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Tissue instruction for migration and retention of T_{RM} cells

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Abstract

During infection, a subset of effector T cells seeds the lymphoid and non-lymphoid tissues and gives rise to tissue-resident memory T cells (T_{RM}). Recent findings have provided insight into the molecular and cellular mechanisms underlying tissue instruction of T_{RM} cell homing, as well as the programs involved in their retention and maintenance. We review these findings here, highlighting both common features and distinctions between CD4 T_{RM} and CD8 T_{RM} cells. In this context we examine the role of memory lymphocyte clusters (MLCs), and propose that the MLCs serve as an immediate response center consisting of T_{RM} cells on standby, capable of detecting incoming pathogens and mounting robust local immune responses to contain and limit the spread of infectious agents.

Introduction

Immunological memory provides the vertebrate host with crucial means to protect against multiple infections by the same pathogen, and forms the basis of vaccines. The cardinal features of immunological memory are that it is specific to the pathogen; it provides more immediate and stronger response to the pathogen and is long lived. Over the past decade, the field of immunology has come to appreciate the existence and the importance of memory T cells that reside in peripheral tissues. Various non-lymphoid organs are seeded by effector T cells during infection or immunization, wherein these cells differentiate and develop into memory T cells with distinct phenotype and function. The tissue microenvironment provides instructive signals for the effector T cells to express molecules that enable long-term residency and survival. Importantly, once established, such tissue-resident memory T cells (T_{RM}) provide protective immunity to infectious microbial agents that enter through the local tissues [1, 2]. Therefore, studying and understanding the biology of T_{RM} will provide important insights into the natural immunological mechanism of host protection at the site of pathogen entry, as well as basis for designing future vaccines against mucosal pathogens.

The differentiation pathway from naïve lymphocytes to T_{RM} is beginning to be understood. In the secondary lymphoid tissue, naïve T cells that are activated in response to infection

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undergo differentiation programs to become effector T cells (Teff) that are capable of migrating to the site of infection to clear the pathogen. The Teff population can be largely divided into short lived effector cells (SLEC) whose primary role is to control infection, and memory precursor effector cells (MPEC) that give rise to long lived memory T cells [3, 4]. SLEC and MPEC are characterized by distinct cell surface expression of $\text{KLRG1}^{\text{hi}}\text{IL-7R}\alpha^{\text{low}}$ and $\text{KLRG1}^{\text{low}}\text{IL-7R}\alpha^{\text{hi}}$, respectively. Both of these Teff populations exit the lymph nodes through the efferent lymph and enter circulation. In the post capillary venules near the site of infection, Teff receive signals to slow down, adhere to the endothelium and to enter the tissue through transendothelial migration. Once inside the tissue, Teff populations migrate towards the infected cells along the chemokine gradient to kill infected cells [5]. Notably while both KLRG1^{hi} and $\text{KLRG1}^{\text{low}}$ Teff cells enter the tissue during the acute phase of infection, only the latter gives rise to the CD8 T_{RM} population in the skin after the resolution of infection [6].

Recent studies have also revealed distinct tissue classes with different degree of access to Teff entry at steady state (Table 1). Some tissues, such as the intestinal epithelium and peritoneal cavity, are seeded by the T_{RM} precursors (Teff) in the absence of local inflammation, while others including the skin epidermis, vaginal epithelium, lung airways, salivary glands and ganglia, require direct infection or inflammation to recruit T_{RM} precursors and maintain T_{RM} [7]. Permissive tissues constitutively express homing molecules that enable Teff to enter and establish residency within the tissue, while restrictive tissues require inflammatory cytokines and chemokines to render endothelial cells permissive to Teff migration. A well-known example of permissive tissue homing molecule is mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) constitutively expressed by the endothelial cells of the intestinal mucosa. Curiously, $\alpha 4\beta 7$, the homing receptor for MAdCAM-1, is upregulated on Teff cells acutely around 4.5 days after infection, which permits Teff a transient access to the intestinal epithelium [8]. While some T_{RM} reside in the epithelial layer in a seemingly random fashion in the absence of their cognate antigens, other types of T_{RM} require microenvironment that provides chemokine and antigenic stimuli. The latter type can be found within a recently reported structure, namely, the memory lymphocyte clusters (MLCs) [9], that are located strategically close to the mucosal surface. Studies have also revealed that the superior protection provided by T_{RM} is based in part on their location of proximity to the invading pathogens, but also on intrinsic and extrinsic factor that govern their immediate effector functions. In this review article, we discuss the role of tissue microenvironment in supporting recruitment, residency and effector functions of T_{RM} . The main topics to be addressed include, 1) the homing receptors and chemokines that orchestrate each step of migration of T_{RM} precursors from circulation into tissue; 2) the retention signals provided by the surrounding cells that support the maintenance of CD8 T_{RM} and CD4 T_{RM} ; 3) discussion of the MLC as a housing complex for T_{RM} ; and 4) the unique T_{RM} features that enable efficient execution of their effector functions.

Rolling signals for T_{RM} precursors

Lymphocyte migration into inflamed tissues occurs through sequential steps involving interaction between specific molecules expressed by lymphocytes and their counter

receptors (see Box 1). The molecules that control each step of activated lymphocytes that seed the T_{RM} pool are being uncovered. Much of this understanding is based on a study of HSV-1 infection in mice, and therefore, may be different for other infections and immunizations. E-selectin ligand is expressed by majority of CD4 Teff after epicutaneous, but not intranasal or subcutaneous HSV-1 infection. Expression of E-selectin ligands on CD8 Teff is more promiscuous, in that all three routes of HSV-1 infection induced its expression. P-selectin ligand is also expressed by CD4 and CD8 Teff following HSV-1 infection through all three routes, although epicutaneous infection appears to imprint higher level of expression on the effector T cells. Therefore, presumably, Teff cells utilize P and E-selectins for rolling on activated endothelial cells [10] (Figure 1). Activation of endothelial cells can be induced by innate cytokines such as IL-1 β and TNF- α [11] during the primary infection, but also by IFN- γ secreted from T_{RM} as described below.

Chemokine activation of T_{RM} precursors

The chemokines that are important in arresting rolling lymphocytes depend on the target tissue. It is well established that leukocyte stimulation by chemokines occurs through chemokines presented by the heparan sulfate glycosaminoglycans that present the chemokines on the apical and basolateral surfaces [12]. A recent provocative study challenged this view for Teff. Using HUVEC cells, the study demonstrated that silencing of exostosin-1 (Ext1), a key enzyme required for heparan sulfate biosynthesis, had no impact on Teff arrest and transendothelial migration whereas it blocked migration of activated neutrophils under shear flow [13]. Thus, Teff might integrate chemotactic signals directly from intra-endothelial chemokine stores rather than from externally deposited chemokines.

In the permissive tissues, endothelial cells constitutively express chemokines that enable arrest of Teff for their eventual entry. In the intestinal mucosa, tissue-homing chemokines such as CCL25 [14] in the small intestine (CCR9 ligand) and CCL28 [15] in the colon (CCR10 ligand) are constitutively secreted by the epithelial cells and are presented on the endothelial cells. This enables Teff expressing the receptors to arrest on the gut endothelium. Almost all small intestinal intraepithelial (IEL) and lamina propria (LP) T cells in mice and humans express CCR9, suggesting that this chemokine receptor is important for localization [14, 16, 17]. In the skin, CCL17 and CCL27 are constitutively expressed by the skin epidermal cells and are also bound on the endothelial surface [18, 19], which enable arrest of T cells expressing the chemokine receptors CCR4 [18, 20], CCR10 [19, 21], respectively. Notably, in healthy skin, CCL27 is expressed in the cytosol of keratinocytes, whereas large amounts of CCL27 protein is released into the papillary dermis in atopic dermatitis or psoriatic patients, suggesting that the regulation of this chemokine is in part mediated by release of stored proteins [19]. In addition to these chemokines, T cells expressing CCR6 [22] or CCR8 [23] respond to CCL20 and CCL8, respectively, expressed in inflamed skin.

In other tissues, chemokines used for arresting CD8 Teff are only induced following infection or inflammation. Inflammatory chemokines responsible for recruitment of CD8 Teff depend on the tissue and the pathogen (Figure 1). In the lung of mice infected with parainfluenza or influenza viruses, CCR5 is required for CD8 Teff cell entry into the airways [24]. CD4 Teff cell entry into the lung following parainfluenza infection instead

relies on CXCR3 [25]. HSV-2 infection in the genital mucosa [26] and HSV-1 infection in the skin [6] induces robust expression of CXCL9 and CXCL10, which enable recruitment of CD8 Teff expressing the receptor CXCR3. CXCR3 expression is induced on T cells stimulated with anti-CD3 plus anti-CD28 monoclonal antibodies in IFN- γ -dependent manner [27]. Following HSV-2 vaginal infection, CD4 Teff cells arrive in the tissue first and start secreting IFN- γ . IFN- γ in turn acts on surrounding cells to induce expression of a handful of chemokines including CXCL9 and CXCL10. Notably, CXCL10 is secreted by tissue cells and endothelial cells in response to type I IFN stimulation, whereas CXCL9 is only induced in response to IFN- γ secretion, likely from dendritic cells and histiocytes [28]. After the CD4 Teff cells pave the way, CD8 Teff cells are able to use CXCR3 to sense the chemokines for recruitment into the vaginal tissue (Figure 1). A recent study indicates that a similar mechanism, whereby IFN- γ secreted from CD4 T cells ushers CD8 Teff cell migration, is at work in the lung, following influenza A virus infection [29]. Exactly which chemokines are required to induce CD4 Teff cell recruitment to the infected tissue remains unclear, although autocrine IFN- γ secretion and responsiveness is required for their entry into the infected vagina [26], suggesting a role for IFN-inducible chemokines in their migration to the tissue.

Arresting signals for T_{RM} precursors

The next step to becoming a resident cell involves chemokine dependent activation of integrins, which enables the lymphocytes to arrest on the endothelial surface [12]. Gut-homing T cells express the integrin $\alpha 4\beta 7$ [30], which mediates T cell binding to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) at steady state [31, 32]. MAdCAM-1 is expressed by intestinal but not cutaneous vascular endothelium [33]. In tissues that are restricted from entry of Teff cells in the absence of local inflammation, there is little constitutive expression of homing addressins. Inducible integrins are likely engaged under inflammatory conditions, as CD8 T cells migrate to the gut independently of $\alpha 4\beta 7$ during rotavirus infection [34]. In the inflamed tissue, a variety of integrin-integrin ligand pairs are known to induce Teff cell arrest. The integrin VLA4 ($\alpha 4\beta 1$) expressed by Teff cells mediates adhesion through binding to its ligand, VCAM-1 expressed by endothelial cells. LFA-1 ($\alpha L\beta 2$) expressed by T cells also enable adhesion to ICAM-1 expressed by endothelial cells activated by proinflammatory cytokines (Figure 1). The $\alpha 1\beta 1$ (CD49a or VLA1) integrin is expressed by the CD8 Teff cells entering the lung airways after influenza infection whereas $\alpha 1\beta 2$ (CD49b or VLA2) is expressed by the CD4 Teff cells that localize in the parenchyma [35]. However, as discussed below, VLA1 is not required for the initial recruitment of CD8 Teff cells, but is critically important in their retention during the memory phase [36].

Transendothelial migration of T_{RM} precursors

The arrested lymphocytes must cross the endothelial cells and their associated cell types (pericytes and perivascular macrophages) in order to gain access into the tissue [12]. The process of transendothelial migration requires sequential and reciprocal signaling between the lymphocytes and endothelial cells involving platelet/endothelial cell adhesion molecule (PECAM) – PECAM (homophilic), CD99– CD99 (homophilic), Junctional adhesion

molecules (JAMs), integrins and VE-Cadherin [37] (Figure 1). Human CD4 Teff cell transendothelial migration requires CD4 Teff LFA1 engagement of endothelial ICAM-1 and JAM1, and Teff CD226 and CD96 engagement of endothelial nectin-2 and poliovirus receptor [38]. Studies using genetically engineered mouse models showed the requirement for PECAM expressed by both T cells and endothelial cells in activated T cell trafficking to various organs [39]. As many of the steps involved in transendothelial migration engages signaling in Teff cell and in endothelial cells, as well as dramatic squeezing of the cell body and nucleus, the impact of transendothelial migration on T_{RM} differentiation and function will be important to determine in future studies.

Retention and survival signals for CD8 T_{RM}

Once inside the tissue, lymphocytes migrate towards the source of chemokines through sensing of the chemokine gradient. In order to remain resident in the tissue, lymphocytes likely respond to positive cues to remain within the tissue, likely via interaction with supportive structures such as extracellular matrix, fibroreticular cells and must also down regulate egress receptors in order to avoid exit cues. In addition to control of migration, memory T cells require growth factor for their survival. Recent studies have shed light on some of the molecular mechanisms and tissue factors that control T_{RM} residency (Figure 2).

Positive retention signals for CD8 T_{RM} cells are provided by the epithelium. TGF- β , a cytokine secreted by a variety of mucosal and skin epithelial cells [40, 41], stimulates CD8 T cells to express the integrin α E β 7 [1]. TGF- β down regulates the expression of T-bet, which releases the repression imposed by T-bet on the CD103 locus (α E) [29]. α E β 7 integrin mediates T cell adhesion to epithelial cells through binding to E-cadherin [42]. CD103 is a frequently used marker of T_{RM}, and CD103 expressed by CD8 T cells is required for their maintenance in tissues including the small intestine epithelium, brain, and skin epidermis [6, 43-45]. However, the requirement for CD103 for CD8 T_{RM} retention is not universal, and can be replaced by other types of retention signals. For example, CD8 T_{RM} cells in the lamina propria do not express CD103 but may rely on macrophages and antigenic stimuli for retention. After oral infection with *Yersinia pseudotuberculosis* two types of CD8 T_{RM} emerge; CD103+ CD8 T_{RM} in the epithelium and CD103- CD8 T_{RM} in the lamina propria. Of note, the latter type (CD103- T_{RM}) does not require TGF- β for development but instead forms clusters with macrophages and CD4 T cells in the lamina propria. In addition, the CD8 T_{RM} cells that develop after LCMV infection in the lamina propria, but not epithelia, show sign of TCR engagement [46]. These results suggest that cells within the clusters may provide the necessary retention signals for T_{RM} residency in the lamina propria.

The avoidance of egress cue for CD8 T_{RM} is provided by CD69-mediated inhibition of the egress receptor sphingosine-1-phosphate (S1P) receptor 1 (S1P1). S1P1 responds to concentrations of S1P, which is highly abundant in blood and lymph but scarce in the tissue [47]. CD69 expression is induced on lymphocytes by TCR signaling and by type I IFN signaling [48]. CD69 binds to the transmembrane region of the S1P1 and promotes its degradation [49]. Transcription factor kruppel like factor 2 (KLF2) is required for expression of S1P1 and is downregulated once Teff cells seed the peripheral tissue [46].

Forced expression of S1P1 by T cells reduces their residency in the skin [46]. KLF2 also promotes the expression of CCR7, which is another egress receptor on tissue lymphocytes required for their migration into the draining lymph nodes [6, 50]. In addition to S1P1, transcriptional profiling of CD8 T_{RM} revealed that S1P5 mRNA is also downregulated in the CD8 T cells isolated from peripheral tissues compared to spleen [6], suggesting that this receptor for S1P also plays a role in egress of memory CD8 T cells out of the tissue into the lymph nodes. In addition to the migratory programming, T_{RM} cells, like any other memory T cell type, must receive survival signals to repopulate and maintain their niche. After skin infection with HSV-1, CD8 T_{RM} cells require IL-15 from radioresistant cells to support their maintenance [6]. Interestingly, CD8 T_{RM} cells are overrepresented in the lymph nodes of IL-15 deficient mice [51]. Thus, IL-15 may be particularly important in the survival of peripheral tissues T_{RM}. In addition to IL-15, CD8 T_{RM} cells rely on aryl hydrocarbon receptor (AhR) for long-term persistence in the epidermis [52]. This study also showed that Langerhans cell depletion had minimal impact on epidermal CD8 T_{RM} maintenance [52], indicating that Langerhans cells are not the source of survival signal (IL-15, AhR ligands). Therefore, CD8 T_{RM} precursors use positive retention signals, avoid egress signals and dwell on survival factor in order to be maintained within the tissue. Important questions in this area relate to the identification of survival mechanisms used by lamina propria CD8 T_{RM}, in particular, the role of supporting cell types in maintaining the number of function of CD8 T_{RM} in the epithelium and lamina propria in various organs.

Retention signals for CD4 T_{RM}

Although much less is understood with regards to signals for maintenance of CD4 T_{RM}, they appear similar to those for lamina propria CD8 T_{RM} but distinct from those for epithelial CD8 T_{RM} as discussed below. As with CD8 T_{RM}, CD4 T_{RM} precursor (CD4 Teff) access is restricted in certain tissues (skin and vagina) and permissive in others (lung). CD4 memory T cells isolated from the lung of mice infected with influenza virus specifically home back to the lung after adoptive transfer, whereas those isolated from the spleen are more promiscuous in their migration into the spleen, liver and the lung [53]. Thus, there appear to be tissue-specific cues that recruits and retains CD4 T_{RM} in the lung at steady state. In contrast, in the restricted tissue such as the skin and the vagina, neither effector nor memory CD4 T cells access and become resident in the absence of inflammation. Following infection with HSV-1, memory CD4 T cells are found predominantly in the dermis and in the hair follicles, and are quite mobile within the dermis while retaining the capacity to migrate to distal areas of the skin [10]. Similarly, in the vagina of mice previously infected with attenuated HSV-2, memory CD4 T cells can be found either scattered throughout the lamina propria, or are localized in organized MLC structure consisting memory CD4 T, CD8 T, dendritic cells and macrophages all of which are tissue-resident [9, 54] (Figure 2).

In contrast to CD8 T_{RM}, CD103 expression is found on only about half of the skin resident CD4 T cells after HSV-1 infection [10] and is detected in about 10% of the CD4 T_{RM} in the vagina after TK– HSV-2 infection [9]. In human skin, CD4 memory T cells in the dermis lack CD103, whereas those in the epidermis are CD103+ [55]. In the mouse vagina, CD4 T_{RM} express high levels of CD44, CD69 and Tbet, whereas expression of CCR7, S1P1, KLF2, and CD62L is low [9]. Therefore, like the CD8 T_{RM}, CD4 T_{RM} express CD69 and

repress KLF2 to block S1P1 and CCR7 expression, which ensures their unresponsiveness to egress cues. In contrast to CD8 T_{RM}, the high Tbet expression on CD4T_{RM} likely inhibits the expression of CD103 [29]. In the absence of CD103, how do CD4 T_{RM} or CD8 T_{RM} in the lamina propria or dermis remain resident within the tissue? Avoidance of egress cues alone is not sufficient to retain T_{RM}, as evidenced by the fact that CD103 deficiency results in loss of CD8 T_{RM} [6, 56]. Recent evidence suggest that one of the ways in which CD103^{neg} T_{RM} could be maintained in peripheral tissue within clusters of cells that provide positive cues within a structure known as memory lymphocyte clusters (MLCs).

Memory lymphocyte clusters as hubs for T_{RM} maintenance

After a local infection or immunization, memory lymphocytes form within the parenchyma of the tissue are maintained in clusters that consist of distinct cell types. Here, we specifically focus on the MLC, and not the clusters that mediate induction of T cells during the primary immunization or infection [57-59]. The MLCs are distinct from tertiary lymphoid organs (TLO) in that MLCs do not have HEVs and are therefore devoid of naïve lymphocytes, they do not have direct lymphatic drainage, and they lack the organized lymphoid compartments found in TLOs (Table 2). Accumulating evidence indicates the existence and importance of MLCs as hubs for T_{RM}, and as immediate response centers that provide protection against invading pathogens within the peripheral tissues.

After intranasal infection with VSV, CD8 T_{RM} form long-lived clusters with CD4 T cells [44]. Similar cluster formation was observed following intracranial injection of antigen-loaded dendritic cells, indicating that direct infection of the tissue cells is not required for MLC formation [44]. Infection by *Toxoplasma gondii* can lead to toxoplasmic encephalitis in patients. Tracking endogenous T cells using two-photon microscopy, a study revealed that a large T cell population is recruited to the areas of the brain associated with areas of *T. gondii* replication [60]. Notably, infection induced a network of reticular fibers that seemed to act as a structural and chemotactic support for T cells in the brain [60]. Future studies are needed to identify whether similar reticular fiber structure supports MLCs in other organs. In the lung, alveolar lymphoid aggregates form after intratracheal infection with LCMV. These aggregates lack HEVs, and are much smaller than the tertiary lymphoid organ, iBALT, which forms bordering large airways [61]. The vast majority of lymphocytes in the alveolar aggregates are inaccessible by antibody injected intravenously, suggestive of their T_{RM} status. In the salivary gland of mice infected with MCMV three weeks prior, CD4 T cells, CD8 T cells, NK cells, B cells and CD11b as well as CD11c expressing cells form clusters [62]. Of note, phagocytic cells loaded with viral antigens can be detected within the lymphocyte clusters in the salivary glands. In the vaginal mucosa, local infection with an attenuated HSV-2 (thymidine kinase mutant; TK-) results in the formation of long-lived MLCs. These MLCs consist of memory CD4 and CD8 T cells, and are supported by CD11b + CD11c- MHC II+ macrophages, which secrete chemokines that retain the memory lymphocytes [9]. Antibody-mediated neutralization of CCL5 reduces the number of HSV-2 specific CD4 and CD8 T cells in the local tissue. Even though CD4 and CD8 T_{RM} express CCR1, CCR5 and CXCR3, antibody neutralization of CXCL9 led to significant reduction in CD8 T_{RM} but left CD4 T_{RM} intact within the MLC, indicating the dependency of the CD8 T_{RM} on both CCL5 and CXCL9 for retention (Figure 2). In situ staining of CCL5 revealed

that the dominant cell type responsible for CCL5 secretion is the CD11b⁺ macrophages. Depletion of CD11b⁺ macrophages dissolves the MLC structure, and renders the animal susceptible to secondary infection with lethal wild type HSV-2. CCL5 secretion from macrophages is likely maintained by low levels of IFN- γ constitutively secreted from the CD4 T_{RM} as antibody blockade of IFN- γ or depletion of CD4 T_{RM} results in ablation of CCL5 expression *in vivo*. The constitutive levels of IFN- γ from CD4 T_{RM} are likely induced by recognition of antigenic peptide presented by local APCs, as antibody blockade of MHC class II molecule diminished CD4 T_{RM} abundance and dissolved MLC structures (Iijima et al., unpublished observations). Altogether, these data support the notion that MLCs represent a self-organizing structure maintained by a positive feedback loop (Figure 2).

The integrins that are activated by the chemokines within the MLC to retain the T_{RM} are unknown. It has been shown that VLA-1 is responsible for retaining CD8 T_{RM} in the lung and other tissues via attachment to the extracellular matrix, as suggested by the fact that CD8 memory T cells are reduced in peripheral tissue after injection of VLA1 blocking antibody at the memory phase of the immune response [36]. Recent transcriptome analysis of CD8 T_{RM} confirmed the selective expression of the $\alpha 1$ integrin in various tissues [6]. On the other hand, the $\alpha 4\beta 1$ integrin is highly expressed on CD4 T_{RM} in the vagina following TK– HSV-2 infection [9], while $\alpha 1\beta 2$ integrin is expressed by lung parenchymal CD4 T cells [35]. Human cervical CD69⁺ CD4 T cells also express $\alpha 4\beta 1$ in addition to $\alpha 4\beta 7$ integrin [63]. Future studies are needed to define the integrins required to retain CD8 T_{RM} and CD4 T_{RM} in their respective homes.

MLCs are present in humans and non-human primates, particularly following infection with live pathogens. Formation of lymphoid aggregates (size from ~60 μ m to over 500 μ m in diameter) comprised of CD8 T cells, B cells and other cells is observed in rhesus macaques immunized intravenously with attenuated SIV (SIV_{nef}). These structures are located throughout the lamina propria from just beneath the epithelium to near the muscularis as well as within the muscularis [64]. At steady state, in the uterus of humans, lymphoid aggregates consisting of a B cell core surrounded by memory CD8⁺ T cells and macrophages form reaching peak size at mid-secretory phase as a part of the normal menstrual cycle [65]. These structures in the uterus possibly represent centers for immune regulation to promote reproductive function and may be quite distinct from the MLCs, which are more stable and are designed to protect the host against infectious agents.

What do the MLCs have in common? First, MLCs occur at the site of primary infection or immunization in an antigen-dependent manner [9, 44]. Second, while no replicating pathogens can be recovered from the MLC, there appears to be a depot of low levels of antigenic peptides that drive the positive feedback loop forward [9, 44, 62]. Third, MLCs contain memory CD8 T_{RM} and CD4 T_{RM}, as well as antigen presenting cells that hold the cluster together [9, 62]. Whether the same set of chemokines are involved in the retention of T cells in different types of MLCs found in various tissues is unclear. We speculate that the MLCs serve as an immediate response center consisting of TRM on standby, capable of detecting incoming pathogens and mounting robust local immune response to contain and limit the spread of infectious agents.

Effector phase of T_{RM} control of pathogens

T_{RM} control invading pathogens more efficiently than circulating memory T cells. The enhanced ability of T_{RM} in antimicrobial function is based on their location and their cell intrinsic and extrinsic capabilities, as discussed further below. Location of T_{RM} with respect to the invading pathogen is likely to provide them with an advantage to respond more quickly than the circulating memory T cell counterpart. CD8 T_{RM}s are localized within the epithelium where the viral infection first takes place [66]. Memory T cells in the MLCs are present right up against the protruding region of the vaginal fold closest to the center of the vaginal lumen where viral infection would likely take place [9]. Circulating memory T cells on the other hand have to undergo the entire process of rolling, adhesion and extravasation in order to enter the tissue at the activated endothelial cells, which are below the epithelial layer of mucosal tissues. Not only are the circulating memory T cells far from the site of infection, but they are also at a disadvantage due to their low frequency in the blood compared to relative high density of T_{RM} in the tissue. To have antiviral impact, circulating memory T cells would have to enter the tissue and proliferate before they can have an antiviral impact. At the site of prior viral exposure, antiviral effector mechanisms against HSV-2 are mediated through IFN- γ secreted from memory CD4 T_{RM} [9, 54, 67, 68]. Analysis of IFN- γ secretion by memory CD4 T cells in the vagina revealed that T_{RM} have a constitutive capacity to secrete IFN- γ , which is enhanced by HSV-2 infection, whereas circulating memory CD4 T cells enter the tissue around 2 days post infection and only start to secrete IFN- γ after 5 days post infection [9], which is long past the peak of viral replication in the vaginal epithelium and is certainly after the establishment of latent infection in the neurons of dorsal root ganglia.

In addition to the effector cytokine 'readiness' of the T_{RM}, there are other cell intrinsic programs that enable T_{RM} to either perform antiviral functions more readily, or to be regulated by surrounding cell types. CD8 T_{RM} isolated from the brain express higher amounts of CTLA4, PD-1, ICOS and granzyme B mRNA transcripts, while TLR1 mRNA amounts were reduced as compared to splenic CD8 memory T cells [69]. Similarly, the expression of genes encoding CTLA-4, and ICOS was elevated in CD8 T_{RM} from gut, lung and skin as compared to splenic memory CD8 T cells, while TLR1 was downregulated in the T_{RM} cells [6]. These data suggest that the CD8 T_{RM} in various tissues have the capacity to not only to serve as effectors (through granzyme B or ICOS) but may also be readily regulated by cells expressing PD-1 ligands (PDL1 and PDL2) or CTLA4 ligands (CD80, CD86) to shut down their activity. Future studies are needed to confirm the expression and functional roles of these surface proteins on T_{RM}. Other genes expressed by CD8 T_{RM} help avoid being infected and killed by the virus they are trying to control. In the lung, CD8 T_{RM} express the interferon-induced transmembrane protein IFITM3 in order to avoid becoming target of influenza virus infection upon secondary challenge [70]. Of note, CD8 T_{RM} express other interferon-stimulated genes such as ISG20 and IFI44 [69], perhaps to protect themselves from a wide range of viruses.

In addition to cell intrinsic effector mechanisms, T_{RM} rely on other cells to promote pathogen control. IFN- γ secreted by T_{RM}s induces enhanced expression of VCAM-1 and ICAM-1 to enable rapid adherence of circulating memory T cells to endothelium and their

recruitment into the vagina following secondary infection with HSV-2 [71]. Similarly, CD8 T_{RM} are able to elicit recruitment of circulating cells (memory T cells, B cells and innate leukocytes) to the site of infection through secretion of IFN- γ and amplification of the immune response to a viral pathogen [72-74]. Therefore, T_{RM} possess locational, temporal and functional advantages over circulating memory T cells in the execution of antimicrobial responses at body surfaces, by serving as first line of defense and through recruitment of other immune cell types.

Concluding remarks

Effector T cells are programmed to proliferate, differentiate and home to various lymphoid and non-lymphoid tissues to become memory T cells. An important part of the programming occurs after the effector T cells migrate and enter their destination site. Thus, T cell memory can be considered to have specificity not only to the molecular details of the pathogen (through recognition of epitopes via antigen receptor), but also to the location of invasion (through the establishment of T_{RM}s).

However, we are only beginning to understand the nature of tissue instruction for memory T cell recruitment and maintenance, with many questions remaining to be addressed (Box 2). Effector T cells express various homing molecules that enable rolling, adhesion and transendothelial migration into inflamed tissues. Once inside the tissue, T_{eff} migrate toward chemokine gradient and activate integrins that enable interaction with extracellular matrix components to remain within the tissue. At the same time, T_{eff} must turn off receptors for egress signal in order to avoid migration out of the tissue. Survival of T_{RM} may be ensured by growth factors released from the surrounding tissue cell types. However, which growth factors are required for different T_{RM} types in various tissues remains to be elucidated. CD8 T_{RM} in the epidermis or mucosal epithelium acquire retention signals from the adjacent epithelial cells. In contrast, CD8 T_{RM} and CD4 T_{RM} in the dermis or lamina propria require retention signals from non-epithelial sources. The MLCs represent an organized long-lived structure beneath the epithelial layer where both CD4 T_{RM} and CD8 T_{RM} maintain their residency and function. A positive feedback loop between resident macrophages and T_{RM} from a self-sustaining relationship that is maintained independently of the circulating pool of lymphocytes. However, not all T_{RM} in the lamina propria reside within the MLCs. The retention signals that maintain CD4 T_{RM} and CD8 T_{RM} in the lamina propria that reside outside of MLCs are unknown.

More general questions in this area relate to the mechanism of execution of the effector function by the T_{RM}, as well as the contributions of circulating memory T cells and B cells in this regard. For instance, T_{RM} in the MLCs must presumably migrate toward the infected cells to mediate killing or secrete antimicrobial cytokines. Mechanisms that release T_{RM} from MLCs and enable their migration towards the infected cells remain to be determined. In addition, MLCs with different memory lymphocyte compositions including CD8 T, CD4 T (Th1, Th2, Th17), and B cells of various isotypes are likely to exist for different types of pathogens. Finally, vaccine strategies that take advantage of MLCs are expected to provide a higher level of protection compared to circulating memory T cells. Current successful vaccines in use in humans rely on antibody-based protection [75]. Vaccine that rely on T-

cell mediated protection offers a great alternative to antibody-based vaccines, as many T cell epitopes are targeted to the core proteins of pathogens that are better conserved than the antibody-recognition of surface antigens which tend to undergo mutations to evade existing antibody pool. Future development of MLC-based vaccine strategies may provide a solution to currently unsuccessful areas of infectious disease prevention and cancer therapy.

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Box 1**Steps in activated lymphocyte migration from blood to tissue**

Lymphocyte migration into inflamed tissues occur through sequential steps involving interaction between specific molecules expressed by lymphocytes and their counter receptors on endothelial cells [11, 76] (Figure 1). In postcapillary venules near the site of inflammation, blood flow rate is sufficiently reduced so as to enable lymphocytes to tether and roll on the activated endothelial cell surface. The rolling step involves selectins expressed by activated endothelial cells and selectin ligands expressed by lymphocytes. Integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$ can also serve as rolling receptors. The next step involves activation of lymphocytes by chemokines on endothelial cells. Chemokine binding to chemokine receptors on lymphocytes triggers Gai-dependent “inside-out” signaling leading to activation of integrins. Activated integrins bind tightly to their ligands on endothelial cells, allowing lymphocytes to arrest on the endothelial surface. Finally, lymphocytes enter the tissue through a series of interactions between molecules expressed on endothelial cells to accomplish transendothelial migration [77].

Box 2**Outstanding Questions**

- Which integrins are activated by chemokines in the MLC for retention of T_{RM} cells?
- How are T_{RM} in the lamina propria outside of the MLCs maintained?
- Do T_{RM} of different CD4 subsets (Th1, Th2, Th9, Th17 and Tfh) exist? If so, what are their residency requirements?
- Do B cells establish tissue-residency and if so, how are they recruited and maintained?
- What are the survival growth factors necessary to maintain T_{RM} in different tissues?
- Can we design better vaccines based on establishment of T_{RM} ?

Highlights

- Effector T cells receive tissues instructions and establish long-term residency.
- T_{RM} are more protective than circulating memory cells based on their location and function.
- Epithelial T_{RM} cells express CD103 and shut off S1P1 and CCR7 to prevent egress.
- Memory lymphocyte clusters (MLCs) retain CD103^{neg} CD4 and CD8 T_{RM} in the lamina propria.

Figure 1. Overview of the steps involved in recruitment of CD8 TRM precursors to the inflamed tissue

(Step 1: Rolling) CD8 Teff slow down and roll on the endothelium of the postcapillary venule at the site of inflammation. P-selectin and E-selectin expressed by endothelial cells bind to P-selectin ligand and E-selectin ligand on CD8 Teff, respectively. **(Step 2: Chemokine activation)** CD4 Teff enter the tissue in response to infection and secrete IFN- γ , which activates dendritic cells to secrete CXCL9 and CXCL10. In addition, endothelial cells in response to type I interferons secrete CXCL10. These chemokines are displayed by the endothelial cells to recruit CD8 Teff. **(Step 3: Arrest)** Chemokine engagement of the receptor CXCR3 activates integrins, VLA-4 ($\alpha 4\beta 1$) and LFA1 ($\alpha L\beta 2$), on CD8 Teff through inside out signaling, and enables the lymphocytes to adhere to the endothelium and come to a complete stop. **(Step 4: Transendothelial migration)** Arrested lymphocytes must cross the endothelial layer in order to get access to the tissue. Teff use molecules such as PECAM, CD99, LFA1 which bind to PECAM, CD99 and ICAM-1/JAM-1 on endothelial cells, respectively, to crawl between the endothelial cells to enter the tissue. **(Step 5: Migration toward infected cells)** Once inside the tissue, Teff are recruited to the site of infection by

migration towards chemokine gradient. Infected cells, as well as type I interferons, can induce secretion of chemokines such as CXCL10 that mediate this last step.

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Figure 2. Model for retention of CD4 T_{RM} and CD8 T_{RM} in the memory lymphocyte clusters
 Once inside the tissue, CD4 Teff and CD8 Teff undergo further differentiation to become T_{RM} . In the epithelial layer, CD8 T_{RM} precursor receives TGF- β signal from the surrounding epithelial cells, which induces CD103 expression. CD103 binds to E-cadherin to mediate stable interaction. CD8 T_{RM} precursors also induce CD69 expression, which binds to S1P1 and degrades it. T_{RM} precursors also downregulate KLF2, a transcription factor required for expression of S1P1 and CCR7. Below the epithelial layer, both CD8 T_{RM} and CD4 T_{RM} are retained in memory lymphocyte clusters (MLCs) consisting of T_{RM} and tissue resident macrophages and dendritic cells. Small amount of antigen is likely retained and presented by macrophages, which induce constitutive secretion of low levels of IFN- γ from CD4 T_{RM} . IFN- γ binds to its receptor on macrophages, inducing expression of CCL5 and CXCL9. CCL5 binds to its receptor on CD4 T_{RM} , enabling their interaction with macrophages through a yet to be identified integrin(s). CXCL9 binds to CXCR3 on CD8 T_{RM} , which helps retain these cells within the MLCs. MLCs are found in the lower female

reproductive tract after HSV-2 infection (humans, mice and guinea pigs) or SIV infection (in primates), in the brain after *T. gondii* infection (mice), in the alveoli after intratracheal LCMV infection and in the salivary gland after MCMV infection (mice).

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Table 1
Permissive vs. restrictive tissues for T_{RM} precursor (Teff) entry

Category	Teff access at steady state	Teff access during inflammation	Examples
Permissive	+	+	Intestinal epithelium, peritoneal cavity
Restrictive	–	+	Skin epidermis, vaginal epithelium, lung airways, salivary glands, ganglia

Modified from reference [7]. Permissive tissues express Teff homing receptor ligands, such as MAdCAM-1, at steady state, while restrictive tissues do not.

Table 2
Distinct features associated with memory lymphocyte clusters (MLCs) vs. tertiary lymphoid organs (TLOs)

	Memory lymphocyte clusters	Tertiary lymphoid organs
High endothelial venules	–	+
Naïve lymphocytes	–	+
Lymphatic vessels	–	+
Germinal centers	–	+
Memory T cells	++	+
B cell zone	–	+
T cell zone	–	+
Infection	Resolved	Chronic