeutic target in cancer because of its role in supporting tumor progression and in inducing immunosuppression. In this regard, it has been shown that targeting the TGF-β pathway inhibits tumor growth by promoting antitumor immunity associated with increased $CD8^+$ T-cell numbers (35). However, the consequence of such cancer immunotherapy approaches on T_{RM} cells, the maintenance of which is dependent of TGF-β, has not been addressed.

T-cell inhibitory receptors are important for maintaining self-tolerance and regulating the immune response in peripheral tissues (36). Among these immune checkpoints, cytotoxic T-lymphocyte-associated antigen (CTLA)-4 and Tim-3 appeared to be associated with tumor antigen-specific $CD8^+$ T-cell dysfunction in melanoma patients (37). $CD103^+$ T_{RM} cells have been shown to express a wide range of inhibitory receptors, such as CTLA-4, Tim-3, and programmed cell death-1 (PD-1), associated with their capacity to maintain peripheral tolerance (25, 38). Data from our group and other groups revealed that intratumoral $CD8^+CD103^+$ T_{RM} cells frequently express PD-1, Tim-3, and Lag-3, which are likely involved in their exhausted state and their dysfunctioning at the tumor site (15, 28, 39, 40). Notably, TGF-β is also involved in PD-1 induction on $CD8^+$ T cells, contributing to T-cell anergy and a sustained tolerance (41). Neutralization of TGF-β results in downregulation of PD-1 expression in T cells causing graft rejection. Mechanistically, PD-1 is regulated by the NFATc1 transcription factor (42), and is enhanced by a TGF-β/SMAD3-dependent signaling pathway (43). Expression of PD-1 on TIL is described as a biomarker of $CD8^+$ tumor-reactive T cells in cancer patients (44). Thus, the $PD\text{-}1^+$ status of tumor T_{RM} cells suggests that they are enriched with antigen-specific $CD8^+$ T cells that may be used as targets in cancer immunotherapy.

Alongside upregulation of genes encoding PD-1, CTLA-4 and Tim-3, $CD8^+$ TIL display increased expression levels of genes encoding transcription factors EGR1 and Nr4a2 (25, 38), as well BATF and NAB1, suggesting a role in T_{RM} establishment in the tumor (28). $CD8^+CD103^+$ TIL also express an increased level of T-bet (45) and the Runx3 transcription factor, which programs their residency in tumors (46). Indeed, *Runx3* deficiency impaired TIL accumulation without affecting migration to the tumor, associated with an increase in tumor growth. By contrast, *KLF2* transcription factor was diminished in T_{RM} cells from human TIL^{hi} tumors (28), while Notch activity appeared to be required for maintenance of $CD103^+$ T_{RM} cells in mouse tumors (23). Therefore, additional studies are needed to better characterize the transcriptional features of $CD8^+CD103^+$ T_{RM} cells in human tumors, and transcriptional factors that govern their residency in malignant tissues. Overall, the T_{RM} cell subset is characterized by a $Runx3^+$, $Notch^+$, $Hobit^+$, $Blimp1^+$, $BATF^+$, $EOMES^{neg}$, and $Tbet^{low}$ transcription factor profile (23, 46–49) and is defined by the surface expression of CD103, CD49a, and CD69 [for reviews see Ref. (50–52)]. It also expresses the inhibitory receptors PD-1, CTLA-4, and Tim-3 (15, 38, 53), and is promoted by particular route of immunization targeting tissue dendritic cells (17, 54, 55) and specific environmental factors mainly TGF-β, IL-33, and IL-15 (56–59).

FUNCTIONAL ACTIVITIES OF INTRATUMORAL T_{RM} CELLS

Thus far, little is known about $CD8^+CD103^+$ T_{RM} functions in tumor tissues. Immune checkpoint expression by $CD103^+$ TIL suggested that $CD8^+$ T_{RM} cells in tumors are enriched with tumor antigen-specific CTL. These T cells were found to express transcripts encoding products linked to cytotoxic functions of $CD8^+$ T lymphocytes, including *IFNG*, *GZMA*, *GZMB*, *SEMA7A*, *KLRB1*, *CCL3*, *STAT1*, *RAB27A*, *IL21R*, and *FKBP1A* (28). Expression of granzyme A, granzyme B, and perforin by $CD8^+CD103^+$ TIL was also observed at the protein level, together with the CD107a (LAMP-1) degranulation marker and the Ki-67 proliferation marker (15, 28, 45, 60).

Functional studies showed that $CD8^+CD103^+$ TIL are able to secrete inflammatory cytokines, including interferon (IFN)γ and TNFα (28, 46). Moreover, interaction of CD103 with E-cadherin on tumor target cells optimizes cytokine release, since siRNA targeting E-cadherin partially inhibited IFNγ production (61). Cytotoxicity experiments indicated that freshly isolated $CD103^+$ TIL were able to kill autologous tumor cells following neutralization of the PD-1–PD-L1 interaction with anti-PD-1 or anti-PD-L1 blocking antibodies (15). This cytotoxic activity is most likely mediated by $CD103^+$ T cells, since anti-CD103 neutralizing monoclonal antibodies (mAb) compromise this function. Consistently, cytotoxicity of $CD103^+$ T-cell clones toward autologous $E\text{-}cadherin^+$ tumor cells is inhibited anti-CD103 blocking mAb (6). Another noteworthy aspect of our contribution to the field is the demonstration that CD103 is an important molecule required for polarization of cytotoxic granules at the immune synapse formed between CTL clones and autologous tumor cells, and that siRNA targeting E-cadherin inhibited TCR-mediated target cell killing (6). Moreover, CD103 contributes to recruitment of $CD103^+$ T_{RM} cells within epithelial tumor islets, and intratumoral early T-cell signaling (30).

A role for the VLA-1 integrin in the differentiation and functions of T_{RM} cells was reported in a mouse tumor model (27). $VLA\text{-}1^+$ T cells, co-expressing or not CD103, secreted high levels of IFNγ upon re-stimulation, and this cytokine production was impaired by anti-VLA-1 or anti-CD103 mAb. Moreover, blockade of VLA-1 or CD103 severely compromised control of tumor growth *in vivo*. Similar studies revealed that $CD8^+CD103^+$ T_{RM} cells accumulate and protect mice against melanoma in a CD103-dependent manner, and these T_{RM} cells play a pivotal role in perpetuating antitumor immunity (22). Conversely, it has been reported that anti-latency-associated peptide (LAP) antibodies targeting the LAP/TGF-β complex induce a decrease in $CD8^+CD103^+$ T cells in mouse spleen and lymph nodes, and that this peculiar T-cell subset displays a tolerogenic feature (62). Murine $CD8^+CD103^+$ regulatory T cells have also been described

in autoimmune diseases where they are induced by TGF-β and display suppressive activities independently of granzyme B (63). Moreover, $CD8^+CD103^+$ T cells are crucial for prevention of chronic GVHD lupus in mice by suppressing T helper and B cell responses through a non-cytotoxic mechanism involving TGF-β and IL-10 signals (64). However, further studies are needed to permit the distinction between human $CD8^+CD103^+$ CTL and $CD8^+CD103^+$ T regulatory cells, even though granzyme B expression appears as a good marker, and determine the exact contribution of both subsets in autoimmune [for a review see Ref. (65)] and cancer diseases.

BIDIRECTIONAL SIGNALING OF CD103 DICTATES ITS ACTIVATION AND FUNCTIONS

Integrins are heterodimeric transmembrane receptors that mediate cell-extracellular matrix adhesion and cell–cell interactions (2). Among a family of 24 members (1), the CD103 integrin, formed by α_E (CD103) and β_7 subunits, is exclusively expressed by leukocytes, in particular IEL (66), psoriatic skin epidermal $CD8^+$ T cells (67), cervico-vaginal antigen-specific CTL (68), and $CD8^+$ T lymphocytes infiltrating various human tumors (6, 18–20, 60, 69). The restricted distribution of the CD103 integrin is attributed to expression of the α_E subunit, since the β_7 subunit is widely expressed in T cells (70).

On naive T lymphocytes, integrins have weak affinity for their ligands. However, stimulation of T lymphocytes through TCR or chemokine receptors initiates an "inside-out" signal that induces integrin activation by triggering integrin-extended conformation and clustering, thereby enhancing their affinity for their ligands. Firm adhesion of integrins to their ligands triggers an "outside-in" signal that has costimulatory functions in TCR signaling, thereby contributing to T-cell activation, migration, and cytotoxicity (71–73). Until recently, the signaling pathways of CD103 integrin and the molecules involved in its bidirectional activation were not clearly elucidated. Like the other integrins, CD103 activation is regulated by TCR engagement. In this context, it has been shown that cross-linking of TCR on IEL or cell treatment with phorbol myristate acetate increased the avidity of CD103 for E-cadherin and provided a mechanism for lymphocyte adherence and activation (74). Furthermore, the CCR9 ligand, CCL25, induced CD103-mediated adhesion of $CD8^+$ IEL to E-cadherin, suggesting a role for this chemokine receptor/chemokine pair in promoting functions of CD103 *via* inside-out signaling (75). Similarly, the CCL7 chemokine has been shown to favor adhesion and retention of CD103-expressing T cells during renal allograft rejection, by promoting the adhesive properties of CD103 (76).

TGF-β is responsible for inducing CD103 integrin in $CD8^+$ T lymphocytes (6, 77) by regulating expression of both *ITGAE* (29, 78) and *ITGB7* (79) genes encoding α_E and β_7 chains, respectively. In addition, in contrast to all other integrins, TGF-β regulates CD103 activation and signaling within epithelial tissues (**Figure 1**). Indeed, we previously demonstrated that the interaction of TGF-β with its receptors TGFBR on the surface of $CD8^+CD103^+$ T cells induces recruitment and phosphorylation of integrin-linked kinase (ILK) by TGFBR1 (activin receptor-like kinase-5) (30). We further showed that phosphorylated-ILK interacted with the CD103 subunit intracellular domain, resulting in phosphorylation of protein kinase B (PKB)/AKT, thereby initiating integrin inside-out signaling leading to activation of CD103 and strengthening of CD103-E-cadherin adhesion.

The mechanism regulating the CD103 outside-in signaling pathway is not fully understood. Studies from our group have shown that CD103-E-cadherin tight adhesion initiates an outside-in signal by promoting phosphorylation of the focal-adhesion-associated adaptor protein paxillin and proline-rich tyrosine kinase-2 (Pyk2), and subsequent binding of phosphorylated-paxillin to the CD103 subunit tail (80). In addition, the adhesive interaction of E-cadherin with CD103 on TIL triggers phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and phospholipase C γ1 proteins, providing intracellular signals that promote CTL effector functions (60). These studies emphasize a unique costimulatory role of the CD103 integrin in activation of tumor-specific CTL, by triggering polarization of cytotoxic granules at the immune synapse and subsequent TCR-mediated cytotoxicity (60), and in proliferation of $CD103^+$ thymocyte cells (81). Engagement of CD103 with E-cadherin also determines cell shape and motility of $CD103^+$ lymphocytes (82), and recruitment of $CD8^+$ T_{RM} cells within epithelial tumor islets, in an actin-polymerization-dependent fashion (30, 80). Moreover, TGF-β enhances T-cell adhesion and movement toward tumor regions by increasing CD103 expression levels and promoting intracellular T-cell signals leading to integrin activation (30). CD103 also contributes to retention of T_{RM} cell subpopulations by interacting with E-cadherin and mediating arrest of T lymphocytes on epithelial tissues (32, 61). Thus, CD103 appears to be a unique integrin for adjusting T-cell adhesion and migratory potential in a TGF-β-rich tumor microenvironment, as well as retention of tumor-specific $CD8^+$ T_{RM} cells and local antitumor effector functions (**Figure 1**).

PROGNOSTIC VALUE OF T_{RM} CELLS IN HUMAN CANCERS

$CD8^+CD103^+$ T_{RM} cells have emerged as predictive markers of patient survival in several malignant diseases, including ovarian, lung, endometrial, and breast cancers (15, 20, 28, 83, 84). Indeed, in a large cohort of high-grade serous ovarian cancers (20) and a cohort of early-stage NSCLC (15), an enhanced $CD103^+$ TIL subset correlated with improved patient survival. $CD103^+$ TIL were also associated with a favorable prognosis in urothelial cell carcinoma of the bladder, and could represent a favorable prognostic predictor of overall and recurrence-free survival (83). In that retrospective study, $CD8^+$ T cells were identified as the principal cellular sources of CD103, and the density of intratumoral $CD103^+$ cells was inversely associated with tumor size. More recent studies also defined the CD103 integrin as a biomarker of good prognosis in cohorts of breast (85) and lung cancer (17, 28, 84). Notably, T_{RM} infiltration in lung cancer correlated with better clinical outcome in both univariate and multivariate analyses, independently of $CD8^+$ T cells (17). In addition, high numbers of intratumoral $CD103^+$ TIL were

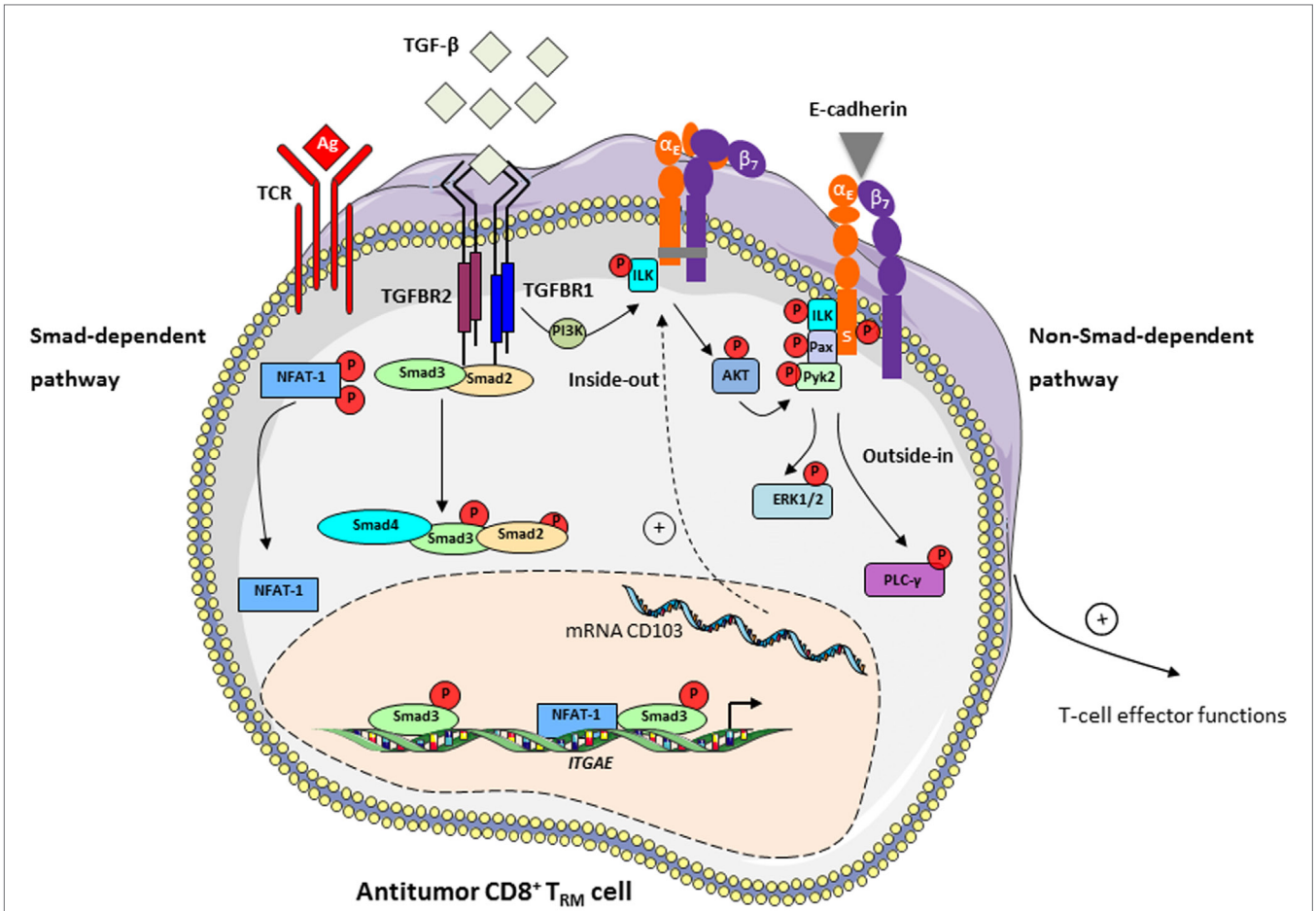

FIGURE 1 | TGF-β induces CD103 expression in tumor-specific T cells and participates in integrin bidirectional signaling. *Left:* TGF-β controls CD103 expression in tumor antigen (Ag)-specific T cells upon interaction of T-cell receptor (TCR) with specific tumor peptide–major histocompatibility complex class I complexes, *via* a Smad-dependent pathway. TGF-β binds to TGFBR at the surface of $CD8^+$ T lymphocytes and leads to recruitment and phosphorylation of Smad2 and Smad3 and their subsequent nuclear translocation. Transcription factors NFAT-1, translocated into the nucleus upon TCR engagement, and Smad2/3 bind to promoter and enhancer elements of the *ITGAE* gene, which encodes the CD103 (α_E) subunit, and activates CD103 expression (29). *Right:* TGF-β participates in CD103 intracellular signaling *via* a non-Smad-dependent pathway. Interaction of TGF-β with TGFBR on $CD8^+CD103^+$ T_{RM} cells induces recruitment and phosphorylation of integrin-linked kinase (ILK). Phosphorylated (P)-ILK interacts with the CD103 subunit intracellular domain, resulting in phosphorylation of protein kinase B/AKT and initiating integrin inside-out signaling leading to activation of CD103 (30). CD103-E-cadherin tight adhesion initiates an outside-in signal by promoting phosphorylation of paxillin (Pax) and Pyk2, and subsequent binding of phosphorylated-paxillin to the α_E subunit tail where a phosphorylatable Ser (S) in the ES^{1163}IRKAQL motif plays an important role (80). Adhesive interaction of E-cadherin with CD103 also triggers activation of PI3K/extracellular signal-regulated kinase (ERK) and phospholipase C/PKC pathways (60), providing intracellular signals that promote $CD8^+$ T_{RM} effector functions, including actin cytoskeleton reorganization, T-cell spreading and migration, cytokine release and polarized exocytosis of cytotoxic granules leading to target cell destruction.

significantly associated with prolonged disease-free survival and overall survival in patients with pulmonary squamous cell carcinoma, but not in those with pulmonary adenocarcinoma (84). The epithelial localization of $CD103^+$ TIL has an even more significant predictive value compared to the stromal location, suggesting that intraepithelial $CD8^+CD103^+$ cells encompass a higher proportion of tumor-specific T_{RM} cells (15, 85). This intratumoral positioning of $CD103^+$ TIL was correlated with expression of E-cadherin on tumor cells in bladder cancer (83), but not in ovarian or breast cancer (20, 85). Moreover, this predominant location in intratumoral regions, rather than in the stroma, was associated with the capacity of CD103 to promote recruitment of TIL in epithelial tumor islets (30). Thus, T_{RM} cells appear to be key components in antitumor immunity, and their presence at the tumor site predicts a favorable prognosis in many cancers of different histological types. Paradoxically, their dominant expression of checkpoint receptors suggests that may be functionally exhausted. However, their location in close contact with tumor cells, their ability to proliferate *in situ*, to produce granzyme B and other cytotoxic molecules and pro-inflammatory cytokines, support the hypothesis that T_{RM} cells are enriched in tumor-specific $CD8^+$ T cells that could trigger specific cytotoxic activity toward target cells in physiological conditions and following neutralization of PD-1–PD-L1 interaction, as we demonstrated *ex vivo* (15) and possibly also during anti-PD-1/anti-PD-L1 cancer immunotherapy. Accordantly, recent studies revealed an expansion of $CD8^+CD103^+$ T_{RM} cells during anti-PD-1 treatment in melanoma (86).

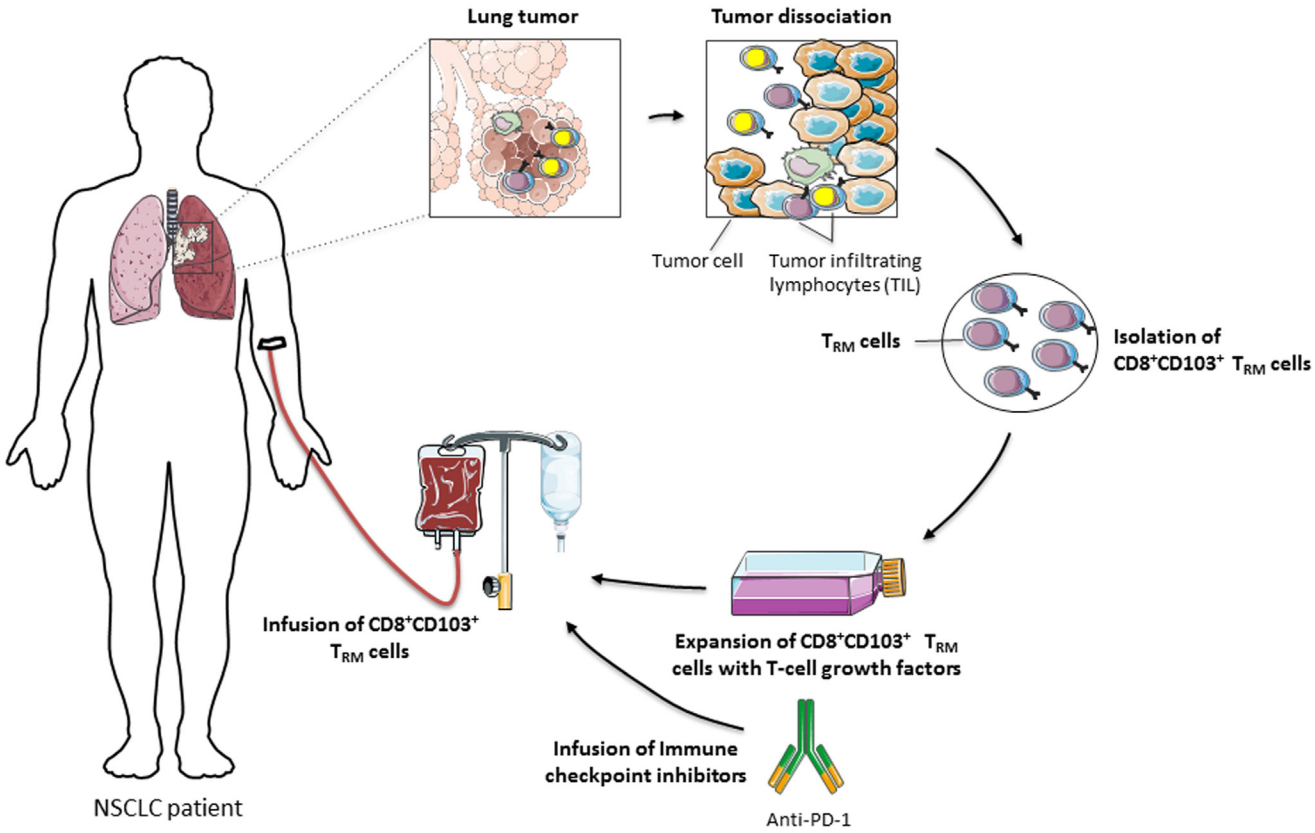

FIGURE 2 | Adoptive transfer of CD8+CD103+ T_{RM} cells for cancer immunotherapy. CD8+CD103+ T_{RM} cells, suspected to express tumor-reactive T-cell receptors, are isolated from tumor-infiltrating lymphocytes (TIL), amplified *ex vivo* in the presence of T-cell growth factors, including IL-2, and then reinjected into cancer patients, in combination or not with immune checkpoint inhibitors, such as anti-programmed cell death-1, to reverse T-cell exhaustion and optimize the antitumor cytotoxic T lymphocyte response.

CONCLUDING REMARKS

Overall, CD8+ T_{RM} cells that accumulate in human tumor lesions appear to be important effectors in antitumor CTL responses. Their retention within the tumor ecosystem may control tumor growth and explain more favorable prognoses in certain cancer patients. Moreover, CD103 emerges as a key molecule in CD8+ T_{RM} activation, the expression of which is probably adjusted in the tumor microenvironment by TGF-β. This integrin not only promotes T-cell adhesion to target cells through interaction with its unique known ligand E-cadherin but also provides positive signals triggering diverse T-cell effector functions, such as spreading, migration, proliferation, and cytotoxicity (**Figure 1**). Nevertheless, additional studies and tools are required to further decipher CD103 structure and bidirectional signaling, and to determine whether this integrin also undergoes conformational changes within the tumor ecosystem in order to control the affinity to its ligand E-cadherin and to regulate its functional properties. In this regard, identification of new partners and associated molecules controlling integrin intracellular signals and regulating the dynamics of CD103 are essential in order to optimize the antitumor reactivity of CD8+ T_{RM} cells. They would also help to determine the true contribution of CD8+CD103+ T_{RM} cells and the identified costimulatory molecules in the success of immune checkpoint blockade immunotherapies in a minor subpopulation of cancer patients, and to improve current T-cell-based cancer immunotherapeutic approaches such as adoptive T-cell therapies (**Figure 2**).

AUTHOR CONTRIBUTIONS

SC, MB, and FM-C coordinated the writing of the manuscript. SC, MB, MK, CN, and FM-C participated in drafting and editing the text and figures. All authors gave final approval to the version submitted.

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Role of Tissue-Resident Memory in Intra-Tumor Heterogeneity and Response to Immune Checkpoint Blockade

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Tissue-resident memory T (T_{RM}) cells are a distinct subset of memory T cells that reside in non-lymphoid tissues for prolonged periods of time without significant recirculation providing continued immune surveillance at these sites. Recent studies suggest that T_{RM} cells are also enriched within tumor tissue. Expression of inhibitory immune checkpoints (ICPs) is particularly enriched on this subset of tumor-infiltrating T cells, suggesting that they are major targets for newer therapies targeting ICPs such as the programmed death-1 pathway. Recent studies suggest that tissue restriction of these cells without recirculation may also lead to heterogeneity of T_{RM} cells within individual metastatic lesions, ultimately leading to inter-lesional diversity. Thus, individual metastatic lesions may contain genomically distinct immune microenvironments that impact both evolution of tumors as well as the mechanisms underlying response and resistance to immune therapies. Understanding the biology of T_{RM} cells infiltrating tumors will be essential to improving immune-based approaches in diverse settings.

Keywords: tissue-resident memory cells, immune checkpoint blockade, tumor heterogeneity, cancer immunotherapy, immunity to cancer

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Immune-based approaches, particularly those based on the blockade of inhibitory immune checkpoints (ICPs) on T cells have emerged as among the most promising new strategies to treat cancer (1). An important aspect of immune therapies is their potential ability to mediate long-term control of tumors. The capacity of the immune system to mediate long-term protection, particularly against pathogens, such as in the context of vaccines, is mediated in large part by immunologic memory (2). Therefore, understanding immunologic memory mediated by T cells is likely to be important for deeper understanding of immune-mediated long-term control of tumors. It is thought that uptake of antigens from dying tumor cells by antigen-presenting cells leads to activation of anti-tumor T cells in the lymph nodes, and resultant effector memory T cells traffic back to the tumor to mediate anti-tumor effects, creating a tumor-immunity cycle (3). Several studies have shown that infiltration of primary and metastatic lesions by immune cells, particularly T cells and myeloid cells impacts outcome (4). Studies in both mice and humans suggest that there are differences in the memory T cell subsets that provide immune surveillance within lymphoid and non-lymphoid tissues (NLTs). As tumor-related mortality in most solid tumors is not due to growth of primary tumors, but rather due to the growth of metastatic tumor cells in NLTs, it is the immune surveillance in NLTs that may be critical for protective tumor immunity. In this review, we discuss newer insights into spatial aspect of immunologic memory and particularly memory T cells within NLTs in the context of tumor immunity. We will discuss emerging evidence suggesting that the biology of these tissue-resident memory (T_{RM}) T cells may not only be critical for understanding and improving clinical responses to ICP

blockade, but may also contribute to the complexity of immune microenvironment by creating inter-lesional heterogeneity in the setting of metastatic cancer.

T_{RM} T CELLS: LOCAL POLICEMEN

Initial models of T cell memory classified effector/central memory (T_{EM}/T_{CM}) T cells, with the effector subset implicated in surveying NLTs (5). Recent studies have identified a third subset, termed T_{RM} T cells that reside for prolonged periods in NLTs and play an important role in protective immunity (6). Mouse T_{RM} cells have been described in diverse tissues, including lung, liver, brain, as well as barrier tissues (6, 7). Murine T_{RM} cells haven been shown to mediate rapid *in situ* protection against viral, bacterial, and parasitic infections and are more effective in this regard than their circulating counterparts, including central memory T cells (7, 8). An important aspect of T_{RM}-mediated immune surveillance is its regional nature. Thus in parabiotic mice that share systemic circulation, T_{RM} cells remain localized within tissues and do not cross over to equilibrate in the paired mouse carrying antigenic stimulus (6). T_{RM} cells express CD69, which is implicated in tissue retention by sequestration of the sphingosine-1-phosphate receptor (9).

Tissue-resident memory cells have also been identified in several human tissues and implicated in tissue-restricted pathology particularly in the skin, such as fixed drug eruptions (10–12). As in the mouse, human T_{RM} cells have been identified by the expression of CD69 on memory T cells within tissues, which is generally lacking in blood memory T cells (13). In humans, CD103 is expressed only in a subset of CD69+CD8+ memory T cells in some barrier tissues, but not by CD4+ memory T cells in any tissue, indicating that CD69 may be a more universal marker distinguishing both CD4+ and CD8+ memory T cells in tissues from their blood counterparts. It is notable that the proportion of T_{RM} cells differs in different tissues, with enrichment in some barrier tissues such as skin. Recent studies have also characterized transcriptional profiles of human T_{RM} cells, which resemble their murine counterparts and also illustrate that these are a distinct subset of human memory T cells (14, 15).

The pathways that regulate generation, recruitment, retention, and long-term maintenance of these T cells in NLTs remain an active area of research. New insights into transcriptional regulation of the T_{RM} differentiation are emerging and may differ between humans and mice. For example, the transcription factor Hobit/ZNF683 is exclusively expressed and required for the generation of murine T_{RM} cells after infection, but expressed at low/negligible levels on human T_{RM} cells (14, 16). In recent studies, we have shown that human and murine T_{RM} cells express NR4A1/nur77, which is also essential for T_{RM} differentiation in several murine tissues (17). Runx3 is another transcription factor that promotes the differentiation of T cells with T_{RM} phenotype (18). Retention and maintenance of T_{RM} cells may also depend on the availability of local antigen, interactions with myeloid cells as well as cytokines like TGFβ and IL-15 in NLTs (19, 20). Tissue distribution of T_{RM} cells, at least against pathogens may depend on the site of initial exposure. For example, human influenza-specific T_{RM} cells are preferentially found in the lung (21) and hepatitis-B specific T_{RM} cells particularly in the liver (22). Human bone marrow may also be a particularly interesting compartment for long-lived memory T cells with phenotype of T_{RM} cells (17, 23, 24).

T_{RM} CELLS IN TUMORS

Several studies have now documented that a large proportion of T cells infiltrating human tumors have T_{RM} phenotype, at least based on the expression of CD69 and CD103 (11, 12, 25–27). In some studies, these T cells were also shown to have genomic signatures consistent with those described for T_{RM} cells (11, 25, 26). This includes altered expression of genes involved in tissue retention/homing (such as downregulation of S1PR1, S1PR5, and KLF2; increase in CD69 and CD103) as well as transcription factors now functionally implicated in this phenotype (such as NR4A1, NR4A2, and Runx3) in several tissues. It is notable that some of the genes (such as Hobit) critically implicated in the biology of murine T_{RM} cells are not expressed at high levels in their human counterparts. It is notable that in mouse models of viral infections such as lymphocytic choriomeningitis virus (LCMV), T cell memory has been largely studied when the underlying viral antigen is depleted. However, the biology in human tumors or other states of persistent viral infection may differ from LCMV models and local antigen may have important implications for T_{RM} biology. Indeed, recent studies suggest that local antigen may drive proliferation of T_{RM} cells *in situ* (28, 29).

While the infiltration of tumors by T cells has in general emerged as a strong indicator of improved prognosis, the presence of T_{RM} cells within tumor-infiltrating lymphocytes (TILs) may be a particular driver of this correlation. The proportion of TILs that have T_{RM} phenotype differs between studies (for example, from 25 to 75%) and may depend in part on the nature of specific markers utilized to identify these cells as well as the specific tissue/organ studied. This subset of cells may also be enriched for tumor reactivity, which is also consistent with other studies showing enrichment of tumor reactivity such as against tumor-associated neoantigens in CD8+ memory T cells with PD1+ phenotype (26, 30). Recent studies in murine models also suggest that these cells are important contributors to protective tumor immunity (31). In this study, the presence of T_{RM} cells was modeled in the setting of autoimmune vitiligo and melanoma-specific T_{RM} cells infiltrating these lesions were shown to mediate strong tumor protection. To date, most of the data relating to the biology of T_{RM} cells in human tumor tissues are largely based on patients with solid tumors. Further studies are needed to better characterize this subset of T cells within hematologic malignancies. Below, we particularly focus on two aspects of the biology of tumor-associated T_{RM} cells, their contribution to clinical responses to ICP blockade therapies and emergence of inter-lesional heterogeneity.

ARE T_{RM} CELLS A CRITICAL TARGET FOR ICP BLOCKADE?

Antibody-mediated blockade of inhibitory ICPs such as programmed death-1 (PD-1) have led to impressive and durable

clinical regressions in several cancers (32). This is remarkable as the expression of ICPs such as PD-1 is limited to only a subset of TILs (33). The principle of ICP blockade is based on the concept of unleashing the activity of pre-existing anti-tumor T cells against the tumor (34). Studies of T cell receptor (TCR) sequencing of T cells from patients receiving anti-PD1 therapy suggests that this therapy leads to *in situ* proliferation of CD8+ T cells within tumors of patients who respond to therapy (35). The ICP expressing T cells were found to include most of the tumor reactive T cells. While such tumor-reactive T cells can be detected in peripheral blood, these cells are predominantly present within the tumor tissue. In recent studies, we and others have shown that T_{RM} cells are the dominant T cell subset expressing ICPs within the tumor microenvironment (11, 25). While most studies have described the presence of T_{RM} cells within adult tumors recent data suggest that T_{RM} cells are also enriched within pediatric tumors like glioma and are the T cell subset within these tumors that predominantly expresses ICPs (36). While T_{RM} cells were initially identified in the tumor tissue based on the expression of classic T_{RM} markers such as CD69 or CD103, gene expression studies confirmed that these T cells are a distinct subset of TILs with a genomic signature overlapping with T_{RM} signature. Importantly, although CD69 is well studied as a T cell activation marker, the genomic profiles of CD69+ T_{RM} cells are distinct from activated T cells and instead enriched for tissue retention genes (25). Therefore, while tumor tissue contains antigens recognized by these cells, and T_{RM} cells express CD45RO consistent with memory T cells, they are genomically distinct from simply activated effector memory T cells. Recent studies in murine tumor models also support the importance of tumor-infiltrating T_{RM} cells in mediating long-term control of melanoma tumors (31). The relationships between T_{RM} cells and other populations such as stem memory cells implicated as targets of proliferative burst after PD-1 blockade need further study (37). Further studies are also needed to better characterize the proportion of tumor infiltrating T_{RM} cells that are truly tumor specific.

The concept that T_{RM} cells may be major targets of ICP blockade therapies is consistent with emerging insights into their functional properties. T_{RM} cells seem to provide a dual role that encompasses both protection and regulation. Thus, while human T_{RM} cells in NLTs can produce higher levels of effector cytokines, such as IFNγ, IL2, and TNF, they also produce higher levels of immune regulatory cytokines such as IL10 (14, 15). Moreover, T_{RM} cells also express higher levels of ICPs, such as CTLA4, PD-1, TIM-3, and LAG-3 (14, 25). T_{RM} cells also seem to have a quiescent phenotype, which may be essential for their ability to survive long-term in tissues, being poised for activation but not harming tissues (17). Antibody-mediated blockade of ICPs such as PD-1, therefore, provides a mechanism for activation of these T cells *in situ*. The precise nature of the activation signal may differ between CTLA4 and PD-1 blockade (or combination thereof) (38).

The concept that T_{RM} cells within tumors may be major targets of ICP blockade has several implications for immune therapies. Vaccines that foster the generation of T_{RM} cells may be best suited for combination with ICP blockade (39). The ability of T_{RM} cells to mediate long-term residence in tissues may help to explain why clinical responses to ICP blockade have been durable. Along these lines, strategies that help to maintain or even enrich these T_{RM} pools may allow enhanced durability of responses. It would also be important to better understand the nature of antigenic targets on tumors recognized by these T cells, and the impact of tumor genetics as well as other cells in the tumor microenvironment on the functional properties and retention of these cells.

DO T_{RM} CELLS CONTRIBUTE TO INTRA-TUMOR HETEROGENEITY OF TUMORS?

Advances in cancer genomics and particularly the capacity to sequence multiple lesions in the same patient or even different parts of the same tumor have demonstrated a complex and heterogeneous landscape with varying sub-clonal architecture; studies have also suggested a potential impact of such intra-tumoral heterogeneity on clinical outcome (40, 41). However, the degree to which the genetics of the microenvironment contributes to intra-tumoral heterogeneity is less clear. Diversity within the immune microenvironment may in principle not only impact the mechanisms underlying response or resistance to immune therapies but also evolution of tumors in individual metastases. Advances in TCR sequencing provide an opportunity to gain some insights into the nature and genetics of T cells infiltrating tumor lesions. While the same antigenic epitope may in principle be recognized by different TCRs, they are likely to differ in terms of their affinity or functional properties.

In the setting of advanced or metastatic cancer, tumor cells grow as discrete lesions in diverse NLTs. These lesions by definition share the systemic circulation of the host and could in principle be likened to the situation in parabiotic mice that share systemic circulation. As discussed earlier, a characteristic feature of T_{RM} cells is tissue residence without recirculation, revealed by lack of equilibration in parabiotic mice. We hypothesized that if T_{RM} cells within individual tissues (e.g., lung or liver or skin lesions) indeed remain local, then dominant TCRs within individual metastatic lesions in the same patient would not equilibrate even if the oncogenic mutations or neoantigen-load were largely shared between these lesions (**Figure 1**). Concurrent sequencing of tumor cells as well as TCRs from individual lesions in patients with advanced melanoma supported this hypothesis; as expected, the inter-lesional diversity of TCRs was mostly accounted for by TCRs from T_{RM} subset of TILs (25). Differences in dominant TCRs between individual lesions from the same patient is consistent with lack of equilibration of TCRs between individual metastatic lesions even though they may share a major component of neoantigen load. However, the mechanisms that limit this equilibration need to be better defined; our current hypothesis is that it may relate to the lack of recirculation of tissue-resident TCRs, or their relative tissue retention, both consistent with T_{RM} biology.

The concept that T_{RM} cells infiltrating tumor tissues may exhibit local residence and little recirculation has several implications for immune therapies, immune monitoring, and cancer biology. If the individual metastatic lesions are established early, and carry different TCRs, then the level of immune pressure in individual lesions may differ and provide a pathway for divergent

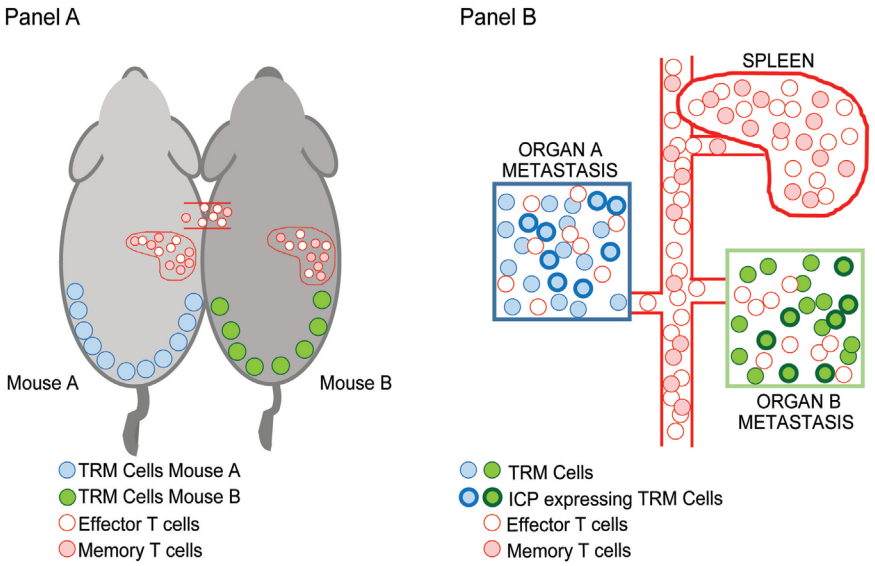

FIGURE 1 | Inter-lesional heterogeneity in metastatic cancer and biology of tissue-resident memory (T_{RM}) cells. T_{RM} T cells were identified in mice based on their restriction to non-lymphoid tissues and lack of recirculation. This was demonstrated using parabiotic mice **(A)** that share the same systemic circulation. Figure shows that T_{RM} cells in the skin (blue/green) do not equilibrate between mice, while other effector/memory T cells (pink/red) do. In the setting of advanced cancer in humans **(B)**, individual metastatic lesions can be observed in diverse tissues that share systemic circulation analogous to the parabiotic mice. Sequencing of T cell receptors (TCRs) in individual lesions from the same patient demonstrated that dominant TCRs in each of the lesions were non-overlapping and that the inter-lesional heterogeneity of TCRs exceeded differences in neoantigens. Importantly, T_{RM} cells were the major contributors to this heterogeneity suggesting that they do not equilibrate between lesions as in parabiotic mice in Ref. (25). A subset of T_{RM} cells that infiltrate these tumors express inhibitory immune checkpoints such as PD1 (shown by bolded outlines).

genomic evolution (42). Along the same lines, it may be important to carefully consider the specific site of tissue biopsy when evaluating the results of immune monitoring. It should be noted, however, that the impact of ICP blockade on T_{RM} homeostasis and redistribution *in vivo* in humans remains understudied and may add additional layers of complexity. Studies harvesting TILs for adoptive transfer are now entering the clinic in diverse cancers. If the dominant TCRs differ between individual lesions, it may be desirable to harvest and pool T cells from more than one lesion to optimize efficacy of such cell therapies. Finally, if the T cells in individual lesions differ, then it raises the potential that multiple mechanisms of immune resistance may be simultaneously operative in the same patient (43); along these lines, isolated progression at a single site in the face of continued regression at other sites may not reflect systemic loss of tumor control in the context of immune therapies. Clinicians have already come to appreciate this difference between immune therapies as compared to chemotherapies and often utilize localized therapies to tackle such lesions.

SUMMARY

In summary, T_{RM} cells within tumor lesions are likely to gain increasing importance as targets of immune therapies as well as deeper understanding of cancer biology and evolution. It is likely that optimal integration of these immune therapies will require attention to the unique biology of these immune cells and exploit their regional nature of enhance tumor immunity with reduced systemic toxicity.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Tumor Resident Memory T Cells: New Players in Immune Surveillance and Therapy

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Tissue resident memory T cells (Trm) are a subset of memory T cells mainly described in inflammation and infection settings. Their location in peripheral tissues, such as lungs, gut, or skin, makes them the earliest T cell population to respond upon antigen recognition or under inflammatory conditions. The study of Trm cells in the field of cancer, and particularly in cancer immunotherapy, has recently gained considerable momentum. Different reports have shown that the vaccination route is critical to promote Trm generation in preclinical cancer models. Cancer vaccines administered directly at the mucosa, frequently result in enhanced Trm formation in mucosal cancers compared to vaccinations via intramuscular or subcutaneous routes. Moreover, the intratumoral presence of T cells expressing the integrin CD103 has been reported to strongly correlate with a favorable prognosis for cancer patients. In spite of recent progress, the full spectrum of Trm anti-tumoral functions still needs to be fully established, particularly in cancer patients, in different clinical contexts. In this mini-review we focus on the recent vaccination strategies aimed at generating Trm cells, as well as evidence supporting their association with patient survival in different cancer types. We believe that collectively, this information provides a strong rationale to target Trm for cancer immunotherapy.

Keywords: tissue resident memory, vaccination, mucosal route, CD103, cancer prognosis, immunotherapy

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INTRODUCTION

Tumor-infiltrating lymphocytes (TILs) frequently remain tolerant or display an exhausted phenotype favored by the tumor microenvironment (1–3). Thus, two of the main challenges of current immunotherapy against cancer are generating specific T cells that may effectively target tumor cells and ensuring the induction of long-term anti-tumor protective immune responses. Therapeutic strategies to promote the development of immunological memory have for the most part focused on circulating memory T cells, such as central memory (Tcm) or effector memory (Tem) but have failed so far to consider resident memory cells (Trm).

Trm cells are a long-lasting population frequently characterized by the expression of CD103, CD69, and CD49a surface markers and by the absence of the lymph node homing receptors CD62L and CCR7. The differentiation toward a residency memory program is known to be regulated by TGFβ and IL-15 cytokines, which promote the expression of the transcription factors Hobbit and Blimp1. The upregulation of these molecules induces the silencing of other transcription factors such as KLF2 and TCF1 and proteins involved in tissue egress such as S1PR1 (4). Trm cells are mainly localized in peripheral lymphoid and non-lymphoid tissues such as lung, skin, gastrointestinal and genitourinary tracts (5). Their permanence in these tissues is

mainly mediated by the expression of the integrins CD103 and CD49a that bind E-cadherin and collagen respectively. The homing properties of Trm cells can vary depending on the tissue and the chemokine receptor expression patterns. The presence of CCR5 and CXCR3, for instance, is essential for the recruitment of CD8+ Trm cells to the lungs in cancer and infection (6, 7).

Trm cells have been broadly studied in infection and inflammation settings (8), where they are considered as the first immune population to become activated even in the absence of *in situ* antigen recognition (9). Their role in immune surveillance of cancer remains unclear, although there is an increase in the number of publications associating the presence of Trm cells in tumor areas with a favorable outcome. Of note, the importance of this subset of lymphocytes has been described in anti-tumor immunity of skin and mucosal tumors (10, 11). Hence, the development of immunotherapeutic strategies favoring Trm cell generation and function could be critical not only in controlling tumor growth but also, and foremost, in preventing tumor recurrences.

In this mini-review we will cover two main aspects of Trm cells in cancer: the importance of the vaccination route to promote Trm cells against tumor antigens and the evidence substantiating an association of their occurrence to patient survival.

VACCINATION ROUTES THAT PROMOTE TRM CELLS IN CANCER

The precise procedures for the generation *ad libitum* of tissue resident memory remain still unknown. A recent study found the existence of a common clonal origin for central and resident memory T cells upon immunizing the skin of mice and humans with different components (protein antigen, hapten and non-replicative virus) (12).

Crosspriming dendritic cells seem to be critical for the generation of resident memory in the tissues. In a preclinical infection model, the priming strength and the durability of antigen presentation were reported to play an important role in Trm generation, in an IL-12, IL-15, and CD24 dependent manner (13). Moreover, the absence of crosspriming dendritic cells results in a preferential reduction of Trm cells over circulating memory T cells in both infection and cancer settings (13, 14).

The imprinting of homing properties of tissue resident T cells is also dependent on the priming event. It has been shown that dendritic cells (DCs) can induce different arrays of chemokine receptors and adhesion molecules, such as integrins or selectin-ligands, in CD8 T cells depending on the sites where they uptake the antigens (15). In a glioblastoma preclinical model, the injection of tumor cells by distinct routes (intraperitoneal, intracranial and subcutaneous) was shown to promote different patterns of integrins on specific T lymphocytes isolated from the respective tumor-draining lymph nodes (15). These results suggest a mechanistic explanation of the impact of the immunization route in the generation of Trm cells (16–23).

Despite the fact that many cancers develop at mucosal sites such as the lungs, oral and genitovaginal cavities or the gastrointestinal tube, most of the preclinical cancer vaccines have been administered subcutaneously or intramuscularly, thus without specific targeting of mucosal sites. This may be relevant in advanced diseases with multiple visceral and cutaneous metastases. Indeed, subcutaneous immunization with DCs pulsed with a human melanoma peptide was found to be sufficient to reject subcutaneous melanomas but not lung metastases. However, systemic intravenous immunization prevented lung metastasis in a mechanism involving CD8 T cells that were retained in the spleen (24).

Recent studies carried out by different groups have assessed vaccination at mucosal sites. By using an orthotopic head and neck tumor model expressing the E7 antigen from HPV (TC-1 cell line), it was demonstrated that intranasal vaccination prevents the tumor growth in the oral cavity and in the lungs. Such an effect was not observed with vaccination by the intramuscular route (25). This anti-tumor outcome was mainly dependent on the presence of E7-tetramer positive CD8 T cells expressing mucosal integrins (CD49a and CD103) that were not only found in tumors but also in mediastinal and cervical lymph nodes (25). In a model of cervicovaginal cancer, the generation of CD8 T cells with a resident phenotype was promoted upon intravaginal viral vector-based vaccination, which also boosted circulating tumor-specific T cells. T cells expressing CD103 and CD69 were shown to produce high levels of IFNγ and TNFα at the tumor site (16, 26). In line with this, the combination of intravaginal HPV-based vaccination administered upon intramuscular E7-expressing DNA vaccine, enhanced tumor-specific CD8 T cells in the mucosa of an HPV-cervicovaginal cancer model. The α4β7 integrin expressed by CD8 T lymphocytes was found to be the main integrin responsible for the migration of these cells to the genital mucosa. Clear evidence shows that DCs present at the mucosal site induce the upregulation of α4β7 integrin on CD8 T cells, favoring homing to the tumor (27). By injecting the same tumor cell line in the bladder, another group showed promising anti-tumor effects in a therapeutic intravaginal vaccine. Although in this model the subcutaneous route showed a better outcome, it was proven that the tumor growth control of intravaginal route was due to presence of tumor-specific CD8 T cells in the bladder mucosa. The fact that these CD8 T cells are detectable at later time points upon vaccination indicates that intravaginal vaccination can give rise to the generation of resident memory T cells (28).

However, intravaginal vaccination was not able to prevent growth of tumors in a transplantable vaginal cancer generated by intravaginal instillation of TC-1 cells expressing the E7 antigen (29). Control of the tumor growth was achieved with subcutaneous and intranasal routes in detriment of intravaginal vaccine administration. The clear discrepancy between these results and the previously mentioned related to the intravaginal vaccination route in cervicovaginal cancer might be explained by the type of vaccines employed in each case. In this study mice were injected with a vaccine based on the E7-peptide combined with different adjuvants, while previous studies focused on viral based-vector vaccines containing E7 antigen. Furthermore, the targeting of different subsets of DCs should be taken into account. Given the fact that crosspriming

dendritic cells are necessary for the generation of Trm cells, a vaccine that potentially targets these specific DCs may induce more Trm and thus, a better outcome in cancer patients.

In fact, the idea of targeting DCs to promote the induction of CD103 on primed CD8 T cells was further explored in a humanized breast cancer model. In this study, DCs that were reprogrammed via dectin-1 favored the generation of a resident phenotype on CD8 T cells in a TGFβ-dependent manner (30). It was also shown that TLR agonists such as poly(I:C; TLR3) or CpG-ODN (TLR9) induced systemic but not resident memory T cells. In contrast, in a genital tumor model, the intravaginal injection of TLR agonists in combination with an E7 peptide-based vaccine, was reported to promote the recruitment of E7-specific T cells into the tumor. Although the increased recruitment of T cells was proposed to be responsible for tumor regression, the generation of Trm cells by this vaccine was not clearly addressed in this study (31).

Even though Trm cells are broadly present in the skin, their generation in this tissue has not been deeply studied in a skin cancer setting. Up to now, two recent reports describe how a prophylactic vaccination through skin scarification enhanced Trm generation in skin, preventing subcutaneous, and intradermal-injected tumor growth (13, 32).

CD103+ EXPRESSION ON TILS IS ASSOCIATED WITH PATIENT SURVIVAL

The integrin αE(CD103)β7 selective for E-cadherin identifies tumor antigen-reactive TILs with more potent effector functions than the CD103 negative TIL subset. Indeed, CD103+ TILs displayed enhanced killing capacity (33) and CD103+ T cells infiltrating glioma had an improved ability to produce granzyme B (34). Upon binding to E-cadherin, CD103+ T cells undergo cytotoxic granule polarization, and degranulation concomitantly to TCR engagement (33, 35). Moreover, the expression of CD103 has been associated with patient survival in diverse cancer types: melanoma (36), non-small cell lung carcinoma (NSCLC) (37, 38), bladder cancer (39), endometrial cancer (EC) (40), breast cancer (41), cervical cancer (42) and high-grade serous ovarian cancer (HGSC) (43–46) (**Table 1**).

At the antigen specific T cell level, a recent study from a phase I clinical trial enrolling melanoma patients vaccinated subcutaneously with a melanoma antigen (Melan-A), analyzed the homing receptors characterizing Melan-A-specific CD8 T cells. The presence of circulating melanoma-specific T cells harboring P-Selectin binding and Very late antigen (VLA-1) correlated with improved patient survival. Moreover, VLA-1+ CD8 T cells were strongly enriched in melanoma metastases (lung, skin and brain) and displayed a Trm phenotype expressing the CD103 and CD69 surface markers (47). Another recent report also focused on melanoma patients, naïve of any treatment or undergoing αPD-1 therapy. They demonstrated an improved survival in 50% of patients with a high number of CD103+ TILs cell compared to 20% in those with lower numbers (36).

A correlation between a high CD103+ TIL density and patient survival was also shown in early stage NSCLC patients. CD103 + CD8 TILs from these tumor biopsies displayed Trm phenotype and expressed high levels of the inhibitory receptors PD-1 and TIM-3 (37). Along the same lines, in NSCLC patients, it was revealed that highly infiltrated tumors ($TILs^{high}$) were enriched for Trm cells and the density of CD103+ CD8 TILs was associated with a favorable outcome (38).

In bladder urothelial cell carcinoma, the density of CD103+ TILs correlated with survival and was inversely linked to the tumor volume (39). Similarly, patients diagnosed with endometrial cancer or cervical cancer had improved survival when a high infiltration of CD103+ TILs was detected (40, 42).

In breast cancer, especially in the basal-like tumor subtype, the patients had improved survival when the tumor was enriched with CD103+ CD8 TILs (41).

The association between CD103+ cells and survival was also established by several studies in high-grade serous ovarian cancer (HGSC) (43–46). It was shown that CD103+ TIL infiltration (46) and the presence of CD103+ or CD3+ intraepithelial lymphocytes (43) correlated with better survival in HGSC patients. Moreover, the stratification of patients according to CD103 or CD3 counts in the tumor, highlighted striking differences according to overall survival: the $CD3^{high}CD103^{high}$ group had a 5-years survival rate at 90%, the $CD3^{low}CD103^{high}$ at 63% and the $CD3^{low}CD103^{low}$ at 0%, thus demonstrating the crucial presence of TILs and the potential power of the CD103 marker to predict patient outcome (43).

CD103+ TIL detection is associated with an improved survival in various mucosal tumor models. However, a study performed with colorectal cancer (CRC) patients did not find any difference in survival comparing intraepithelial CD103+ cell density in the whole cohort. In addition, analysis of high CD103+ density in KRAS WT CRC patients, a subgroup of the cohort, defined a group with unfavorable outcome (48).

Taken together, the presence of high levels of CD103+ TILs is associated with improved patient survival in the majority of the cancer types described here, albeit with the possible exception CRC. This prominent exception remains to be confirmed, including the distinction of CRC subtype. Moreover, the possible mechanistic basis for this unique disconnect may offer further insights into the role of Trm cells in different tumor microenvironments. Of note, Tregs may also express CD103 (49), thus calling for a complete subset analysis of TILs. In this regard, novel high content immunohistochemical technologies may help in providing high resolution TIL analyses.

TRM CELLS AS TARGETS FOR CANCER IMMUNOTHERAPY

CD8+ CD103+ TILs have been shown to express high levels of PD-1 in various cancers: melanoma (36), lung cancer (37, 38), endometrial adenocarcinoma (40) and HGSC (44, 46). CD8+ CD103+ PD-1+ TILs displayed potent cytokine

TABLE 1 | Association of patient survival with CD103+ expression on tumor infiltrating lymphocytes (TILs) in different cancer types.

Tumor	CD103+ TILs correlate with survival	Treatment	Trm cells phenotype	Cancer grade	No. of patients	References
Glioma	N.A.	(–)	CD103+, Granzyme B+	(–)	6–7	(34)
Melanoma	N.A.	Vaccination	CD103+, CD69+, VLA-1+	III/IV	18	(47)
	Yes	+/–αPD-1	CD103+, CD69+, PD-1^{high}, Granzyme B+, KLRG1^{low}	III	44	(36)
NSCLC	Yes	Surgery	CD103+, CD69+, PD-1^{high}	Early stage	101	(37)
	Yes	(–)	CD103+, CD69+, CD49a+	Early stage	36 and 689	(38)
Bladder	Yes	Surgery	CD103+	Ta-T4	302	(39)
Endometrial	Yes	Surgery	CD103+, PD-1+	FIGO I-IV	305	(40)
Cervix	Yes	Surgery and/or radio(chemo)	CD103+	FIGO IA2-IVA	304	(42)
Breast	Yes	Surgery and radiation or chemotherapy	CD103+	FIGO I-III	424	(41)
HGSC	Yes	Surgery and chemotherapy	CD103+, PD-1+	FIGO I-III	210	(46)
	Yes	Surgery and chemotherapy	CD103+	FIGO II-III	135	(43)
	Yes	Surgery and chemotherapy	CD103+, PD-1+	FIGO IIb	186	(44)
	Yes	Surgery and chemotherapy	CD103+	FIGO I-IV	135	(45)
Colorectal	No	Surgery	CD103+	T1-T4	239	(48)

N.A., not analyzed; (–), not known.

production (IFNγ and TNFα) after PMA/ionomycin stimulation, thus representing an interesting target for immunotherapy (50). Indeed, it was demonstrated that αPD-1 treatment led to CD103+ TIL expansion in the majority of melanoma patients showing improved survival, confirming that the use of checkpoint blockade may effectively boost this T cell population leading to favorable patient outcomes (36).

Interestingly, the expression of other inhibitory receptors varies in CD8+ CD103+ TILs within different cancer types. For instance, in melanoma CD103+ CD69+ TILs were PD-1+ and LAG-3+ but negative for CTLA-4 (36); in NSCLC CD103+ TILs expressed PD-1 and TIM-3 but with negligible CTLA-4 expression (37) and in HGSC CD103+ TILs did not express TIM-3, CTLA-4 or LAG-3 (50). The different expression profiles of inhibitory receptors on CD103+ TILs warrant further detailed characterization that may serve to guide the selection of the specific immune checkpoint blockade administration, such as monotherapies or combinations.

It was demonstrated that human Influenza-specific Trm cells possess strong proliferative potential after CFSE labeling (51). In addition, secondary effector cells derived from CD8+ CD103+ CD69+ Trm cells displayed enhanced polyfunctionality, since IFNγ, TNFα, and granzyme B production was improved compared to effectors differentiated from CD103+ CD69- or CD103-CD69+ subsets (51). Therefore, it would be of great interest to sort, re-expand, and adoptively transfer (ACT) this T cell population in patients. However, when ACT of Trm cells was attempted in a mouse model, it was met with poor success (52). This failure may be explained in part by poor homing capacities of Trm cells. An approach to counteract the homing intrinsic limitations of Trm cells could be the transfer of this population directly at the required mucosal site. On the other hand, the use of ACT of Tcm cells in mice efficiently led to the generation of a Trm cell population after infection or tumor challenge (13). Thus, the potential of ACT with Tcm cells due to their plasticity could be exploited to favor Trm cell differentiation.

CONCLUDING REMARKS

The growing interest in CD8+ Trm cells is illustrated in the number of recent studies aimed at understanding the optimal way to promote their formation in preclinical cancer models. According to reports detailed above, the vaccination route is crucial to boost Trm cell formation at the required site. In several models, the intramucosal vaccination route demonstrated an enhanced potential to generate Trm cells, compared to the conventional intramuscular or subcutaneous routes.

It has also been demonstrated that CD8+ Trm cells may be key players in successful cancer immunotherapy, since their presence in tumor areas is frequently associated with better survival in the majority of cancer types. Due to their localization, mostly at mucosal sites, and surface expression of several inhibitory receptors, such as PD-1, CD103+ TILs represent an appealing target for immunotherapy. Nevertheless, it remains unclear whether the adoptive transfer of these cells would succeed due to their poor homing capacities. However, the enhancement of Trm proliferation and function seems to be critical in combating mucosal

cancers where Trm are prevalent. Promising results in cancer vaccination indicate that this approach may be the most productive way to target Trm cells in the clinic, and their performance in combination with immune checkpoint blockade, or other immunotherapy modalities, awaits evaluation in clinical trials.

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Resident Memory-Like Tumor-Infiltrating Lymphocytes (TIL_{RM}): Latest Players in the Immuno-Oncology Repertoire

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Resident memory T cells (T_{RM}) are a recently identified subset of long-lived memory T cells that are characterized in terms of their unique surface phenotype combined with a non-recirculating pattern of localization to non-lymphoid, peripheral tissues. T_{RM} have quickly become a key area of focus in understanding immune responses to microbial infection in so-called "barrier" tissues, and appear to be particularly critical for protection against repeat exposure at the same site. More recently, tumor-infiltrating T cells with canonical T_{RM} features are being identified in human cancers, in particular cancers of epithelial origin, and their presence is broadly found to be associated with favorable long-term prognosis. Moreover, recent studies have shown that these "resident memory-like" tumor-infiltrating lymphocytes (referred to herein as TIL_{RM}) are uniquely activated in melanoma patients undergoing PD-1 directed checkpoint blockade therapy. Accordingly, there is much interest at present regarding the biology of these cells and their precise role in anti-cancer immunity. Herein, we review the current state of the literature regarding TIL_{RM} with a specific emphasis on their specificity, origins, and relationship to conventional pathogen-specific T_{RM} and speculate upon the way(s) in which they might contribute to improved prognosis for cancer patients. We discuss the growing body of evidence that suggests TIL_{RM} may represent a population of bona-fide tumor-reactive T cells and the attractive possibility of leveraging this cell population for future immunotherapy.

Keywords: resident memory T cells, CD8, CD103, tumor-infiltrating lymphocytes, prognosis

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BRIEF INTRODUCTION TO RESIDENT MEMORY T CELLS (T_{RM}) AND THE T_{RM}-DEFINING SURFACE MARKER CD103

In recent years, there has been growing recognition of the importance of a peripheral, non-recirculating component of the immune system known as T_{RM} [for review see Ref. (1–3)]. T_{RM} have historically been defined by their peripheral tissue localization and lack of circulatory activity. More recently, there is increasing understanding of the unique surface phenotype(s) of T_{RM} and how the specific molecules that comprise this phenotype contribute to their (non-)circulatory nature. Although this phenotype can vary somewhat between tissues, disease states, and CD4 versus CD8 subsets, most T_{RM} in skin, lung, and GI tract typically express CD69, a molecule widely considered to be an indicator of recent activation, but which is also involved in downregulation of the receptor

for sphingosine 1 phosphate (S1P1), thereby inhibiting the ability of T_{RM} to traffic out from peripheral tissue in response to S1P1 gradients (4). Likewise, T_{RM} frequently lack surface expression of CCR7, preventing them from trafficking in response to gradients of CCL19 and CCL21 (5). In addition, surface expression of CD103 (the α_E component of the α_E/β_7 integrin molecule) (6, 7) is now widely considered to be a canonical marker of T_{RM}, and although T_{RM} populations can be comprised of variable proportions of both CD4 and CD8 cells, CD103 appears to be uniquely overexpressed by CD8 T_{RM} (8). CD103 expression is also biologically relevant to the non-recirculating phenotype of T_{RM}, as the ligand for α_E(CD103)/β_7 integrin is E-cadherin expressed on epithelial cells (9). Although chemokines are thought to be the initial mediator of T cell recruitment into peripheral sites of inflammation, adhesive interactions between α_E(CD103)/β_7 and E-cadherin is thought to be responsible for the long-term "retention" of antigen-specific T_{RM} at relevant sites (10, 11). This phenomenon is particularly well studied in the context of mucosal tissue infection, where the long-term retention of T_{RM} at the site of an initial infection is thought to provide durable and rapid protection against repeat attack by the same organism. Indeed, once T_{RM} populations are established, they can be retained at the original site of infection for months or even years, even in the complete absence of relevant antigen (12–14). This T_{RM} phenomenon can also be exploited by vaccination strategies that involve delivery of vaccine to the relevant mucosal tissue (15). Indeed, the historical field of "mucosal" immunity and the newer field of "T_{RM}-mediated" immunity are rapidly merging in terms of the memory T cell components.

In addition to mediating adhesion and T_{RM} formation, both α_E(CD103)/β_7 and E-cadherin are also capable of intracellular signaling. For example, the intracellular domain of E-cadherin interacts with β-catenin which in turn interacts with the actin cytoskeleton, affecting cell shape and motility (16). Likewise, cross-linking of surface-expressed α_E(CD103)/β_7 impacts the shape and motility of lymphocytes (17), enhances T cell proliferation and induces lysis of target cells (18). Thus through the combination of "inside-out" and "outside-in" signals, α_E(CD103)/β_7 has the potential to profoundly impact T_{RM} effector function, in addition to augmenting peripheral memory formation.

MECHANISM OF CD103 UPREGULATION ON T_{RM}

TGF-β has long been known to play a key role in the regulation of α_E(CD103)/β_7 surface expression on T lymphocytes (19, 20). Although TGF-β is often considered solely as an immunosuppressive factor, it is, in reality, a highly pleiotropic cytokine that is expressed in a multitude of (primarily peripheral) tissue types and has biological activities that are context specific (21). Interestingly, although TGF-β is required for upregulation of α_E(CD103)/β_7 surface expression, TGF-β exposure alone is not sufficient (18, 22). Rather, it is the combination of TGF-β plus concurrent signaling through the TCR that results in dramatic and rapid α_E(CD103)/β_7 expression. Indeed, the combination of these two signals makes perfect sense biologically as it would allow for large numbers of lymphocytes (with diverse specificities) to transiently traffic through TGF-β-rich sites of peripheral infection, but result in the α_E(CD103)/β_7-mediated retention of only those T cells with relevant specificity. This model of T_{RM} formation is supported by the finding that in CD103 knockout mice, numbers of T_{RM} are substantially reduced (10). Likewise, dysregulation of the SMAD signaling pathway downstream of the TGF-β-receptor results in reduced numbers of T_{RM} (23).

Although TGF-β-mediated upregulation of CD103 clearly plays an important role in the establishment of T_{RM}, it is certainly not the only mechanism. For example, it has also been reported that the formation of T_{RM} populations can be enhanced through signaling *via* the homeostatic cytokine, interleukin-15 (IL-15) (24, 25). However, dependency upon IL-15 for T_{RM} formation varies from tissue to tissue (26), implying that the requirement for IL-15 is not absolute and may be more complex than that of TGF-β. Moreover, as described above, $CD4^+$ T_{RM} populations, in general, express much lower levels of CD103 than do $CD8^+$ T_{RM}, thus they must maintain residency in a CD103-independent manner (27, 28).

T_{RM} IN THE CANCER SETTING

In recent years, there has been growing appreciation that T_{RM} biology/immunology is not unique to the infectious disease setting. Indeed, it has long been speculated that T_{RM} play a key role in both allograft rejection and autoimmunity. For example, α_E(CD103)/β_7 is expressed on the majority of tissue-infiltrating $CD8^+$ T cells during transplant rejection (20, 29, 30) and graft versus host disease (22). In CD103-deficient mice, T cells are not able to infiltrate allogeneic islet cell transplants and allografts persist for long periods *in vivo* (30, 31) often surrounded by a characteristic "halo" of CD103-deficient CD8 T cells. In the autoimmune disease setting, islet infiltrating cells in both human diabetic patients (32, 33) and mouse models of autoimmune diabetes (34) are enriched for α_E(CD103)/β_7-expressing T_{RM}. Presumably, in each of these settings T_{RM} are derived *via* the same TGF-β plus concurrent TCR signaling mechanism described above for infectious diseases.

α_E(CD103)/β_7-expressing tumor-infiltrating T cells (TIL) are also now turning up, with increasing regularity, in various cancer settings, particularly in cancers of epithelial origin. This should really not be surprising considering the relationship between TGF-β and α_E(CD103)/β_7 and the frequent expression of TGF-β in cancers of various types. TGF-β overexpression in cancer has been broadly considered as an immunosuppressive mechanism of tumor escape from immunological pressure (21, 35). However, an alternate hypothesis could be that TGF-β production by tumors is not so much an acquired trait as it is an amplification of the TGF-β that is expressed as part of the "normal" biology of epithelial tissues. Regardless of the mechanism, when tumor-reactive T cells enter these TGF-β-rich environments and then become activated through the TCR, there is full reason to assume they would upregulate α_E(CD103)/β_7 on the cell surface, in the same manner that conventional T_{RM} do.

However, as described above, CD103 expression is only one part the larger phenotypic profile that defines T_{RM}. Whether CD103-expressing TIL are phenotypically identical to conventional pathogen-specific T_{RM}, or whether they are simply closely

related cousins is an issue that remains to be determined. For example, the phenotypic features that are known to be shared among conventional T_{RM} populations, regardless of their specificity and/or tissue location, are reported to be driven by the T_{RM} master transcriptional regulators Blimp-1 and Hobit (36). However, the expression of Blimp-1 and Hobit in tumor-infiltrating T_{RM} is yet to be reported. By contrast, the transcription factor Runx3, which influences the downregulation of mRNA transcripts associated with cellular migration (S1pr1, Klf2, and Ccr7) appears to be expressed in both conventional and tumor-infiltrating T_{RM} (37). Moreover, conventional pathogen-specific T_{RM} are thought to be retained in peripheral tissue after resolution of infection, acting as a vanguard against future re-exposure. In this context, a large proportion of conventional T_{RM} are likely persisting in peripheral tissue in an antigen-free manner, until such time as they become re-challenged through re-exposure. By contrast, tumor-infiltrating T_{RM} (assuming they are tumor-specific) are resident within active tumor tissue and would thus be continuously exposed to antigen, which would likely result in a phenotype distinct from conventional "resting" T_{RM}. For these reasons and because the precise relationship between conventional T_{RM} and tumor-infiltrating T_{RM} is yet to be well-defined in the literature, in our laboratory we have adopted the term "TIL_{RM}" (resident memory-like TIL) to delineate these CD103-expressing tumor resident cells from conventional pathogen-specific T_{RM}.

Until recently, broader investigation into the global nature of TIL_{RM} infiltration in human tumors was severely hampered by the lack of an anti-human CD103 antibody that was suitable for IHC of formalin-fixed tissues. This situation changed in 2013 when a new antibody was, ironically, developed for diagnosis of hairy cell leukemia (38), a setting where CD103 is ectopically overexpressed. Since the introduction of this reagent, TIL_{RM} have now been reported to be present in at least eight different tumor settings including lung, breast, ovarian, endometrial, cervical, melanoma, colorectal, pancreatic, and bladder cancer (39–53) (see **Table 1**). In the majority of these reports, CD103 is used as a marker to delineate "intraepithelial" TIL, and more importantly, the presence of $CD103^+$ TIL is associated with favorable prognosis.

TABLE 1 | Summary of studies examining $CD103^+$ TIL_{RM} as a prognostic indicator in solid cancers.

Tumor histology	Summary	Reference
Bladder	A large proportion of TIL in the urothelium co-express $CD8^+$ $CD103^+$. Carcinoma stromal tissue was highly enriched for $CD8^+$ $CD103^+$ TIL but not associated with increased E-cadherin expression	Cresswell et al. (50)
Colorectal	Microsatellite instable tumors show increased infiltration of $CD8^+$ $CD103^+$ TIL compared to microsatellite stable tumors	Quinn et al. (47)
Colon	CD103 expression is enhanced by antigen recognition and TGF-β signaling. T cell activation in the presence of TGF-β induces CD103 expression	Ling et al. (49)
Ovarian	$CD103^+$ TIL were found to be abundant across all major ovarian cancer subtypes but highly enriched in high-grade serous cancer (HGSC), and their presence correlates with improved survival	Webb et al. (55)
Lung	$CD103^+$ TIL correlate with improved early stage patient survival in non-small cell lung cancer (NSCLC) and intraepithelial TIL density. $CD103^+$ TIL show enhanced effector function against autologous tumor	Djenidi et al. (39)
Ovarian	CD103 demarcates intraepithelial $CD8^+$ TIL which co-express PD-1 and appear quiescent in the tumor microenvironment	Webb et al. (41)
Breast	High abundance of $CD103^+$ TIL in ER negative (basal-like subtype) tumors within intraepithelial regions correlates with good prognosis	Wang et al. (40)
Melanoma	Interlesional TIL populations show an enriched gene signature indicative of a resident memory phenotype which is responsive to immune checkpoint blockade	Boddupalli et al. (48)
Endometrial	Abundance of $CD8^+$ $CD103^+$ TIL in endometrial tumor epithelium is a strong prognostic indicator in endometrial adenocarcnoma	Workel et al. (42)
Ovarian	$CD103^+$ TIL collected from HGSC co-express PD-1 and CD27. TIL activated in the presence of HGSC upregulate CD103	Komdeur et al. (43)
NSCLC and head and neck squamous cell cancer	Cytotoxic T lymphocytes have an enriched resident memory gene signature. $CD8^+$ $CD103^+$ TIL co-express checkpoint receptors such as PD-1 and CTLA-4. Higher density of resident memory T cells (T_{RM})-like TIL are associated with improved patient outcome	Ganesan et al. (46)
Cervical	CD103 gene expression is associated with effector T cell function. Abundance of intraepithelial $CD8^+$ $CD103^+$ TIL correlates with improved patient survival	Komdeur et al. (44)
Pancreatic	Increased ratio of $CD8^+$ $CD103^+$ TIL to $CD8^+$ $CD103^-$ TIL correlates with improved patient survival	Lohneis et al. (51)
Melanoma	Presence of $CD8^+$ $CD69^+$ $CD103^+$ TIL correlates with improved patient survival in melanoma. $CD103^+$ TIL show high levels of expression of the inhibitory markers PD-1 and LAG-3	Edwards et al. (45)
Lung	Single-cell RNA sequencing of lung TIL showed distinct pre-exhausted and exhausted TIL phenotypes. Tumor resident T cells expressed high levels of CD69 and CD103 overall	Guo et al. (52)
Breast	Single-cell RNA sequencing of breast TIL revealed high TIL abundance was characterized by a T_{RM}-like phenotype and associated with improved patient survival in triple negative breast cancer	Savas et al. (53)

TIL_{RM} CELLS IN THE GYNECOLOGIC CANCER SETTING

Our group first noted the presence of TIL_{RM} cells in the ovarian cancer (OvCa) setting during a flow cytometry-based survey of immune cells present in OvCa patient ascites (54). Interestingly, some but not all, ascites specimens contained CD103-expressing T cells, specifically within the CD8 subset and sometimes comprising as much as 80% of the cells in that compartment. The presence of these cells in a fluid-based tissue (ascites) initially seemed inconsistent with them being a T_{RM} population as T_{RM} are normally restricted to solid tissues. However, the ascites compartment in ovarian patients can contain large numbers of free-floating tumor cells plus abundant amounts of TGF-β. Thus it should not be surprising that tumor-specific T cells present in this fluid compartment could adopt a T_{RM} phenotype more typical of solid tissues. We have also found that these cells have a unique phenotype that includes upregulation of HLA-DR, Ki67, and PD-1, but a lack of CD69, CD137, or intracellular cytokines suggesting that they have been recently activated, but are not actively "engaging" with targets at the time of analysis. Although the cells were PD-1 positive (41), they lacked other markers of exhaustion and were capable of robust cytokine production after stimulation with PMA/ionomycin, *ex vivo*, suggesting that they were not terminally exhausted. These initial findings regarding CD103-expressing TIL_{RM} in OvCa were limited to flow cytometric analysis of small numbers of ascites specimens. However, once an IHC-suitable antibody was available, we followed up by analyzing larger cohorts of patients using tissue microarray technology and showed that CD103-expressing TIL_{RM} cells were also present in the solid tumors of some, but not all, OvCa patients (55). Moreover, we also demonstrated that infiltration of tumors by TIL_{RM} correlated strongly with a favorable 5-year disease-specific survival advantage in high-grade serous cancer (HGSC), the most lethal of OvCas (55, 56). This finding has now been replicated in three additional cohorts of OvCa patients (43, 57, 58) as well as in endometrial (42) and cervical cancers (44). Clearly, TIL_{RM} cells are playing an important role in the gynecologic tumor setting, as they are in other epithelial tumor settings.

EVIDENCE IN SUPPORT OF TIL_{RM} CELLS BEING "TUMOR-SPECIFIC"

Based upon their significant prognostic benefit and unique surface phenotype, we and others speculate that TIL_{RM} in OvCa as well as other cancers are highly likely to be tumor-specific (56). Unfortunately, at present there is a paucity of well-characterized tumor antigens in the HGSC setting to directly test this hypothesis. Nonetheless, our group has previously characterized the cellular immune response to the cancer/testis tumor antigen (NY-ESO-1) in a small cohort of HGSC patients (59) by IFN-γ ELISPOT. The specificity of one such patient was mapped to a well-known HLA-A2-restricted epitope (NY-ESO-$1_{157–165}$) for which MHC tetramer reagents are available. Combining tetramer staining with CD103 staining revealed that NY-ESO-1-specific $CD8^+$ cells in this tumor sample were indeed $CD103^+$ (54), confirming that tumor-specific cells fell within the TIL_{RM} compartment in this patient. However, the NY-ESO-1-specific cells in this sample comprised only a tiny proportion of the entire TIL_{RM} population, which had otherwise unknown specificity.

Similar results regarding tumor specificity of TIL_{RM} have been obtained in other cancer settings. One of the first studies to demonstrate tumor specificity of TIL_{RM} was in the non-small cell lung cancer setting wherein the authors found that $CD8^+CD103^+$ TIL selectively upregulated CD107a and granzyme B in the presence of autologous tumor cells and also specifically lysed autologous tumor cells when co-cultured in the presence of an anti-PD-1 blocking antibody (39). More recently, TIL_{RM} populations have been identified in the melanoma setting and have been shown to contain cells that stain with melan A-specific tetramers (45), again confirming the presence of tumor-specific T cells in the TIL_{RM} subset. Likewise, TIL_{RM} have been demonstrated to play a role in anti-tumor immunity in various murine tumor models. For example, using a murine model of melanoma it was reported that CD103 was required for establishment of gp100-specific TIL_{RM} populations at the tumor site (60). Interestingly, in this model gp100-specific TIL_{RM} cells even remained at the site after tumor resolution and provided long-term immunity against rechallenge, but also caused permanent vitiligo in the dermis. On a somewhat related note $CD103^+$ TRM have also recently been reported to be abundant in human vitiligo specimens (61).

Despite the abundance of evidence supporting the likely tumor specificity of TIL_{RM}, one should also consider the alternate hypothesis, that because many of these epithelial tumor types originate from a tissue that could be directly or indirectly considered a mucosal barrier tissue, the TIL_{RM} populations could actually be conventional pathogen-specific T_{RM} "bystander" populations that have been amplified during tumor outgrowth. Indeed, this possibility has been raised in a very recent study designed to characterize the phenotypes of authentic tumor-specific TIL versus bystander virus-specific TIL present in human colorectal and lung tumors (62). Interestingly, in this study both the tumor-specific and bystander T cells were found to express features of T_{RM}, including CD103, whereas CD39 was found to be a more reliable marker for distinguishing between the two. Although this study does not contradict earlier findings demonstrating CD103 expression by tumor-reactive TIL, if correct, it suggests that TIL_{RM} populations may actually be more heterogeneous than previously thought. Indeed this might be particularly relevant in the gynecologic cancer setting as HSV-2 reactive T cells with a typical T_{RM} phenotype have been reported to be present in the cervical tissue of women with known HSV-2 infection (63) and the numbers of typical T_{RM} in the fallopian tube are reported to increase with age (64). Perhaps these pathogen-specific TIL_{RM} populations in previously healthy gynecologic barrier tissues simply "come along for the ride" once the tissue becomes cancerous, and perhaps even co-exist with nascent tumor-specific TIL_{RM} populations. Clearly, it remains a challenge to the field to more precisely define the specificity of TIL_{RM}.

THE "PARADOX" OF THE PROGNOSTIC EFFECT OF TIL_{RM}

As described above, the significant prognostic benefit conferred by TIL_{RM} in HGSC and other cancers implies that they are likely

to be tumor-specific or at least encompass tumor-specific populations. However, at the same time, this interpretation is somewhat paradoxical as these cells are present in tumor specimens that have been obtained from patients who have required clinical intervention (in the form of surgical de-bulking in the case of HGSC). This scenario suggests that if TIL_{RM} are indeed tumor-specific, they have ultimately lost the ability to control growth of the primary tumor. In recent years, it has become readily apparent that this paradox can be explained, at least in part, by various mechanisms of immune suppression and/or immune exhaustion. Indeed, the tumor microenvironment in OvCa, much like other cancers, has long been considered to be highly immunosuppressive due to the presence of soluble immune-inhibitory factors including IL-10, TGF-β, IDO, and PGE-2 (65). Likewise, the master immune-inhibitory switch molecule CTLA-4 has also been shown to be upregulated in the OvCa setting (66). In addition, inhibitory cells such as $CD4^+$ $Foxp3^+$ regulatory T cells (67), immunosuppressive $B7\text{-}H4^+$ tumor-associated macrophages (68), and myeloid-derived suppressor cells (69) have all been reported to be present in OvCa. More recently, the PD-1 immune checkpoint pathway has also been found to play a potential role in OvCa (70), as it has in many other cancer settings.

As mentioned above, our group has recently made the observation that the $CD103^+$ TIL_{RM} in HGSC tumors (and ascites) are almost universally positive for PD-1 surface expression (41). By contrast, PD-1 surface expression does not seem to be a universal

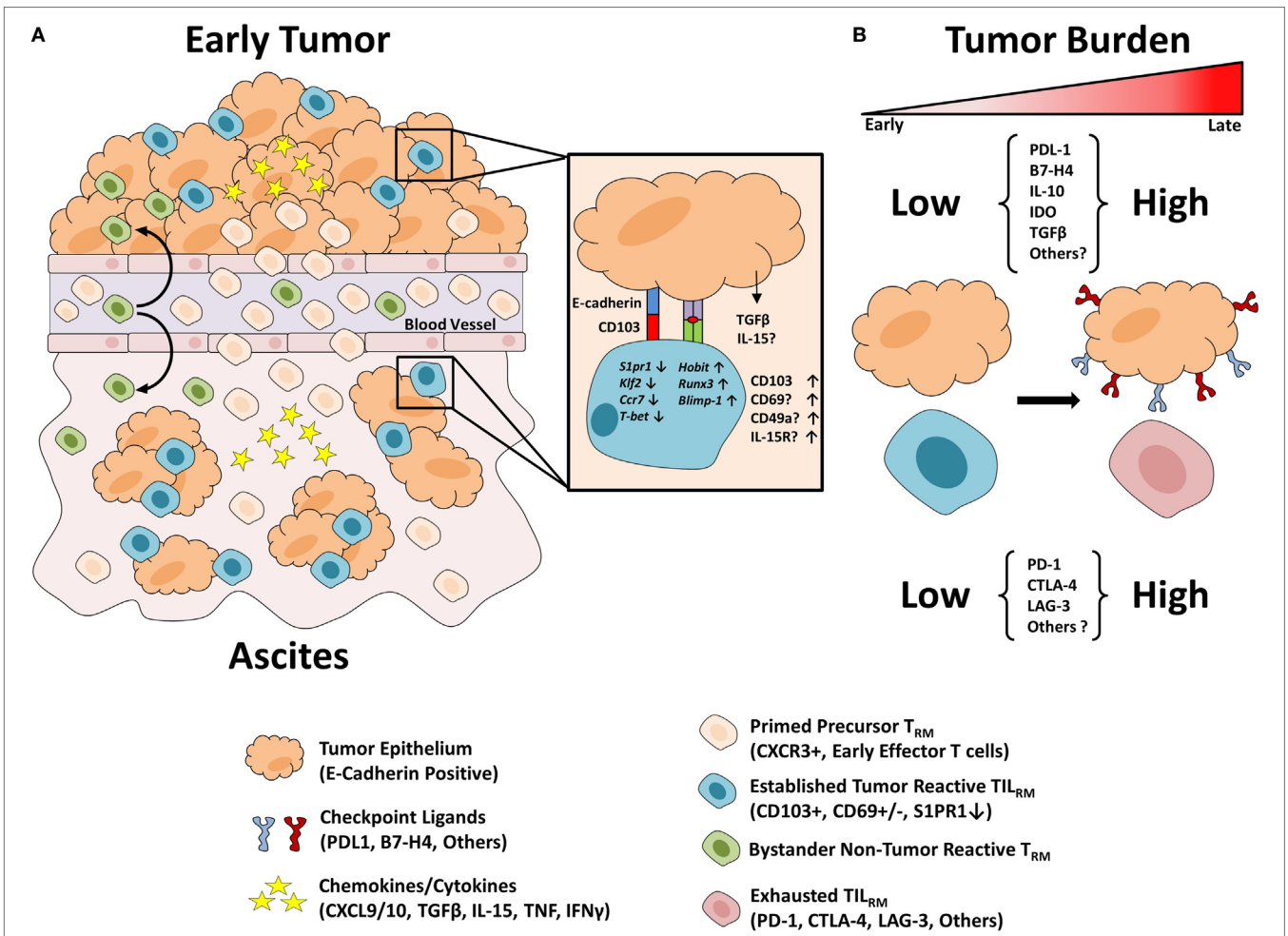

FIGURE 1 | Proposed model of TIL_{RM} formation. **(A)** Precursor resident memory T cells (T_{RM}) populations are composed of previously activated $CXCR3^+$ T cells which are attracted to the chemokines CXCL9/10 in the inflamed tumor environment. Within the epithelial tumor tissue, cells encounter TGF-β which promotes CD103 expression. In response to TCR engagement cells may express increased CD69 which in turn disrupts S1PR1 expression leading to a breakdown in the chemoattractant signal from S1P concentrations in the blood. TIL_{RM} cells bind to their target tumor cells with increased strength due to CD103 binding to its ligand E-cadherin, thus promoting their residency in the epithelial tissue. Similarly, precursor T_{RM} may traffic to the inflamed ascites environment and interact with epithelial tumor cells leading to TIL_{RM} formation. Finally, bystander precursor T_{RM} populations may traffic to the inflamed tumor and/or ascites environment and develop T_{RM}-like characteristics but with irrelevant antigen specificity. **(B)** Throughout cancer progression, the tumor microenvironment becomes increasingly inhospitable with increased tumor burden. Tumor cells upregulate immunosuppressive checkpoint receptors to avoid immune eradication. Following T cell activation and prolonged antigen stimulation T cells upregulate a variety of immune checkpoints which act to suppress anti-tumor immunity. TIL_{RM} may be inhibited due to the high expression of such checkpoint receptors and thus are likely candidates to respond to immune checkpoint blockade therapy.

characteristic of conventional T_{RM} where expression of PD-1 is reported to be dynamic and perhaps even restricted to certain tissue types (71, 72). This finding would suggest that unlike conventional T_{RM}, intra-tumoral TIL_{RM} may have become partially (or permanently) exhausted likely due to chronic stimulation with tumor antigen over a period of weeks to months. Indeed, we speculate that although CD103 expression may initially be beneficial to TIL_{RM} function by promoting retention within the tumor, CD103 may actually be detrimental in the longer term by causing T cells to become "trapped" within the tumor, thereby exacerbating the phenomenon of chronic Ag stimulation (see **Figure 1**). This scenario is supported by a recent finding in melanoma, wherein $CD103^+$ TIL_{RM} selectively and specifically became activated and started expanding in patients who were undergoing anti-PD-1 immunotherapy (45). This finding suggests that TIL_{RM} may be critical players in dictating responsiveness to checkpoint blockade therapy, a topic which is currently undergoing intense scrutiny. Thus, more fully understanding the biology of TIL_{RM} becomes paramount in that context.

CONCLUSION AND FUTURE PERSPECTIVES

Resident memory T cells have rapidly gained a reputation as sentinels of peripheral immunity, primed to prevent infection *via* re-exposure to a previously encountered pathogen. However, the biology of T_{RM} is now spilling over into the field of oncology where T_{RM} are being detected in an increasing number of tumor settings. Whether all the functions and characteristics of conventional T_{RM} directly translate into the unique, dynamic and often hostile microenvironment of tumors has yet to be fully elucidated. Furthermore, what role TIL_{RM} play in preventing disease recurrence after standard treatments such as radiation and chemotherapy is essentially unknown territory. Clearly much remains to be learned about these cells. What is certain is the prognostic benefit that comes along with the presence of TIL_{RM}, implying that at best, they play a direct role in anti-tumor immunity, or at minimum, they are a surrogate indicator of a separate phenomenon that leads to favorable outcomes for patients with TIL_{RM} positive tumors. Future studies should explore the potential utility of these cells in cancer immunotherapy strategies, including checkpoint blockade, cancer vaccination, and cellular therapies. Of particular interest would be understanding methodologies to convert immunologically "cold" tumors to "warm" ones by coaxing the formation and putative anti-tumor activity of TIL_{RM}. One can even imagine that the TIL_{RM} phenomenon could be applied to the rapid growing field of chimeric antigen receptor (CAR) T cell technology as it transitions into the solid tumor setting, by facilitating the retention of CAR T cells in solid tumor targets. Clearly, we are still in the early days of understanding TIL_{RM} biology, but the potential implications for immuno-oncology are significant.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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METHODS
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In Vivo Blockade of Murine ARTC2.2 During Cell Preparation Preserves the Vitality and Function of Liver Tissue-Resident Memory T Cells

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On murine T cells, GPI-anchored ADP-ribosyltransferase 2.2 (ARTC2.2) ADP-ribosylates the P2X7 ion channel at arginine 125 in response to nicotinamide adenine dinucleotide (NAD^+) released during cell preparation. We have previously shown that chronic gating of P2X7 by ADP-ribosylation reduces the vitality and function of regulatory T cells and natural killer T cells that co-express high levels of ARTC2.2 and P2X7. Here, we evaluated the expression of ARTC2.2 and P2X7 by effector and memory T cells in the liver of naïve mice and after infection with *Listeria monocytogenes* (Lm). We found that $KLRG1^-/CD69^+$ tissue-resident memory T cells (Trm) in the liver of naïve mice and 7 weeks after infection with Lm express high levels of ARTC2.2 and P2X7. Isolation of liver Trm and subsequent incubation at 37°C resulted in cell death of the majority of $CD4^+$ and $CD8^+$ Trm. Injection of the ARTC2.2-blocking nanobody s+16a 30 min prior to organ harvesting effectively prevented ADP-ribosylation of P2X7 during cell preparation and thereby prevented NAD-induced cell death of the isolated Trm upon subsequent incubation at 37°C. Consequently, preserving Trm vitality by s+16a injection enabled a highly sensitive *in vitro* cytokine expression profile analyses of FACS sorted liver Trm. We conclude that *in vivo* blockade of ARTC2.2 during cell preparation by nanobody s+16a injection represents a valuable strategy to study the role and function of liver Trm in mice.

Keywords: ADP-ribosylation, P2X7, tissue-resident memory T cells, nanobodies, ARTC2.2

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INTRODUCTION

Mammalian ecto-ADP-ribosyltransferases (ecto-ARTs) are a family of toxin-related enzymes that use extracellular (NAD^+) to attach an ADP-ribose group to arginine residues of cell surface proteins. In mice, the ecto-ARTs family comprises six family members (ARTC1–5) including two isoforms of ARTC2, termed ARTC2.1 and ARTC2.2 that are encoded by two closely linked genes (*Art2a* and *Art2b)* (1). ARTC2 isoforms are expressed on immune cells. While ARTC2.1 is expressed mainly by innate immune cells such as macrophages, dendritic cells, and microglia, ARTC2.2 is the major ecto-ART expressed by T cells (2–4). The ARTC2 enzymes ADP-ribosylate various target proteins and thereby modulate their function. One well-characterized target of ARTC2.2-mediated ADP-ribosylation is the adenosine triphosphate (ATP)-gated P2X7 ion channel (5, 6). Two differentially spliced isoforms of P2X7 are expressed by murine immune cells (7, 8). P2X7a is expressed by innate

immune cells and plays a critical role in inflammasome formation and the release of mature interleukin (IL)-1β from these cells. P2X7k is expressed by T cells where ADP-ribosylation of P2X7 at R125 can trigger gating of P2X7k at much lower concentrations of NAD^+ compared to ATP (9). ATP and ADP-ribosylation-mediated gating of P2X7 on T cells induces the rapid influx of calcium, activation of cell surface metalloproteases, cleavage of cell surface ecto-domains of CD62L (10) and CD27 (11), externalization of phosphatidylserine, and ultimately cell death (5).

Several studies have shown that the ecto-ART substrate NAD^+ can be released from endogenous sources, e.g., *via* cell lysis or, in a more controlled fashion, *via* connexin hemichannels (12, 13). We have previously demonstrated that NAD^+ is released during the passage of cell culture cells and the preparation of primary leukocytes from murine spleen, lymph nodes, or the liver (12, 14). Of note, ARTC2 is catalytically active and ADP-ribosylates cell surface proteins, including P2X7, even if cells are prepared at 4°C (12). Gating of P2X7 by ADP-ribosylation, however, requires temperatures above 24°C, i.e., functional effects of P2X7 ADP-ribosylation on T cells are manifested during reincubation of isolated T cells at 37°C. This commonly results in cell death of a substantial fraction of T cells (12), in particular T cell populations that co-express high levels of ARTC2.2 and P2X7 such as regulatory T cells (Tregs) and natural killer T cells (NKTs) (14, 15). ADP-ribosylation of P2X7 during cell preparation affects the vitality of these cells and makes it difficult to use them for further *in vitro* functional assay or for adoptive transfer experiments (16). We recently described an experimental approach to prevent preparation-related ADP-ribosylation by systemic injection of the ARTC2.2-blocking nanobody s+16a, a 15 kDa small single domain antibody derived from llama heavy chain antibodies (14, 17). Injection of s+16a 30 min prior to sacrificing the mice prevents the detrimental effects of preparation-related P2X7 ADP-ribosylation and facilitates the use of freshly prepared Tregs and NKTs for functional assay and adoptive transfer experiments.

Tissue-resident memory T cells (Trm) comprise a population of T cells, which stays in peripheral tissues after an immune response against invading pathogens, forming a rapid first-line defense against recurring infection (18). Trm are characterized by cell surface expression of CD69 and lack of cell surface expression of the killer cell lectin-like receptor subfamily G member 1 (KLRG1) (19). A recent study suggests that cell preparation affects the vitality and function of this T cell population in the context of a malaria mouse model (20). In our present study, we analyzed liver Trm from naïve mice and from mice 7 weeks after *Listeria monocytogenes* (Lm) infection in order to increase the number of Trm in the liver. In both, we analyzed the expression of ARTC2.2 and P2X7. We tested the impact of the ARTC2.2-blocking nanobody s+16a on the vitality of Trm vitality and on the functional capacity of freshly prepared Trm to secrete cytokines. Our results demonstrate that $CD8^+$ and $CD4^+$ liver Trm co-express high levels of ARTC2.2 and P2X7 and that preparation of primary Trm from liver causes ADP-ribosylation of P2X7 resulting in cell death in the majority of isolated $CD4^+$ and $CD8^+$ Trm upon incubation at 37°C. Systemic injection of nanobody s+16a preserved Trm vitality and allowed sensitive monitoring of otherwise unnoticed cytokine expression.

MATERIALS AND METHODS

Mice

C57BL/6 mice were used for all experiments. ARTC2ko mice (21) and P2X7 mice (22) were backcrossed onto the C57BL/6J background for at least 12 generations. Splenocytes from RAG1ko mice (23) were used as feeder cells in some experiments. All mice were bred at the animal facility of the University Medical Center (UKE). All experiments involving tissue derived from animals were performed with approval of the responsible regulatory committee (Hamburger Behörde für Gesundheit und Verbraucherschutz, Veterinärwesen/Lebensmittelsicherheit, G17/17). All methods were performed in accordance with the relevant guidelines and regulations.

Lm Infection

C57BL/6 mice were intravenously (i.v.) infected with a Lm strain recombinant for ovalbumin (2×10^4 bacteria in 200 µl PBS) (24). Mice were housed under specific pathogen-free conditions in individually ventilated cages, received food and water *ad libitum* and were controlled on a daily basis during the experiment.

Nanobody s+16a Treatment

s+16a was recombinantly produced by transfecting HEK-6E cells with the pCSE2.5 vector containing the coding region of s+16a. Mice were injected i.v. with 50 µg of the ARTC2.2-blocking nanobody s+16a solved in 100 µl NaCl 30 min prior to sacrificing the mice in order to prevent ADP-ribosylation of P2X7 during cell preparation.

Preparation of Liver Trm

Mice were anesthetized by CO_2/O_2 exposure and sacrificed by cervical dislocation. The preparation of single-cell suspensions from liver was performed throughout at 4°C. Liver lobes were gently mashed through a metal sieve using a syringe piston. Purification of liver leukocytes was achieved by running a Percoll gradient. For this, cells were resuspended in 5 ml 33% Percoll/PBS in a 15-ml Falcon tube, and centrifuged at 1,600 rpm, 12°C, for 20 min without breaks. The pellet was collected, and cells were washed once in PBS (ThermoFisher). Contaminating erythrocytes were lysed using ACK erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2). For FACS analyses or sorting, cells were washed and resuspended in FACS buffer containing PBS, 1 mM EDTA (Sigma), and 0.1% bovine serum albumin (Sigma).

Antibodies and Flow Cytometry

The following antibodies were used for flow cytometric analyses: anti-ARTC2.2 (clone Nika109; UKE), anti-CD3 (clone 145-2C11, BioLegend), anti-CD4 (clone RM4–5, BioLegend), anti-CD8 (clone 53-6.7, BioLegend), anti-CD45 (clone30-F11, BioLegend), anti-CD69 (clone H1.2F3, BioLegend), anti-KLRG1 (clone 2F1/KLRG1, BioLegend), and anti-P2X7 (clone RH23A44, UKE). PE-labeled CD1d-tetramer (PBS-57-loaded) was kindly provided by the NIH tetramer core facility. Flow cytometric analyses were performed on a BD Fortessa (Beckton Dickinson) or a BD

FACS CantoII (Beckton Dickinson). Liver Trm were identified as $CD4^+CD69^+KLRG1^-$ or $CD8^+CD69^+KLRG1^-$, and tissue residency was probed by applying the anti-CD45 *in vivo* labeling technique (25). For this, 2 µg of fluorochrome-labeled anti-CD45-perCP solved in 100 µl PBS were intravenously injected into mice, which were sacrificed 3 min after injection. After cell preparation, all leukocytes were labeled with anti-CD45-PE-Cy7 and blood vessel resident cells were identified as CD45-PE-$Cy7^+$CD45-$perCP^+$. For some experiments, liver Trm were sorted at the FACS Core Facility at the University Medical Center Hamburg-Eppendorf (UKE) on a BD FACSAriaFusion (Beckton Dickinson). Analysis of flow cytometric data was performed using FlowJo X (Flowjo, LLC).

Monitoring P2X7-Induced Cell Death

$CD4^+CD69^+KLRG1^-$, $CD4^+CD69^-KLRG1^+$, $CD8^+CD69^+KLRG1^-$, and $CD8^+CD69^-KLRG1^+$ cells were FACS sorted and 1×10^4 cells were resuspended in 200 µl complete IMDM medium containing IMDM (ThermoFisher) + 5% FCS, β-mercaptoethanol (50 µM, ThermoFisher), and gentamicin (50 µg/ml, ThermoFisher). For some experiments, FACS sorted Trm were cultured in the presence of 2×10^5 $eFluor^{670}$-labeled feeder cells obtained from RAG1ko mice in a ration of 1:20. Cells were incubated for 2 h at 4°C on ice or at 37°C in a cell culture incubator in the presence of propidium iodide (PI, 2.5 µg/ml, ImmunoChemistry Technologies, LLC). PI uptake was used to determine cell death by flow cytometry directly after incubation.

Cytokine Secretion Assay

$CD4^+CD69^+KLRG1^-$ and $CD8^+CD69^+KLRG1^-$ Trm were isolated by FACS sorting from mice 7 weeks after infection with Lm. Isolated Trm were cultured in 200 µl IMDM (ThermoFisher) + 5% FCS, β-mercaptoethanol (50 µM, ThermoFisher), and gentamicin (50 µg/ml, ThermoFisher) at a cell density of 20,000 ($CD4^+$ Trm) or 10,000 ($CD8^+$ Trm) cells per well for 20 h in the presence of Phorbol 12-myristate 13-acetate (PMA, 20 ng/ml, Invivogen) and ionomycin (1 µg/ml, Invivogen) to induce cytokine expression. Levels of IFN-γ, TNF-α, IL-2, IL-4, IL-21, IL-22, IL-17A, IL-17F, IL-10, IL-9, IL-5, and IL-13 were measured in the supernatants of stimulated Trm by using the LEGENDplex mouse Th cytokine 13-plex (BioLegend) according to the manufacture's instruction.

RESULTS

Liver Trm Co-Express ARTC2.2 and P2X7

Tissue-resident memory T cells (Trm) are a population of non-circulating $CD4^+$ and $CD8^+$ T cells that stay in peripheral tissues after infection to build a rapid first-line defense against recurring pathogen invasion (18). A recent study suggests that liver $CD8^+$ Trm are affected by NAD^+ released during cell preparation (20), however, these cells have not yet been fully characterized toward their expression of ARTC2.2 and P2X7. Since co-expression of ARTC2.2 and P2X7 potentially renders cells susceptible toward NAD^+-induced cell death (5), we set out to measure ARTC2.2 and P2X7 expression on these cells. In order to increase the frequency of liver Trm, we infected mice i.v. with Lm and analyzed liver $CD4^+$ and $CD8^+$ Trm 7 weeks after infection (**Figure 1A**). $CD4^+$ and $CD8^+$ Trm were identified as $CD3^+CD1d^{tet-}$ T cells that express CD69 but lack KLRG1 expression. Conversely, $CD69^-KLRG1^+$ T cells were identified as effector memory T cells (Tem). The remaining $CD69^-KLRG1^-$ T cells were termed "double negative" (DN) including naïve and memory T cells (**Figure 1B**). In order to distinguish vascular T cells and from tissue-resident T cells, we injected anti-CD45-perCP antibodies 3 min prior to sacrificing the mice. Due to the fenestrated endothelium of the liver sinusoids, anti-CD45-perCP *in vivo* labeling (termed $CD45^{blood}$) led to a low-level CD45 staining of all $CD45^+$ liver leukocytes (termed $CD45^{all}$). However, when comparing $CD8^+$ and $CD4^+$ Trm with Tem or DN T cells, only Tem and DN T cells contained a substantial fraction of cells that were strongly labeled by the i.v. injected anti-CD45-perCP antibody, confirming that $CD69^+KLRG1^-$ Trm reside deeper in the liver tissue (**Figure 1C**). Seven weeks after Lm infection, the frequencies of $CD8^+$ Trm and Tem as well as of $CD4^+$ Trm were significantly increased compared to naïve mice (**Figure 1D**). We next, analyzed these three subpopulations of $CD8^+$ and $CD4^+$ T cell populations obtained from naïve mice and 7 weeks after Lm infection for expression of ARTC2.2 and P2X7 using specific monoclonal antibodies (26, 27). In naïve mice, a substantial fraction of $CD8^+$ and $CD4^+$ Trm co-express high levels of ARTC2.2 and P2X7 (**Figure 1E**). By contrast, most $CD8^+$ and $CD4^+$ Tem and DN cells express ARTC2.2 but lack P2X7 expression. Seven weeks after Lm infection, we found that the majority of $CD8^+$ and $CD4^+$ Trm co-express high levels of ARTC2.2 and P2X7. By contrast, most $CD8^+$ and $CD4^+$ Tem express only low levels of ARTC2.2 and P2X7. Furthermore, DN $CD8^+$ and $CD4^+$ T cells do not express substantial levels of P2X7 but a major fraction of $CD8^+$ (>80%) and $CD4^+$ (>60%) DN T cells expresses high levels of ARTC2.2. In summary, $CD8^+$ and $CD4^+$ liver Trm co-express high levels of ARTC2.2 and P2X7, especially when isolated 7 weeks after Lm infection (**Figure 1F**), and therefore are potentially sensitive toward NAD^+ released during cell preparation.

Injection of s+16a Preserves the Vitality of Isolated Liver Trm

T cells co-expressing high levels of ARTC2.2 and P2X7, such as Treg and NKT cells, are highly susceptible to NAD^+-released during cell preparation, resulting in reduced vitality and function of the isolated cells (14, 16). Extracellular NAD^+ released during cell preparation serves as substrate for ARTC2.2 catalyzing the ADP-ribosylation of R125 of P2X7, even when cells are prepared at 4°C (**Figure 2A**). When cells are brought back to 37°C, e.g., for functional assays or adoptive transfer, ADP-ribosylation of P2X7 triggers channel gating leading to influx of Ca^{2+} and ultimately to cell death which can be visualized by PI uptake. ADP-ribosylation of P2X7 during cell preparation can be prevented by injection of the ARTC2.2-blocking nanobody s+16a 30 min prior to organ harvesting. Since liver Trm co-express high levels of ARTC2.2 and P2X7, we hypothesized that preparation-related ADP-ribosylation of P2X7 reduces the vitality of these cells upon reincubation at 37°C. To test this, we first isolated Trm from the liver of naïve WT, ARTC2ko, and P2X7ko mice *via* FACS and incubated the isolated cells for 2 h in IMDM + 5% FCS in the presence of PI at 37°C or kept the cells at 4°C on ice.

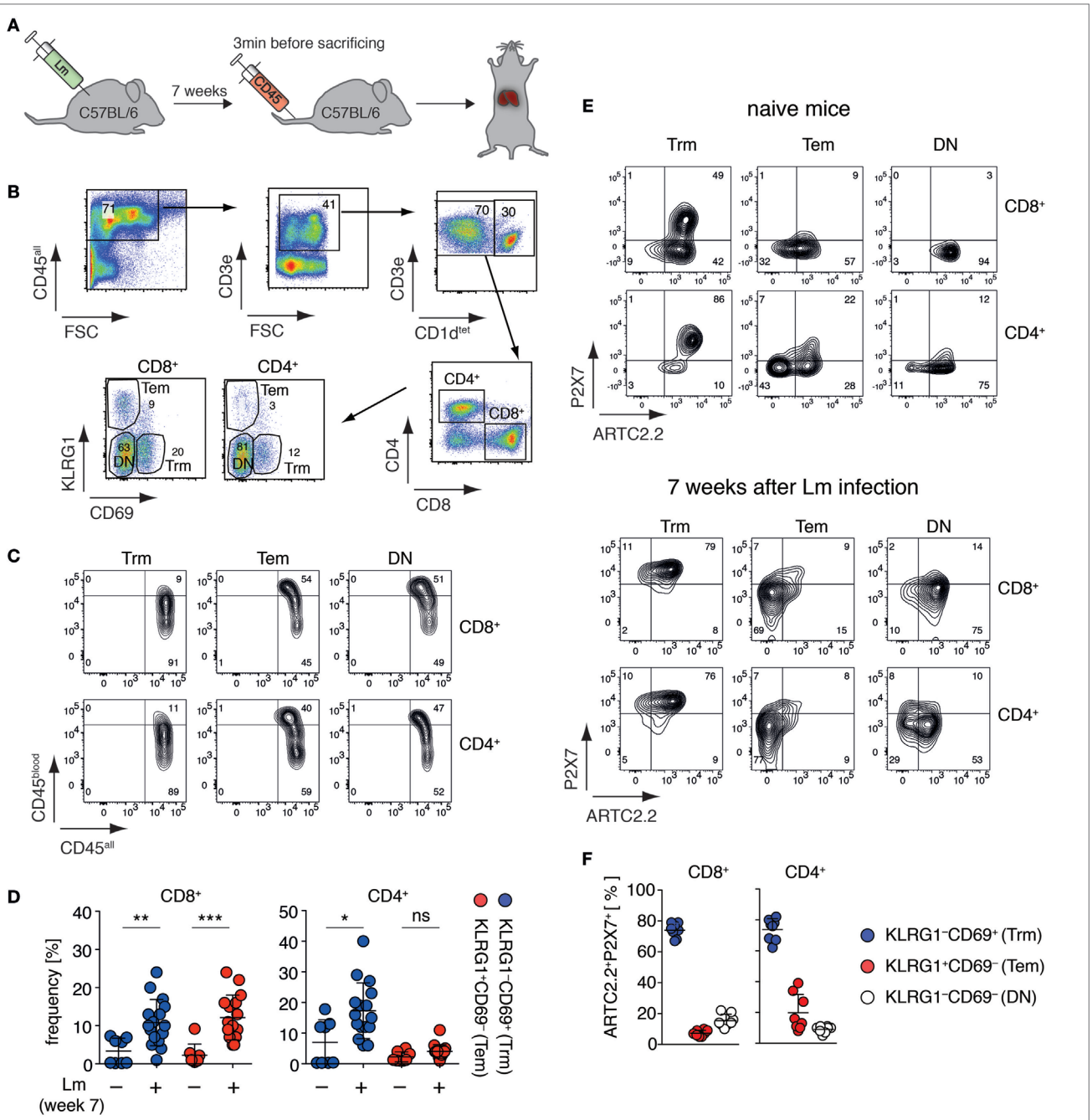

FIGURE 1 | Liver Trm co-express high levels of ARTC2.2 and P2X7. **(A)** C57BL/6 mice were infected i.v. with 2×10^4 *Listeria monocytogenes* (Lm). Seven weeks after infection, mice were treated with perCP-labeled anti-CD45 3 min before sacrificing to label vascular leukocytes. The liver of treated mice was harvested for Trm analyses. **(B)** Gating strategy: within the $CD3^+CD1d^{tet-}$ T cell pool Trm were identified as $CD8^+CD69^+KLRG1^-$ or $CD4^+CD69^+KLRG1^-$ and effector memory T cells (Tem) as $CD8^+CD69^-KLRG1^+$ or $CD4^+CD69^-KLRG1^+$; double negative (DN) marks $CD8^+$ or $CD4^+$ T cells that were $CD69^-KLRG1^-$. **(C)** *In vivo* anti-CD45 labeling ($CD45^{blood}$) of Trm, Tem, and DN in relation to *ex vivo* anti-CD45 labeling ($CD45^{all}$). **(D)** Frequencies of $CD8^+$ and $CD4^+$ Trm and Tem in the liver of naïve and Lm infected mice. Two groups were compared using Student's *t*-test ($n = 8$–16) with $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **(E)** FACS analyses of ARTC2.2 and P2X7 expression on Trm, Tem, and DN cells from naïve mice (upper panel) and mice 7 weeks after infection with Lm (lower panel). **(F)** Frequency of ARTC2.2 and P2X7 co-expressing cells from mice 7 weeks after Lm infection are quantified as % of $CD4^+$ or $CD8^+$ T cells. The shown data represent results from at least two independently performed experiments.

PI uptake by Trm was subsequently analyzed by flow cytometry as a measure for cell death. We observed that the majority of WT $CD8^+$ and $CD4^+$ Trm died upon incubation at 37°C as shown by incorporation of PI. This discrepancy in vitality upon incubation at 4 and 37°C was virtually absent when analyzing $CD8^+$ and $CD4^+$ Trm from ARTC2ko and P2X7ko mice (**Figure 2B**). To

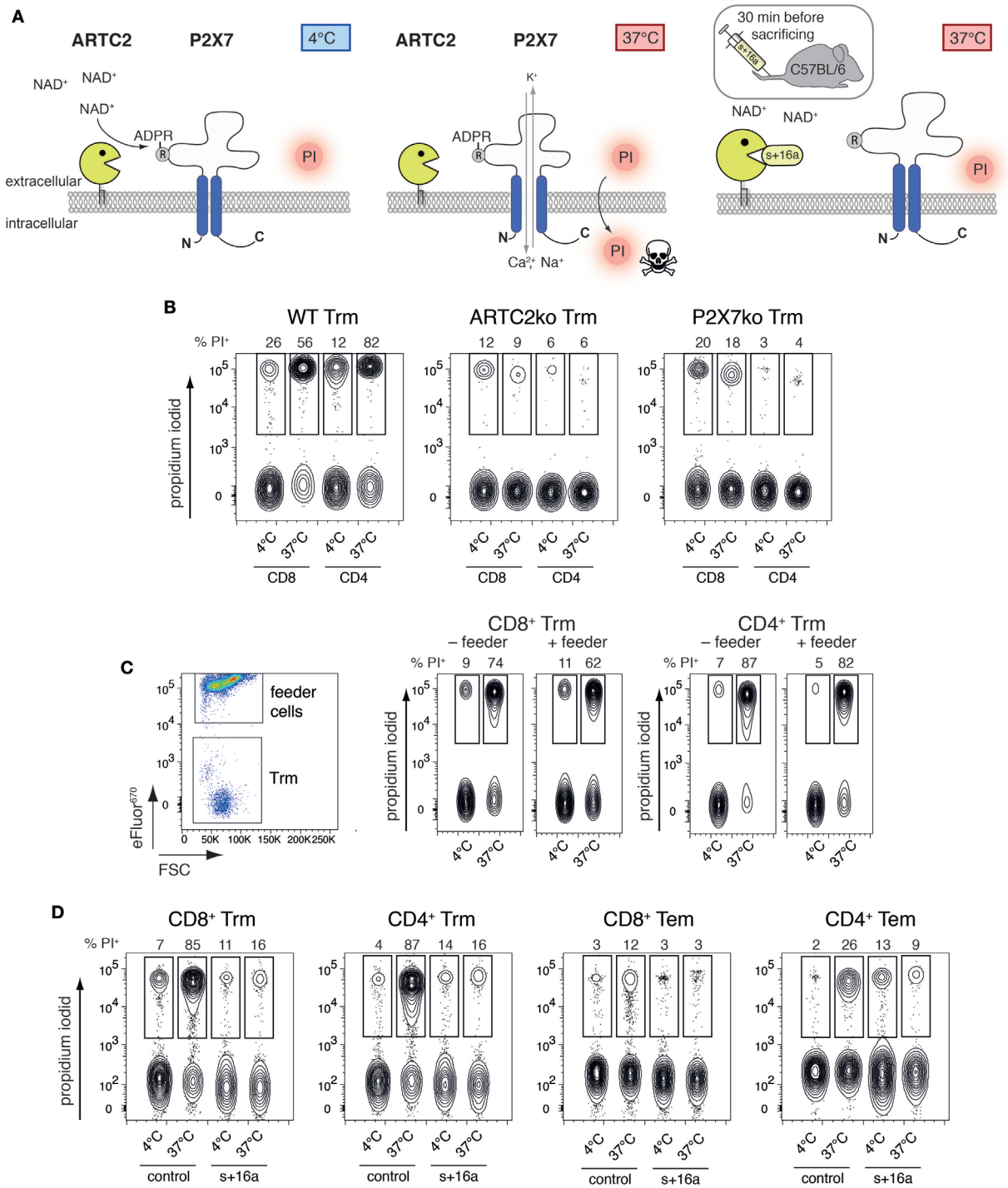

FIGURE 2 | ADP-ribosylation of Trm during cell preparation induces cell death upon 37°C incubation. **(A)** NAD^+ is released during cell preparation and serves as substrate for ARTC2.2 to ADP-ribosylate P2X7 at R125, even if cells are prepared at 4°C. ADP-ribosylation-mediated gating of P2X7 occurs when cells are brought back to 37°C, inducing Ca^{2+} influx and ultimately cell death. ADP-ribosylation of P2X7 during cell preparation and subsequent activation of P2X7 at 37°C can be prevented by injection of the ARTC2.2-blocking nanobody s+16a 30 min prior to sacrificing the mice. **(B)** $CD8^+$ and $CD4^+$ Trm were isolated *via* FACS from the liver of naïve WT, ARTC2ko, and P2X7ko mice. Cells were incubated at 37°C for 2 h and propidium iodide (PI) uptake was measured by flow cytometry as marker for cell death. **(C)** Isolated $CD8^+$ and $CD4^+$ Trm from the liver of naïve WT mice were cultured in the presence or absence of $eFluor^{670}$-labeled feeder cells in a ration of 1:20. Cells were incubated at 37°C for 2 h and PI uptake by Trm was measured by flow cytometry as marker for cell death. **(D)** $CD8^+$ and $CD4^+$ Trm and effector memory T cells (Tem) were isolated *via* FACS from the liver of Lm infected mice 7 weeks after infection. One group of mice was treated with s+16a prior to organ harvesting and the second group was left untreated as control. Cells were incubated at 37°C for 2 h and PI uptake was measured by flow cytometry as marker for cell death. The shown data represent results from two independently performed experiments.

further test whether the presence of feeder cells could improve the vitality of Trm, we co-incubated FACS-sorted WT CD8$^+$ and CD4$^+$ Trm with eFluor^{670}-labeled splenocytes obtained from RAG1ko mice (**Figure 2C**). The results show that the presence of feeder cells does not improve the vitality of CD8$^+$ and CD4$^+$ liver Trm, when incubated at 37°C. Together, this suggests that the observed loss of Trm vitality upon incubation at 37°C is triggered *via* ARTC2.2-mediated ADP-ribosylation of P2X7. In order to further probe this conclusion, we compared the vitality of isolated CD8$^+$ and CD4$^+$ Trm and Tem obtained from mice 7 weeks after Lm infection and analyzed the impact of injecting the ARTC2.2-blocking nanobody s+16a 30 min before sacrifice on cell vitality. For this, one group of mice was injected i.v. with 50 µg s+16a 30 min prior to sacrifice, the other group was left untreated as control. As shown before for liver Trm from naïve mice, we observed that the vast majority (85%) of CD8$^+$ and CD4$^+$ Trm of the untreated control group died during the 37°C incubation, compared to only 12% of CD8$^+$ Tem and 26% of CD4$^+$ Tem (**Figure 2D**). By contrast, CD8$^+$ and CD4$^+$ Trm and Tem sorted from the s+16a-treated mice exhibited a preserved vitality with only 16% dead cells after incubation at 37°C. Together, our results reveal the detrimental effects of preparation-related P2X7 ADP-ribosylation on liver Trm vitality upon incubation at 37°C.

Injection of s+16a Allows Cytokine Profiling of Liver Trm

Our findings that the majority of isolated liver Trm succumb to cell death upon incubation at 37°C raises the question whether this affects functional assays that involve incubation steps at 37°C and impinges on the quality of the obtained data. To test this, we analyzed the cytokine expression profile of freshly sorted liver CD8$^+$ and CD4$^+$ Trm from s+16a-treated mice and untreated control mice 7 weeks after infection with Lm in a proof-of-principle experiment. We restimulated 1–2 × 10^4 isolated cells *in vitro* with PMA/ionomycin for 20 h and analyzed the cytokine expression profile in the cell supernatants using a 13-plex bead-based immunoassay designed to quantify T cell-specific cytokine responses. By this, we detected low concentrations of IFN-γ, TNF-α, and IL-2 in the supernatants of stimulated CD8$^+$ Trm and CD4$^+$ Trm isolated from control mice (**Figure 3A**). Strikingly, CD8$^+$ and CD4$^+$ Trm isolated from s+16a-treated mice produced more than 100-fold higher concentrations of

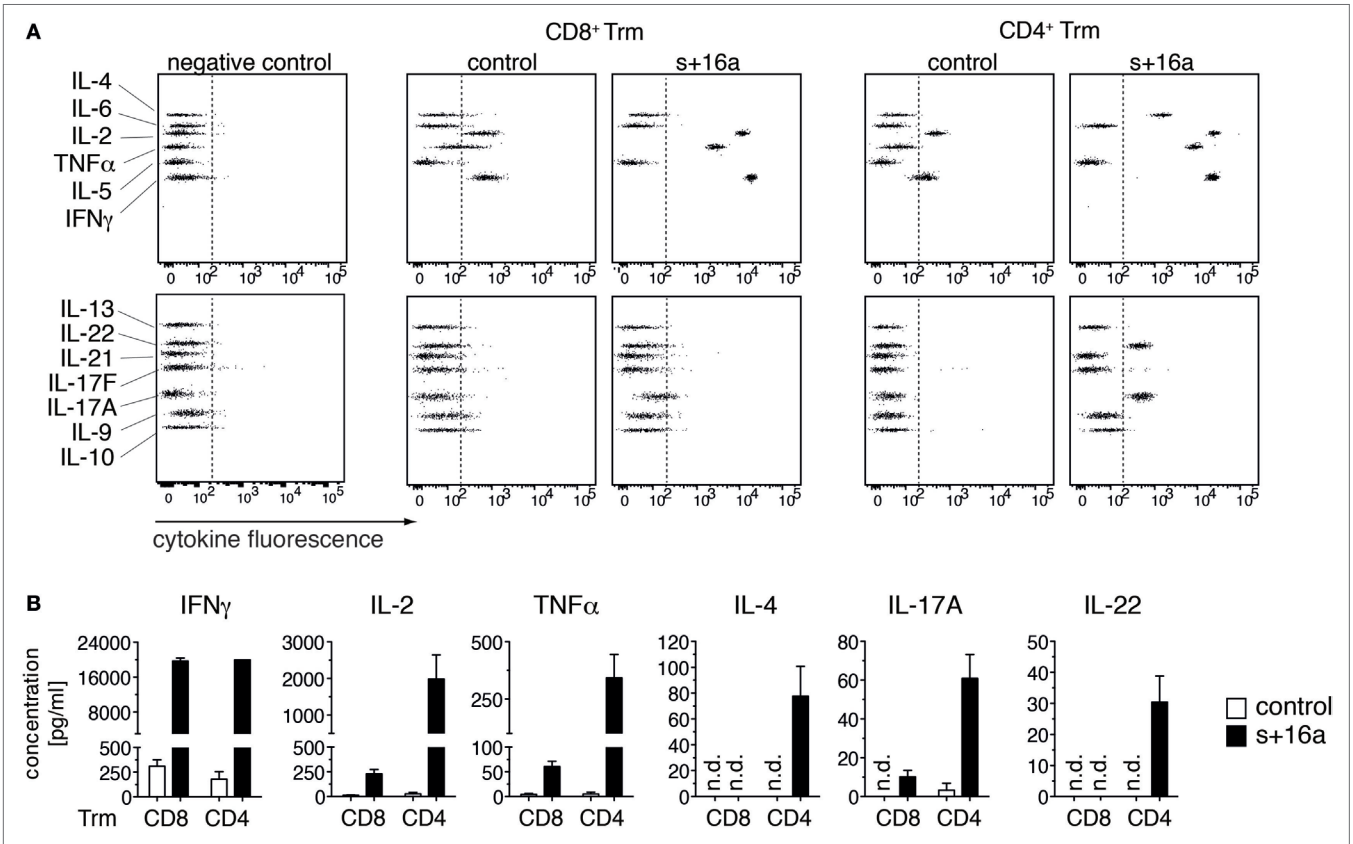

FIGURE 3 | *In vitro* cytokine expression profile of PMA/ionomycin stimulated liver Trm. **(A)** CD8$^+$ (1 × 10^4 cells) and CD4$^+$ (2 × 10^4 cells) Trm from the liver of Lm infected mice were FACS sorted 7 weeks after infection. One group of mice was treated with s+16a prior to organ harvesting and the second group was left untreated as control. Cells were stimulated with PMA/ionomycin for 20 h and T cell cytokines [IFNγ, TNFα, interleukin (IL)-2, IL-4, IL-21, IL-22, IL-17A, IL-17F, IL-10, IL-9, IL-5, and IL-13] were measured in the supernatants using a bead-based 13-plex assay. **(B)** The concentration of cytokines in the supernatants of stimulated CD4$^+$ and CD8$^+$ Trm from s+16a-treated and control mice (n = 3) was quantified.

IFN-γ, TNF-α, and IL-2 (**Figure 3B**). Furthermore, no IL-4 or IL-22 and only very low levels of IL-17A were detectable in the supernatants of stimulated $CD4^+$ Trm isolated from control mice. By contrast, $CD4^+$ Trm harvested from s+16a-treated mice showed robust expression of IL-4, IL-22, and IL-17 upon PMA/ionomycin stimulation (**Figures 3A,B**). Interestingly, IL-17A was also detectable in the supernatants of stimulated $CD8^+$ Trm from s+16a-treated mice. These results demonstrate that ADP-ribosylation of P2X7 during cell preparation reduces the vitality of Trm and therefore blunts their cytokine secretion during 37°C PMA/ionomycin stimulation. This can limit the detection of low-level expressed cytokines, as demonstrated for IL-17A, IL-22, and IL-4.

DISCUSSION

Our results show that liver tissue-resident memory T cells (Trm) co-expresses high levels of ARTC2.2 and P2X7. The high ARTC2.2 expression enables ADP-ribosylation of Trm cell surface proteins during cell preparation from the liver in response to NAD^+ released during cell preparation. ARTC2.2 ADP-ribosylates the P2X7 ion channel even if cells are prepared at 4°C. Subsequent incubation of the isolated Trm at 37°C then induces P2X7 activation, resulting in cell death and making it difficult to use these cells during further *in vitro* assays. The consequences of preparation-related ADP-ribosylation on Trm resemble those of ARTC2.2 and P2X7 co-expressing Tregs and NKT cells, i.e., dramatic loss of cell vitality during *in vitro* culture (14). Our rescue approach, i.e., injection of the ARTC2.2-blocking nanobody s+16a prior to sacrificing the mice, markedly preserves the vitality of Trm at 37°C incubation and allows sensitive *in vitro* cytokine expression profiling.

Several studies have shown that both, ARTC2.2 and P2X7 are differentially expressed among T cell subpopulations (15, 28–31). Downregulation of ARTC2.2 is observed by T cells upon T cell receptor activation (32). Similarly, recently activated T cells express lower levels of P2X7 compared to their naive counterparts (33). Both findings fit to the phenotype of liver $CD69^-KLRG1^+$ Tem observed in the Lm infected mice. The physiological role of ARTC2.2 and P2X7 on Trm remain unclear. High P2X7 expression on Trm could be beneficial to boost T cell receptor signaling toward eliciting a T cell memory response during pathogen reencountering since P2X7 can act as receptor for autocrine ATP stimulation to enhance IL-2 production (34). However, massive tissue damage during liver infection accompanied by the release NAD^+ would probably kill most of the liver Trm and thereby delay pathogen clearance. Therefore, further studies are needed to investigate the interplay between ARTC2.2 and P2X7 on Trm *in vivo* during a second course of infection. Furthermore, it needs to be investigated whether Trm from other organs also exhibit a high co-expression of ARTC2.2 and P2X7 and are vulnerable to NAD-induced cell death (NICD). A recent study by Yoshizawa et al. describes the transcriptome characterization of $CD69^+$ Trm in the lung of influenza infected mice. Interestingly, the authors found a differential P2X7 expression in two Trm populations directed against the two immunodominant epitopes $PA_{224–233}$/H-$2D^b$ and $NP_{366–374}$/H-$2D^b$. $PA_{224–233}$/H-$2D^b$ Trm exhibited pronounced P2X7 mRNA expression in $CD103^-$ and, at slightly lower level, in $CD103^+$ cells. By contrast, P2X7 mRNA expression was virtually absent in $CD103^-$ and $CD103^+$ $NP_{366–374}$/H-$2D^b$ Trm (35). Another study demonstrated that $CD4^+$ T cells from the small intestine of naïve mice exhibit P2X7 expression on $CD69^+$ cells (36). The latter were susceptible toward NICD as demonstrated by *in vivo* depletion after injection of NAD^+, indicating that ARTC2.2 is co-expressed by these cells.

In general, it is advised to check the expression levels of ARTC2.2 and P2X7 when working with murine T cell populations. As a first approach, this can be done by querying public mRNA sequencing databases such as www.immgen.org (37). The results of such analyses reveal that Tregs, $CD4^+$ memory T cells, and NKT cells from spleen and liver express high mRNA levels of *Art2* and *P2rx7*. These cells can then be characterized for ARTC2.2 and P2X7 cell surface expression by flow cytometry or by functional assays that monitor P2X7-related effects such as ecto-domain shedding of CD27 and CD62L or phosphatidylserine externalization and PI uptake upon 37°C incubation (16). It is important to note that the human ARTC2 gene is non-functional due to premature stop codons and therefore ARTC2-related effects observed in mice are not directly transferrable to humans (38).

ARTC2.2 ADP-ribosylates multiple targets on T cells, including CD25, the α-chain of the high affinity IL-2 receptor (39). Here, ADP-ribosylation reduces binding of IL-2 and subsequent STAT5 signaling in Tregs. For $CD8^+$ cytotoxic T cells, it has been shown that ARTC2.2-catalyzed ADP-ribosylation of CD8 diminishes the binding to MHC class I (40). Therefore, the beneficial effects of systemically injected ARTC2.2 blocking nanobody on Trm vitality and cytokine secretion may be mediated also in part by preventing ADP-ribosylation of these and other targets. A recently published study describes a mass-spectrometry-based approach to identify ADP-ribosylated proteins (41). This technique has been applied by our group to identify ADP-ribosylated cell surface target proteins on microglia (4) including several cell adhesion molecules. This technique could be utilized to analyze the ADP-ribosylome of T cells in order to identify other target proteins that are potentially influenced in their function by ADP-ribosylation.

One limitation of our approach to prevent ADP-ribosylation during T cell preparation using the s+16a nanobody is that it needs to be injected i.v. or i.p. into mice which may be technically challenging and requires the approval to perform animal experiments. A recent study suggests to use the P2X7 antagonist KN62 as alternative substance that can be used *in vitro* during the preparation of single-cell suspensions from lymphoid organs in order to protect T follicular helper and regulatory cells from NICD and further during *in vitro* culture of T cells (42). Indeed, this preserved the vitality of ARTC2.2 and P2X7 expressing T follicular helper and regulatory cells. However, P2X7 blockade by KN62 does not prevent the ADP-ribosylation of other membrane proteins during cell preparation. Moreover, blockade of P2X7 might also interfere with T cell function, as autocrine ATP stimulation upon T cell receptor activation enhances the production of IL-2 *via* P2X7 activation (34). Furthermore, though KN62 is a highly potent non-competitive P2X7 antagonist (IC50:

15 nM), it is also cell permeable and a selective inhibitor for Ca^{2+}/calmodulin-dependent protein kinase II (IC50: 500 nM), which plays a role in T cell receptor mediated IkB kinase activation (43). Therefore, even though systemic injection of the s+16a nanobody approach is technically more elaborative, blocking of ARTC2.2 during cell preparation probably interferes less with T cell function compared to P2X7 blockade.

In a proof-of-principle experiment, we demonstrated that injection of s+16a allows a sensitive *ex vivo* cytokine expression analyses of isolated Trm in response to polyclonal PMA/ionomycin stimulation. By this, we could detect low-level cytokines expressed by $CD4^+$ Trm such as IL-4, IL-17A, and IL-22 that were undetectable when $CD4^+$ Trm were isolated from control mice that did not receive s+16a treatment. It is likely that the reduced vitality of the isolated control $CD4^+$ Trm is responsible for this, however, we cannot rule out that tissue-resident T-helper type 1 (Th1), 2 (Th2), 17 (Th17), and 22 (Th22) cells are differentially affected by NICD. Indeed, P2X7 is highly expressed by intestinal Th1 and Th17 cells and injection of NAD^+ induces the depletion of these cells as it did for intestinal Tregs (36).

We conclude that when working with murine liver Trm, one needs to be aware that T cells expressing high levels of ARTC2.2 and P2X7 succumb to preparation-related ADP-ribosylation of P2X7 and other cell surface proteins that affects Trm vitality and function. Using our nanobody-based approach to block ARTC2.2 *in vivo* minimizes cell loss, paving the way for sensitive Trm cytokine expression profiling and other functional analyses.

ETHICS STATEMENT

This study was carried out in accordance with the German animal welfare law. The protocol was approved by the Hamburger Behörde für Gesundheit und Verbraucherschutz, Veterinärwesen/Lebensmittelsicherheit (approval number G17/17).

AUTHOR CONTRIBUTIONS

BR, ML, and FR performed the experiments and analyzed the data. TM, H-WM, and FK-N supervised the experiments and assisted with data interpretation and manuscript preparation. BR assembled the figures and wrote the manuscript, which has been reviewed by all authors.

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